Heat and pressure resistance of *Escherichia coli*

by

Hui Li

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science
University of Alberta

ABSTRACT

Strains of *Escherichia coli* may survive heat or pressure stress, acquire specific virulence genes and cause severe human diseases. The locus of heat resistance (LHR) has been identified as an important heat resistant element in *E. coli*. The objective of this thesis was to explore the role of the LHR on heat and pressure resistance of *E. coli*, as well as its relationship with protein folding and aggregation, and evaluate the effects of food matrix and antimicrobials on pressure resistance of *E. coli*.

To explore the heat and pressure resistance related to protein folding and aggregation, the role of LHR was investigated in *E. coli* MG1655 expressing *ibpA-yfp* fusion. A total 10 proteins of LHR were detected through proteomic analysis using mass spectrometry, including two small heat shock proteins, two heat shock proteases, proteins of the YfdX family, thioredoxin, and a sodium/hydrogen antiporter. Microscopic observations showed that LHR reduced the inclusion bodies after heat or the pressure treatment, indicating that LHR proteins function to re-fold and turnover aggregated proteins. The proteomic analysis confirmed that the phenotype of pressure resistance of LHR occurred through stress regulation, mitigation of protein aggregation and reduction of oxidative stress.

To evaluate the effect of food matrix on the pressure resistance of *E. coli*, the resistance of two five-strain cocktails to pressure in bruschetta, tzatziki, yogurt and ground beef was compared. Pressure reduced *E. coli* in plant and dairy products by

more than 5 logs (cfu/mL) but not in ground beef. Food components calcium, magnesium, glutamate, caffeic acid and acetic acid exhibited a protective effect on *E. coli* after pressure treatment and during storage at 4 °C.

Further study assessed the combined effect of antimicrobials and pressure on enterohaemorrhagic *E. coli* (EHEC) in pressure treated beef steaks. The thiol-reactive allyl-isothiocyanate (AITC) and cinnamaldehyde exhibited synergistic activity with pressure on *E. coli*. However, the membrane-active essential oil components carvacrol and thymol showed antagonistic or no synergistic effect with pressure.

In conclusion, the LHR confers pressure resistance of *E. coli*, and the resistance is related to protein folding and aggregation. Moreover, pressure resistance of *E. coli* is affected by food matrix and antimicrobials.

PREFACE

A version of Chapter 2 of this thesis is a literature review and has been published as Li, H., and Gänzle, M. (2016). Some like it hot: hear resistance of *Escherichia coli* in Food. *Frontiers in Microbiology*, 7, 1763.

The proteomic analysis in Chapter 3 was carried out in Bavarian Biomolecular Mass Spectrometry Center in Germany. Stephanie Heinzlmeir contributed to this work, and Drs Michael Gänzle, Jürgen Behr and Rudi F. Vogel supervised this work. I participated in part of the experiments and data analysis of this work under the guidance of Stephanie Heinzlmeir. Dr. Abram Aertsen from KU Leuven provided the two strains of *E. coli* MG1655 *ibpA-gfp* and LMM1010 *ibpA-gfp*.

A version of Chapter 4 of this thesis has been published as Li, H., Garcia-Hernandez, R., Driedger, D., McMullen, L.M., Gänzle, M. (2016). Effect of the food matrix on pressure resistance of Shiga-toxin producing *Escherichia coli*. *Food Microbiology*. 57, 96-102. Part of the experimental work in this chapter including Figure 3-2C, Figure 3-5 and Table 3-2 were performed by Rigoberto Garcia-Hernandez under the supervision of Dr. Michael Gänzle and Dr. Lynn McMullen.

A version of Chapter 5 of this thesis has been published as Li, H., and Gänzle, M. (2016). Effect of hydrostatic pressure and antimicrobials on survival of *Listeria monocytogenes* and enterohaemorrhagic *Escherichia coli* in beef. *Innovative Food Science and Emerging Technologies*. 38, 321-327. Haihong Wang provided marinades.

Financial supports for this thesis were provided by the Alberta Livestock and Meat Agency, Alberta Crop Industry Development Fund, Alberta Innovates Biosolutions, and the Beef Cattle Research Council. Hui Li is supported by China Scholarship Council.

ACKNOWLDEGMENTS

I would like to express my gratitude to all those people who greatly supported me for my PhD study during the last four and half years.

I would like to express my sincere and special gratitude to my supervisor Dr. Michael Gänzle for his guidance, support, patience, and humor. I really appreciate every discussion, talk, and quick email exchanges with him. I got to know how to think about research from him, and became more and more confident in the last several years under his encouragement.

I would also like to express my appreciation to my supervisory committee members Dr. Lynn McMullen and Dr. Feral Temelli, for all of their guidance and support. Their patience and lovely words always encouraged and motivated me very much. I would also like to extend my thanks to Dr. Tracy Raivio and Dr. Hosahalli Ramaswamy for agreeing and taking time to be reviewers of my thesis.

I would like to express my gratitude to people in Agri-Food Discovery Place (AFDP), particularly Ken Fahner, Ross Lowe, Patrick Ward, Linda Ho and Laurie Heidebrecht, who helped me a lot when I worked in the meat wing of AFDP. As well, I am very grateful for the support from Jody Forslund, for all her patience and answers to my questions or concerns. My sincere thanks also goes to Stephanie Heinzlmeir, Dr. Jürgen Behr and Dr. Christina Ludwig, for all of their help on my lab work and daily life during my visit to Germany.

I am so grateful for all my lab mates and friends in our department, for all of their help on my lab work, and for the great times we spent together. I would like to thank Ryan Mercer, Rigoberto Garcia-Hernandez, Oanh Nguyen, Yuan Fang, Xiaohong Sun, Jiandong Ren, Qing Li, Liang Chen, Xin Zhao, Anna Nilson, Yuanyao Chen, Yalu Yan, Ying Hu, Ziyi Hu, and Arisha Seeras. I would like to specially thank my great friends, particularly Gary Short, Kathy Short, Shichao Yue, Junhua Kang and Lucia Hodulová for being so supportive and helpful especially during my hard time in Edmonton.

Finally, I would like to thank my husband and my parents for their great love. I would not have come so far without their support, understanding and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 **Introduction and objectives**

High hydrostatic pressure has been commercially used as an effective intervention to improve food safety (Balasubramaniam et al., 2015; Georget et al., 2015). The pressure resistance of *E. coli* is related to the regulation of sigma factors σ^E and σ^S, as well as the proteins that protect pressure-induced oxidative stress (Gänzle and Liu, 2015). Remarkably, pressure resistance is also related to the disassembly of inclusion bodies and reassembly of protein aggregates, and is regulated by the inclusion body binding proteins IbpA and IbpB and heat shock proteins including DnaK and DnaJ through the heat shock response and assembly and segregation of protein aggregates (Charoenwong et al., 2011; Robey et al., 2001; Aertsen et al., 2004; Govers et al., 2014; Gänzle and Liu, 2015).

Some heat resistant strains of *E. coli* are highly resistant to pressure (Garcia-Hernandez et al., 2015; Liu et al., 2015), and pressure resistant mutant strains of *E. coli* are also resistant to heat (Hauben et al., 1997). Heat-induced alterations of *E. coli* cells include membrane, cytoplasm, ribosome and DNA, particularly protein misfolding and aggregations (Chapter 2). Resistance systems of *E. coli* act against these alterations, mainly through gene regulations of heat shock response including EvgA, heat shock protein, σ^E and σ^S, to refold of misfolded proteins, and achieve antagonism to heat stress (Chapter 2). Thus heat shock proteins functioning to refold the misfolded

proteins, and proteins against oxidative stress may contribute to both heat and pressure resistance of *E. coli*.

A ~14 kb genomic island named the locus of heat resistance (LHR) was identified in highly heat resistant strains and is present in approximately 2% of *E. coli* strains (Mercer et al., 2015). LHR contains 16 open reading frames (ORFs) (**Figure 1-1**), which encode for two small heat shock proteins (Orf2 and Orf7), two proteins of the YfdX family with unknown function (Orf8 and Orf9), three heat shock proteases (Orf3, Orf15 and Orf16), a thioredoxin (Orf12), and a sodium/hydrogen antiporter (Orf13) (Mercer et al., 2015). LHR was found to confer heat resistance in sensitive strains of *E. coli*, and loss of the LHR was found to diminish the heat resistance (Mercer et al., 2015; Pleitner et al., 2012).

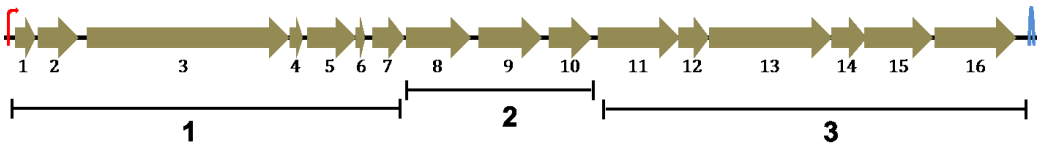

Figure 1-1 The locus of heat resistance. The entire genomic island was designed into 3 fragments for constructing a plasmid-borne copy of the LHR. Figure was adopted from Mercer et al. (2015).

Even though there has been no conclusive evidence linking heat and pressure resistance of *E. coli*, both have been proposed to be involved in the regulation of protein folding and aggregation. Currently, high pressure alone is not sufficient for inactivation of resistant *E. coli* strains. The objective to improve high pressure processes necessitates an understanding of the role of matrix constituents, and an exploration of adding new hurdles such as antimicrobials. Moreover, further knowledge on the

mechanisms of heat and pressure resistance of *E. coli* is necessary for optimization of food processing.

The hypothesis of this thesis research was that the LHR mediates pressure resistance of *E. coli* by alteration of protein folding and aggregation and the pressure resistance of the LHR positive *E. coli* is affected by food matrix and antimicrobials.

To test this hypothesis, the research aimed to meet the following objectives:

1) To explore the mechanisms of heat and pressure resistance of *E. coli* relating to protein folding and aggregation (Chapter 3).
2) To compare the resistance of shiga-toxin producing *E. coli* (STEC) to high pressure treatment in different food products to determine the effect of food matrix on pressure resistance (Chapter 4).
3) To evaluate the synergistic effect of antimicrobial compounds with pressure on *E. coli* in a model system and meat (Chapter 5).

1.1 References

Aertsen, A., Vanoirbeek, K., Spiegeleer, P.D., Sermon, J., Hauben, K., Farewell, A., Nyström, T., and Michiels, C.W. (2004). Heat shock protein-mediated resistance to high hydrostatic pressure in *Escherichia coli*. *Appl. Environ. Microbiol.* 70, 2660-2666.

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

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

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

Gänzle, M.G., and Liu, Y. (2015). Mechanisms of pressure-mediated cell death and injury in *Escherichia coli*: from fundamentals to food applications. *Front. Microbiol*. 6, 599.

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

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

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

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

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

Pleitner, A., Zhai, Y., Winter, R., Ruan, L., McMullen, L.M., and Gänzle, M.G. (2012). Compatible solutes contribute to heat resistance and ribosome stability in *Escherichia coli* AW1.7. *Biochim. Biophys. Acta*. 1824, 1351-1357.

Robey, M., Benito, A., Hutson, R.H., Pascual, C., Park, S.F., and Mackey, B.M. (2001). Variation in resistance to high hydrostatic pressure and *rpoS* heterogeneity in natural isolates of *Escherichia coli* O157:H7. *Appl. Environ. Microbiol*. 67, 4901-4907.

CHAPTER 2 **Literature Review**

2.1 Introduction

Pasteurization and domestic cooking are common interventions for reducing the numbers of vegetative bacterial cells including pathogens in food. Heat kills vegetative bacterial cells by inactivation of cellular components, particularly membranes, proteins, and ribosomes (Lee and Kaletunc 2002; Mackey et al., 1991; Mohácsi-Farkas et al., 1999; Tsuchido et al., 1985). Thermal food processing has an excellent record of establishing and maintaining food safety. However, consumer preferences for raw or minimally processed food, and the aim to minimize thermal degradation of nutrients are incentives to reduce the intensity of thermal processing. Moreover, fresh foods including meats and produce cannot be heated to temperature that are lethal to all pathogens, and bacterial pathogens are highly resistant to thermal processing in the dry state (Santillana Farakos et al., 2014; Syamaladevi et al., 2016). In addition, the heat resistance of pathogens is variable and heat resistant strains may withstand thermal processes that are lethal to the majority of strains of the same species (Dlusskaya et al., 2011; Murphy et al., 1999; Ng et al., 1969).

E. coli has been generally considered to be a relatively heat sensitive organism; however, specific strains of *E. coli* belong to the most heat resistant vegetative foodborne pathogens (**Figure 2-1**; Jay et al., 2008; Doyle and Beuchat, 2013). Heat resistant *E. coli* have D_{60} value of more than 6 min (**Figure 2-1**, Mercer et al., 2015; Liu et al., 2015), and their resistance matches or exceeds that of *Salmonella* Senftenberg

755 with D_{60} of 6.3 min (Ng et al., 1969; Baird-Parker et al., 1970) and *Staphylococcus aureus* with D_{60} of 4.8-6.5 min (Kennedy et al., 2005; Jay et al., 2005; Doyle and Beuchat, 2013).

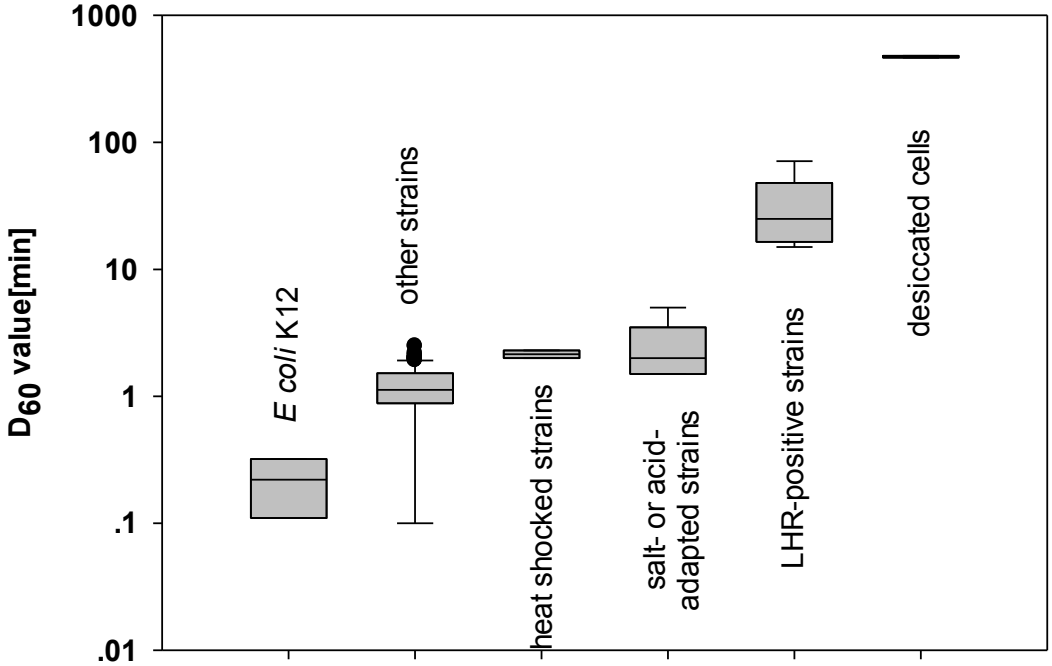

Figure 2-1 Heat resistance of *E. coli.*Data shown are $\log_{10}$ value of D_{60} (min) of 144 strains collected from related publications: 3 values of K-12 strains (Chuang et al., 2007; Jin et al., 2008; Dlusskaya et al., 2011), 125 of other strains of *E. coli* (Liu, 2015; Enache et al., 2011; Juneja and Marmer, 1999; Mercer et al., 2015; Dlusskaya et al., 2011; Pleitner et al., 2012), 2 D-values of strains after overexpression of heat shock proteins (HSP) (Ruan et al., 2011; Hauben et al., 1997), 7 D-values of strains after adaptation to salt or acid stress (Pleitner et al., 2012; Buchanan and Edelson, 1999; Garcia-Hernandez et al., 2015), 5 D-values of LHR positive strains (Pleitner et al., 2012; Mercer et al., 2015), and 2 D-values of strains treated by dry heat (Meetoo et al., 2010; Kim et al., 2015).

Foodborne disease due to *E. coli* has been linked to the consumption of meat and meat products as well as fruits and fresh produce (Greig and Ravel, 2009; Yeni et al., 2015; Frenzen et al., 2005; Karch et al., 2005). Optimized heat treatments for effective microbial decontamination and minimum organoleptic deterioration of foods (Klaiber

et al., 2005; Rajic et al., 2007; Woodward et al., 2002) necessitate knowledge of the heat resistance of target foodborne pathogens as well as factors influencing heat resistance. This review aims to provide an overview of the current knowledge on the mechanisms of heat resistance of *E. coli* to provide novel perspectives on conventional and novel thermal processing of foods. Major mechanisms of heat resistance are active in all strains of *E. coli*; however, relatively few studies elucidated genetic determinants for strain-specific acquisition of heat resistance. A recently identified genomic island termed locus of heat resistance (LHR) substantially increases the heat resistance of about 2% of strains of *E. coli* (Mercer et al., 2015). Where appropriate, *E. coli* will be compared to *Salmonella enterica*, a closely related organism exhibiting comparable resistance to heat.

2.2 Variability of resistance of strains of *E. coli* to heat

The D_{60}-value of *E. coli* K12 is reported as 0.1 to 0.3 min (Chuang et al., 2007; Jin et al., 2008; Dlusskaya et al., 2011); however, a majority of strains of *E. coli* exhibits D_{60}-values exceeding that value by up to 10-fold (**Figure 2-1**). Heat resistance is not related to the phylogenetic group, the serotype, or the virotype of *E. coli* (Mercer et al., 2015; Liu et al., 2015). Highly heat resistant strains of *E. coli* exhibit D_{60} values exceeding 10 min (Dlusskaya et al., 2011; Garcia-Hernandez et al., 2015). Genetic determinants of the variability of heat resistance between strains are only partially understood. An overview of the isogenic mutant strains of *E. coli* and their heat

resistance is shown in **Table 2-1**. Genes that are related to the heat shock response, including the

Table 2-1 Effect of gene disruption or overexpression on heat resistance of *E. coli.*

***Escherichia coli* serotype or strain number**	**Heat (Temp., time)**	**Lethality ($logN/N_0$)**	**Medium / products**	**References**
MC4100 (parental strain) KY1601 (Δ*rpoH*)	57°C, 2 min	<0.1 >3.5	M9 medium	Jenkins et al., 1991
AB1157 (parental strain) JI132 (Δ*sodA sodB* strain)	48°C, 2 h	<0.5 >6	LB broth	Benov and Fridovich, 1995
ATCC 43895 (parental strain) FRIK 816-3 (Δ*rpoS*)	55°C, 7 min	<1 >4	fermented sausage	Cheville et al., 1996
MC4100 MC4100 (Δ*ibpA/B*)	50°C, 4 h	<2 >3	LB broth	Kuczyńska-Wiśnik et al., 2002
W6-13 (parental strain) M4020 (Δ*wca*)	60°C, 5 min	3.3 6.6	minimal glucose broth	Mao et al., 2001
AW1.7 AW1.7 (Δ*cfa*)	60°C, 30 min	2.0 3.1	LB broth	Chen and Gänzle, 2016
MG1655 MG1655 (Δ*cfa*)	57°C, 15 min	1.3 2.2	LB broth	Chen and Gänzle, 2016
BL21 overexpression of IbpA/IbpB	50°C, 30 min	1.5 0.7-0.9	M9 medium	Kitagawa et al., 2000
E. coli W3110 overexpression of EvgA	50°C, 2 h	5 1.5	TY broth	Christ and Chin 2008
GGG10 overexpression of NmpC	60°C, 1 min	3.5 0.5	LB broth	Ruan et al., 2011
AW1.7 (pRK767) AW1.7 ΔpHR1 (pRK767) AW1.7 ΔpHR1 (pLHR)	60°C, 5 min	<1 >8 <1	LB broth	Mercer et al., 2015

LB: Luria-Bertani; TY: Tryptone-yeast extract.

alternative sigma factors σ^H and σ^E, the heat shock proteins IbpA/B, the alternative sigma factor σ^S regulating the general stress response, the oxidative stress response regulated by SodA/B, and genes related to envelope properties including synthase of colanic acid, cyclopropane fatty acids (CFAs), NmpC and EvgA relate to heat resistance (**Table 2-1** and references therein). *E. coli* strains deficient of in σ^H, σ^S, SodA/B, IbpA/B, and colanic acid as well as CFAs were more sensitive to heat compared to their isogenic parental strains. Overexpression of EvgA increased heat resistance (**Table 2-1**). The locus of heat resistance (**Table 2-1**) mediates extreme heat resistance with D_{60}-values of 10 min or higher (**Table 2-1**). The heat resistance of strains of *E. coli* also depends on the food matrix (**Table 2-2** and **Table 2-3**). The resistance of *E. coli* LTH5807 to heating on mung bean, radish, or alfalfa seeds differed substantially (**Table 2-2**). The survival of the LHR-positive *E. coli* AW1.7 in beef patties cooked to 71°C provides further evidence that the heat resistance of *E. coli* depends on the food matrix. Heat treatments that are considered to be lethal to *E. coli* thus may fail to safely eliminate *E. coli* (**Table 2-2** and **Table 2-3**).

Table 2-2 Examples of lethality of *E. coli* strains in food.

***S*erotype or strain number**	**Heat (T/time)**	**Lethality ($logN_0/N$)**	**Medium /products**	**References**
LTH5807 (O157:H^-; stx^-)	60°C, 10 min 60°C, 3 min 60°C, 4 min	>7.2 >7.2 5.9	mung bean radish alfalfa	Weiss and Hammes, 2005
204P (O157:H7)	50°C, 300 min 55°C, 30 min	3-5 2-4	pork sausage (7-30% fat)	Ahmed et al., 1995
AW1.7 AW1.7 ΔpHR1 GGG10	Internal 63/71°C	3-5#/3.5 4-7#/5 4.5/UDL	beef patties	Liu et al., 2015
MG1655 (K12), LMM1030	Internal 63°C	5-6#	beef patties	Liu et al., 2015
O26, O104, O111, O121, and O157	Internal 63°C	2-NC	beef patties	Liu et al., 2015
O26, O104, and O121	Internal 71°C	6-NC	beef patties	Liu et al., 2015
O157:H7 (VTEC) Non-O157 (VTEC)	Internal 49-71°C	3.2-4.1 2.5-4.5	beef steaks^	Luchansky et al., 2012
8- strain VTEC cocktail**	191.5°C, ≤1.25 min 1.5-2.5 min	1.6-5.1 UDL	single cubed beef steaks	Swartz et al., 2015
8- strain VTEC cocktail**	≤3.0 min 3.5 min	0.8-5.3 UDL	double cubed beef steaks	Swartz et al., 2015

UDL: cell counts after treatment were under detection limit.

NC: no surviving cells after enrichment.

#Reductions depend on fat content from 15% to 35% in ground beef.

^Thickness of beef steaks is 2.54 or 3.81 cm; initial cell counts are around 5.50 cfu/g.

**Temperature is the surface temperature; cooking time refers to the time per side; initial cell counts are around 6.3-6.8 cfu/g.

Table 2-3 Examples of *D* values of *E. coli* strains in food.

***S*erotype or strain number**	**Temperature**	***D* value (min)**	**Medium /products**	**References**
O157:H7 E0139 SEA 13B88	57°C	8.2/9.1 6.2/7.9	Cantaloupe/watermelon juice	Sharma et al., 2005
Heat resistant strains of 7 VTEC serotypes (O26, O45, O103, O111, O121, O145 and O157)	56°C 60°C 62°C	2.1-4.5 0.4-1.0 0.2-0.5	apple juice	Enache et al., 2011
ATCC25922	55°C	10.9	goat milk	Pereira et al., 2006
380-94 (O157:H7)	58°C 60°C 62°C	14.4 6.1 2.5	postfermented pepperoni	Riordan et al., 2000
4-strains cocktail of EDL-931, A 9218-C1, 45753-35, 933 (all are O157:H7)	55°C 60°C 65°C	11.5-12.0 1.9-2.0 0.3-0.4	Ground turkey, lamb and pork	Juneja and Marmer, 1999

2.3 Mechanisms related to outer membrane and membrane fluidity.

Cell surface structures and appendages provide the first line of defense to environmental stress. An overview of heat stress responses related to cell membranes and the periplasm is provided in **Figure 2-2**. Most strains of *E. coli* secrete extracellular polysaccharides, including colanic acid, which forms a thick mucoid matrix on the cell surface (Whitfield and Valvano, 1993; Mao et al., 2001). A colanic acid-deficient mutant of *E. coli* M4020, obtained by insertional disruption of the *wsc* genes required

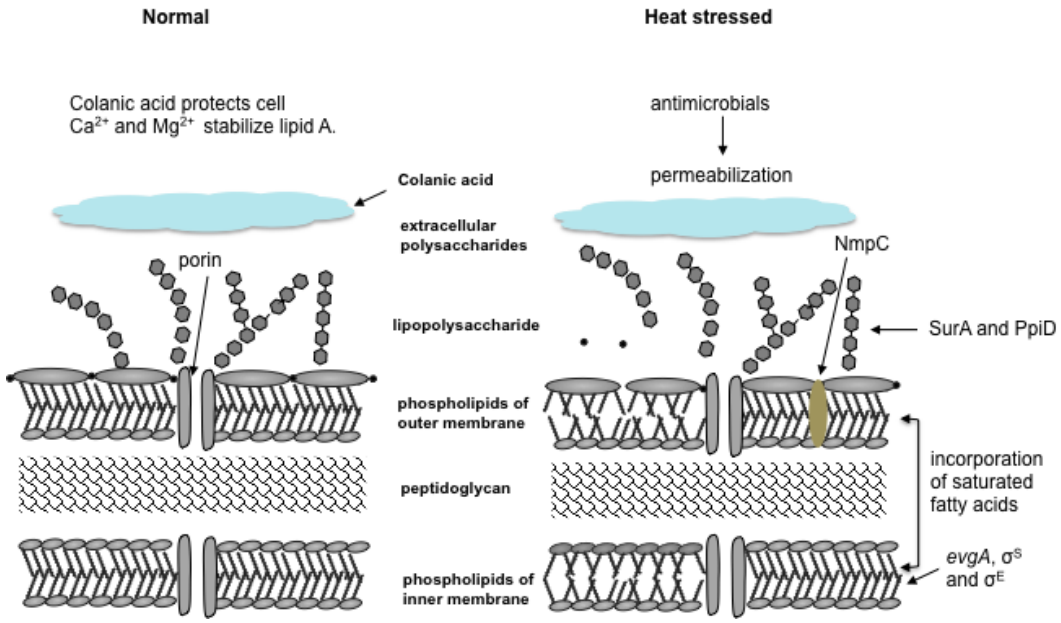

Figure 2-2 Heat effects on cell membranes and attributes to heat resistance of *E. coli.* Extracellular polysaccharides including colanic acid forms a thick mucoid matrix on cell surfaces and provide protection of cells (Whitfield and Valvano, 1993; Mao et al., 2001). Lipopolysaccharide is a barrier to prevent rapid penetration of hydrophobic molecules, and is stabilized by divalent cations Mg^{2+} and Ca^{2+} against heat or pressure stress (Hitchener and Egan, 1977; Vaara, 1992; Hauben et al., 1998; Gayán et al., 2013; Chapter 4). The solute transport proteins and the outer membrane porin NmpC contribute to heat resistance of *E. coli* AW1.7 (Ruan et al., 2011). Addition of antimicrobials including chitosan decreased the heat resistance due to the more profound permeability of outer membrane (Liu, 2015). A master transcriptional regulator *evgA* increased heat resistance due to that it activates genes involved in periplasmic functions (Christ and Chin 2008). Cytoplasmic proteins σ^S and σ^E also influence the properties of cell envelope (Lange and Hengge-Aronis, 1991; Bukau, 1993). Lipopolysaccharide proteins SurA and PpiD lead to overall reduction in the level and folding of outer membrane proteins, consequently induce the periplamic heat shock response (Missiakas et al., 1996; Dartigalongue and Raina, 1998). Incorporating more saturated fatty acids such as palmitic acid and cyclopropane fatty acids into membrane lipids antagonizes the heat-induced increase in fluidity and achieves an ideal physical state of membrane (Katsui et al. 1981; Ruan et al., 2011; Chen and Gänzle, 2016). Disruption of *cfa* coding for CFA synthase induced accumulation of the unsaturated fatty acid in membrane lipids, consequently reducing the heat resistance of them (Chen and Gänzle, 2016).

for colanic acid biosynthesis, was less tolerant to exposure to 55 and 60 °C than its parental strain *E. coli* O157:H7 W6-13 (**Table 2-1**), indicating that colanic acid confers

heat resistance to *E. coli* O157:H7 (**Figure 2-2**) (Mao et al., 2001). Lipopolysaccharide (LPS) serves as a barrier to prevent rapid penetration of hydrophobic molecules, and is stabilized by divalent cations, particularly Mg^{2+} and Ca^{2+} (**Figure 2-2**) (Hitchener and Egan, 1977; Vaara, 1992; Hauben et al., 1998; Gayán et al., 2013; Chapter 4). Expression of the outer membrane porin NmpC increased survival of *E. coli* GGG10 at 60°C by 50- to 1,000-fold (**Figure 2-2**) (Ruan et al., 2011). The outer membrane permeabilizing polysaccharide chitosan decreased the heat resistance of *E. coli* in apple juice at 60 °C (Liu, 2015). The pronounced effect of chitosan on heat resistance occurred on EHEC when combined with rutin or resveratrol in beef patties, due to the greater bacterial destruction from outer membrane to cytoplasmic membrane (Nair et al., 2016).

The fluidity of the membrane influences its function (Zhang and Rock 2008). The adjustment of membrane lipid composition and membrane fluidity by homoviscous adaptation is a major contributor to the bacterial resistance to heat stress (Sinensky 1974; Arneborg et al. 1993; Yuk and Marshall 2003; Denich et al. 2003; Yoon et al. 2015). Many of the adaptive systems responding to heat stress in *E. coli* contribute to the stabilization of membrane-bound enzymes, and affect physical properties of the cytoplasmic membrane (Torok et al. 1997; Beney and Gervais, 2001). Remarkably, heat resistance induced by slow heating of *E. coli* was related to adaptation of the membrane fluidity rather than protein synthesis (Guyot et al., 2010). Heat-adaptation increased the heat resistance of *E. coli* strains by the maintenance of the membrane in

the liquid-crystalline state. The incorporation of saturated fatty acids into membrane lipids reduces membrane fluidity (Nakayama et al. 1980; Katsui et al. 1981) and consequently antagonizes the heat-induced increase in fluidity (**Figure 2-2**) (Quinn 1981; De Mendoza et al., 1983; Suutari and Laakso 1994; Mejía et al., 1995; Yuk and Marshall 2003). The heat resistant *E. coli* AW1.7 was characterized by a higher proportion of saturated and cyclopropane fatty acids (CFAs) in the cytoplasmic membrane when compared to heat sensitive strains of *E. coli* (**Figure 2-2**) (Ruan et al., 2011). A contribution of cyclopropane fatty acids to heat resistance of *E. coli* was confirmed by disruption of *cfa* coding for CFA synthase (Chen and Gänzle, 2016). The *cfa* deficient derivatives of *E. coli* AW1.7 and MG1655 did not produce cyclopropane fatty acids; the unsaturated fatty acids C16:1 and C18:1 replaced cyclopropane fatty acids in the membrane lipids and the mutant strain was less resistant to heat when compared to the parent strains (**Figure 2-2**) (Chen and Gänzle, 2016).

2.4 Regulation of heat response by EvgA, heat shock proteins, and σ^E

Cytoplasmic mechanisms of heat resistance relate to the effect of heat shock proteins and compatible solutes on protein folding, and to oxidative stress (**Figure 2-3**). The regulation of the heat shock response of *E. coli* is governed by the two alternative sigma factors σ^H and σ^E (**Figure 2-3**A). The heat shock response is induced by temperatures around the growth / no-growth interface which aggravate protein misfolding but permit gene expression and protein synthesis (Govers et al., 2014; Lindner et al., 2008; Winkler et al., 2012; Lee et al., 2016). σ^H and σ^E are encoded by

rpoH and *rpoE*, regulate transcription of heat-shock regulons coping with protein misfolding in the cytoplasm and the periplasm, respectively, and mediate cytoplasmic stress and envelope stress responses (Bukau, 1993). Heat shock proteins including chaperones and proteases function by holding partially unfolded proteins to prevent aggregation of heat-denatured proteins, and disaggregation of denatured proteins to allow refolding or proteolytic degradation (Parsell and Lindquist, 1993; Landini et al., 2014; Lee et al., 2016). The small heat shock proteins IbpA and IbpB are holdases; DnaK, DnaJ, GrpE facilitate protein folding during translation, and guide aggregated proteins to the disaggregase ClpB. ClpP and other heat-shock proteases degrade aggregated proteins. The expression of heat shock proteins is induced by σ^H under sublethal heat stress and increases heat resistance of *E. coli* (Arsène et al., 2000). A σ^H deletion in *E. coli* eliminated synthesis of heat shock proteins including DnaK, GroEL and HtpG and the resulting strain was very sensitive to exposure to 57^{o}C (**Table 2-1**). Starvation significantly enhanced the heat resistance of this strain (Jenkins et al., 1991). Small heat shock proteins prevent protein aggregation by heat (Jakob et al., 1993; Lee et al., 1997; Kitagawa et al., 2000; Mogk et al., 2003). Overexpression of IbpA and IbpB increased resistance not only to heat but also to superoxide (Kitagawa et al., 2000; **Table 2-1**). Small heat shock proteins IbpA and IbpB prevent the aggregation of denatured endogenous proteins (Laskowska et al., 1996; Veinger et al., 1998; Kuczyńska-Wiśnik et al., 2002). The DnaK system also prevented protein aggregation induced by heat. This disaggregation is more efficient when DnaK acts in concert with

ClpB (Mogk et al., 1999, 2003). However, disruption of *clpA*, *htpG*, and *ibp* in *E. coli* did not affect the viability at 50°C (Thomas and Baneyx, 1998). The pressure resistant strains *E. coli* LMM1010, LMM1020, and LMM1030 exhibit an increased basal expression of heat shock proteins including DnaK, Lon, and ClpX; this increased expression may also account for the moderate increase of heat resistance of these strains (Aertsen 2004, Hauben 1997). Overall, the inducible heat shock response is a key contributor for growth of *E. coli* at temperature exceeding the optimum temperature of growth, but it makes only a modest contribution to the strain-specific differences of the resistance to lethal heat challenge.

Four key proteins are involved in the regulation of σ^E-dependent envelope stress response, including RseA, RseB, DegS, and Yael (Alba and Gross, 2004). The activity of σ^E is modulated by the expression of outer membrane proteins and outer membrane proteins induce σ^E activity (Mecsas et al., 1993). Moreover, deletions of lipopolysaccharide proteins SurA and PpiD lead to an overall reduction in the level and folding of outer membrane proteins, and to the induction of the periplasmic heat shock response (**Figure 2-2**) (Missiakas et al., 1996; Dartigalongue and Raina, 1998).

A master transcriptional regulator *evgA* activates genes involved in periplasmic functions, as well as in membrane and permeability functions. Its overexpression significantly increases heat resistance of *E. coli* (Christ and Chin, 2008; **Table 2-1**; **Figure 2-2**). The response regulator EvgA is part of a two-component regulatory system with sensor kinase EvgS, binding the intergenic region of *evgAS* and *emrKY*

coding for efflux pump, and regulating the expression of both operons (Kato et al., 2000). Comparison of the genome-wide transcription profile of EvgA-overexpressing and EvgA-lacking strains revealed that EvgA conferred acid resistance to *E. coli* (Masuda and Church, 2002). EvgA controls the expression of a wide range of genes, including *gadABC*, *hdeAB*, *emrKY*, *yhiUV*, and *yfdX* which are related to acid resistance, osmotic adaptation, drug resistance and other functions (Nishino et al., 2003).

2.5 Regulation of heat resistance by σ^S, and cross-resistance to acid, oxidative, and high pressure stress

Stationary phase cells are generally more resistant than exponential phase cells, mainly because of the increased expression of σ^S (**Figure 2-3**A) (Kaur et al., 1998; Cheville et al., 1996). The σ^S regulon contributes to the general stress response and increase acid, heat, and / or osmotic resistance of *E. coli* (Allen et al., 2008, Cheville et al., 1996; Hengge-Aronis, 2002, Landini et al., 2014; Robey 2001). Adaptation to acid stress provides cross-protection to heat stress (Ryu and Beuchat, 1998; Buchanan and Edelson, 1999; Ryu and Beuchat, 1999; Mazzotta, 2001; Yuk and Marshall, 2003). For example, adaptation of enterohemorrhagic *E. coli* to pH 4.6 increased its heat resistance at 58°C 2-4 fold when compared to cells grown at pH 7.0 (Buchanan and Edelson,

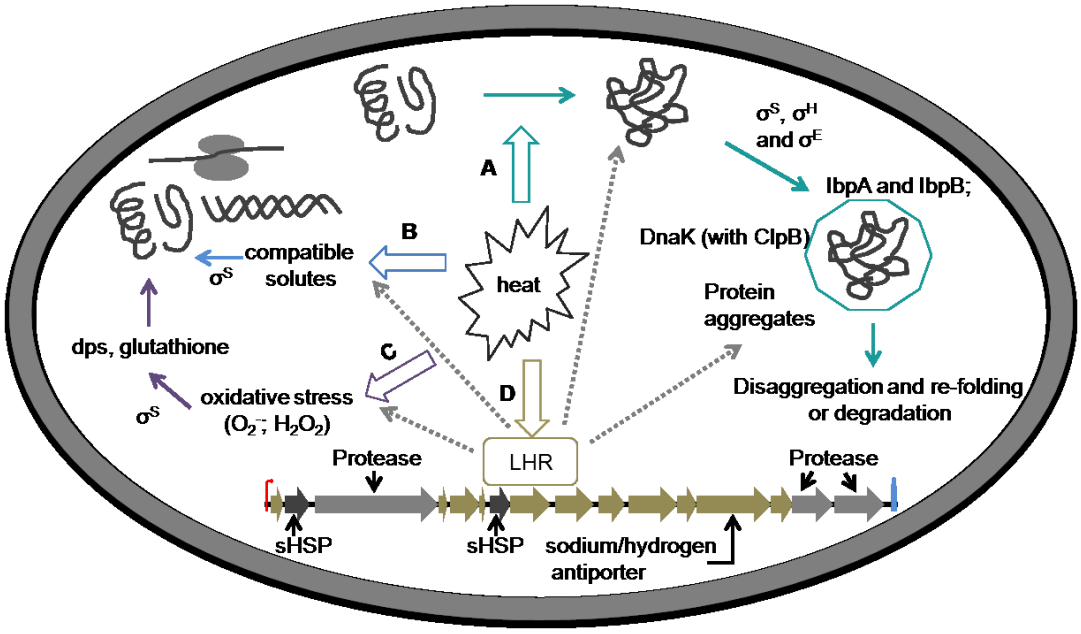

Figure 2-3 Cytoplasmic determinants of heat resistance in *E. coli.*A: Preventions of protein aggregation. Heat enhanced misfolding of proteins, consequently induce aggregations. General stress response factors σ^S, σ^H and σ^E, as well as some small heat shock proteins can suppress protein aggregation (Parsell and Lindquist, 1993; Landini et al., 2014). Small heat shock proteins IbpA and IbpB bind to misfolded proteins and thus contribute to disaggregation of during sublethal heat shock (Laskowska et al., 1996; Veinger et al., 1998; Kuczyńska-Wiśnik et al., 2002). The DnaK system acting together with ClpB prevents proteins aggregation induced by heat (Mogk et al., 1999, 2003). B: Compatible solutes accumulation induced by salt contributes to heat resistance through overcoming osmotic stress and stabilizing ribosomes (Ramos et al., 1997; Lamosa et al., 2000; Pleitner et al., 2012). Accumulation of amino acids including glycine betaine and proline as major cytoplasmic solutes, and the accumulation of carbohydrates including glucose and trehalose occurred in response to the addition of NaCl in *E. coli*, resulting in increased thermal stability of ribosomes during heat treatment (Pleitner et al., 2012). Mannosylglycerate and diglyerol phosphate protect proteins during heat treatment (Ramos et al., 1997; Lamosa et al., 2000). C: Mitigation of oxidative stress. Accumulation of oxidative stress induced by heat damages intracellular components including proteins, ribosomes and DNA. The general stress response factor σ^S and the DNA binding protein dps acts against oxidative stress (Landini et al., 2014; Choi et al., 2000). Pyruvate and catalase contribute to recovery of sublethally injured cells after heat treatments (Czechowicz et al., 1996; Mizunoe et al., 2000). D: Regulation of the locus of heat resistant (LHR). LHR was a recently identified unique set of genes contributing to extreme heat resistance in *E. coli* (Mercer et al., 2015). LHR contains 16 predicted open reading frames (ORF) encoding small heat shock proteins (sHSP, Orf2 and Orf7), hypothetical proteins yfdX family (Orf8 and Orf9), proteases (Orf3, Orf15 and Orf16), thioredoxin (Orf12), and sodium/hydrogen antiporters (Orf13), accordingly

contributing to heat shock response, osmotic stress response, turnover of misfolded or disaggregation proteins, oxidative stress response, osmotic and heat stress response, respectively (Mercer et al., 2015; Lee et al., 2016). Predicted functions of LHR are linked with dash lines.

1999). Induction of acid resistance in *E. coli* O157:H7 increases levels of cyclopropane fatty acids in the cytoplasmic membrane (Brown et al., 1997), which stabilize cells against several environmental stressors including heat (Grogan and Cronan, 1997; Chen and Gänzle, 2016). Moreover, σ^S dependent gene expression increased the heat resistance of *E. coli* O157:H7 after adaptation to temperatures above the optimum growth temperature (Cheville et al., 1996; Yuk and Marshall, 2003; **Table 2-1**). Starvation of *E. coli* O157:H7 substantially increased D_{52}-values; this enhanced heat resistance was related to the expression of starvation-induced proteins UspA and GrpE (Zhang and Griffiths, 2003).

Heat induces production of O_2^- in *E. coli* under aerobic conditions, possibly by disruption of the electron transport systems of the membrane, and consequently induces the manganese-containing superoxide dismutase (Privalle and Fridovich, 1987). Accumulation of reactive oxygen species after exposure to sublethal stress results in lethal damage to DNA, RNA, proteins, and lipids (Aertsen et al., 2005; Aldsworth et al., 1999; Cabiscol et al., 2000). The general stress response factor σ^S also protects against oxidative stress (**Figure 2-3**C) (Landini et al., 2014). The σ^S-regulated DNA binding protein dps binds DNA as homo-dodecamer and prevents DNA damage by oxidative stress or low pH (Choi et al., 2000). The synthesis of cyclopropane fatty acids in *E. coli* also increases resistance to oxidative stress (Grogan and Cronan, 1997). Proteins that

alter the resistance of *E. coli* to pressure-induced oxidative stress, including systems for thiol-disulfide redox homeostasis and proteins containing iron-sulfur clusters, probably also contribute against oxidative stress induced by heat (Malone et al., 2006; Charoenwong et al., 2011; Imlay, 2013; Gänzle and Liu, 2015).

Oxidative stress induced by sublethal thermal damage may also account for the phenomenon termed "viable but nonculturable state" (VBNC). VBNC cells cannot be detected by standard culture techniques but can be resuscitated under favorable conditions (Bogosian et al., 2000; Gupte et al., 2003; Morishige et al., 2013). Addition of sodium pyruvate recovered cells of *E coli* after heat-induced sublethal injury. This protective effect was related to the ability of pyruvate to degrade hydrogen peroxide (Czechowicz et al., 1996; Mizunoe et al., 2000). Addition of sodium pyruvate or catalase to medium agar also resuscitated VBNC *Salmonella* Enteritidis or *Vibrio vulnificus* cells, respectively, which had become sensitive to hydrogen peroxide (Bogosian et al., 2000; Morishige et al., 2013).

2.6 Effects of salt or sugar addition in high moisture foods

The water activity of a food product and particularly the salt content influence the heat resistance of *E. coli*. *E. coli* responds to an increase of the osmotic pressure by accumulation or synthesis of compatible solutes, small organic solutes that balance the osmotic pressure without interfering with cytoplasmic functions (Kempf et al., 1998). High cytoplasmic concentrations of compatible solutes increase heat resistance of *E. coli* and other bacterial cells by stabilizing ribosomes and proteins through a

mechanisms referred to as “preferential hydration” (**Figure 2-3**B) (Ramos et al., 1997; Lamosa et al., 2000; Pleitner et al., 2012). A reduction in water activity from 0.995 to levels between 0.98 and 0.96 in salt or sucrose solutions significantly enhanced the heat resistance of *E. coli* (Kaur et al., 1998). The heat resistance of several strains of *E. coli* was also increased by the addition of 2 – 6% of NaCl (Garcia-Hernandez et al., 2015). Addition of 2% NaCl resulted in the accumulation of amino acids, including glycine betaine and proline as major cytoplasmic solutes; accumulation of carbohydrates, including glucose and trehalose occurred in response to the addition of 6% NaCl (Pleitner et al., 2012). The accumulation of solutes corresponded to an increased heat resistance of *E. coli,* and a higher thermal stability of ribosomes (Pleitner et al., 2012). The effect of NaCl addition on solute accumulation and heat resistance of *E. coli* is observed at concentrations that are typical for food systems. A critical concentration of NaCl in ground beef, about 2.7-4.7%, substantially increased heat resistance of *E. coli* O157:H7 at 55-62.5°C (Juneja et al., 2015). In addition, pre-exposure to 5% NaCl at room temperature for 24 h increased the heat resistance of *E. coli* O157:H7 at 55°C (Bae and Lee, 2010).

The effect of the fat content on heat resistance of *E. coli* is controversial. An increased fat content in food products increased the heat resistance of *E. coli* in some studies (Line et al., 1991; Huang et al., 1992; Ahmed et al., 1995; Smith et al., 2001; Liu et al., 2015), while other studies reported decreased resistance, no effect, or strain-specific effects (Liu et al., 2015; Kotrola and Conner, 1997; Vasan et al., 2014).

The potential direct effects of fat on heat resistance of *E. coli* are confounded by the strong effect of fat on heat transfer in solid foods. Reduced heat transfer increases the heating times to a certain target temperature and thus profoundly affects process lethality.

2.7 Locus of heat resistance (LHR) and extreme resistance to heat.

Extreme heat resistance of *E. coli* is conferred by the locus of heat resistance (LHR) (**Figure 2-3**D, Mercer et al., 2015). The LHR is a genomic island of about 14 kbp which encodes for 16 genes; 6 of these genes are unique to heat resistant strains of *E. coli* (Mercer et al., 2015). Acquisition of the LHR increases survival after exposure to 60°C for 5 min by more than 7 log(cfu/mL); the LHR is thus one of the most powerful mediators of heat resistance in *E. coli* (**Table 2-1**; Mercer et al., 2015). Loss of the LHR also reduces the pressure resistance in *E. coli* AW1.7 (Garcia-Hernandez et al., 2015; Liu et al., 2015; Mercer et al., 2015). Remarkably, the presence of a truncated LHR in wild type strains of *E. coli*, or cloning of fragments of the LHR had little effect on heat resistance, indicating that the 16 genes act in concert to provide heat resistance in LHR-positive strains (Mercer et al., 2015). A genomic island with high similarity to the LHR, the *Pseudomonas aeruginosa* clone C-specific genomic island (PACGI-1) was characterized in *Pseudomonas* (Lee et al., 2015).

The 16 predicted open reading frames (ORF) within LHR encode small heat shock proteins (Orf2 and Orf7), proteins of the YfdX family with unknown function (Orf8 and Orf9), heat shock proteases (Orf3, Orf15 and Orf16), thioredoxin (Orf12), and a

sodium/hydrogen antiporter (Orf13) (Mercer et al., 2015). According to the predicted function of proteins encoded by the LHR, the genomic island may thus contribute to the turnover of misfolded or aggregated proteins, the osmotic stress response, and mitigate oxidative stress (Mercer et al., 2015). The contribution of genes encoded by the LHR to protein folding and protein turnover was confirmed in the homologous gene cluster PACGI-1 in *P. aeruginosa* (Lee et al., 2015). The small heat shock proteins sHsp20c and $ClpG_{GI}$ contribute to thermotolerance in *P. aeruginosa* through their function as holdases and disaggregating chaperones (Lee et al., 2015 and 2016). Cloning of the homologous LHR proteins in *E. coli*, however, had no influence on the heat resistance in *E. coli* (Mercer et al., 2015), demonstrating that the effect of LHR-encoded genes is species specific, and that extreme heat resistance in *E. coli* necessitates heat shock proteins acting in concert with other biochemical functions.

2.8 Heat resistance of desiccated *E. coli*.

Desiccated strains of *E. coli* and *Salmonella* are characterized by extreme resistance to physical and chemical stressors including heat (Beuchat and Scouten, 2002; Studer et al., 2013; Beuchat et al., 2013; Syamaladevi et al., 2016). Parameters for the heat inactivation of dry bacterial cells are comparable to the moist heat inactivation of bacterial endospores rather than pasteurization (Podolak et al., 2010; Brandl et al., 2008; Du et al., 2010). Hot air roasting of almonds even at very high temperature (130-150 °C) results in less than a 4 log (cfu/g) reduction of *Salmonella* on almonds (Yang et al., 2010). Similarly a 2 log (cfu/g) reduction of *Salmonella* on dry

alfalfa seeds required 10 d of treatment at 60 °C; an equivalent bactericidal effect was achieved after 5 min of treatment with wet heat at 60°C (Neetoo and Chen, 2011; Jaquette et al., 1996).

Mechanisms of dry heat resistance are best understood for *Salmonella* (Podolak et al., 2010; Finn et al., 2013). The heat resistance of *Salmonella* at 75 °C in meat and bone meal was higher at a_W 0.77 than at a_W 0.88 (Riemann, 1968). Comparable to the effect of NaCl in high-moisture foods, the heat resistance of dry cells is related to the intracellular concentration of compatible solutes, including K^+, glutamate and trehalose. The up-regulation of σ^S, σ^E, fatty acid catabolism, and formations of Fe-S clusters and filaments also contribute to the resistance to dry conditions (Finn et al., 2013). It was speculated that the extent and strength of the vibration of water molecules in dry bacteria are limited substantially because of the very low water contents. The low water content thus prevents denaturation of cytoplasmic and membrane proteins even at very high temperatures (Archer et al., 1998; Earnshaw et al., 1995). This mechanism was proposed in analogy to bacterial endospores, where the decreased core water reduces the amount of water associated with proteins, thus preventing thermal denaturation (Nicholson et al., 2000). Desiccation of bacterial cells may also stabilize ribosomal units (Syamaladevi et al., 2016).

Several studies demonstrate that concepts and mechanisms that were identified in *Salmonella* are also relevant in *E. coli*. Desiccated verotoxigenic *E. coli* (VTEC) survived at 70°C for 5 h, thus exhibiting almost the same level of heat resistance as

Salmonella (Hiramatsu et al., 2005). The lethality of treatments of radish seeds at 60 °C against *E. coli* O157:H7 increased as the a_W increased from 0.25 to 0.65 and 1.0 (Kim et al., 2015). However, information on the dry heat resistance of *E. coli* remains limited when compared to the information on the wet heat resistance of the organisms.

2.9 Conclusion

The resistance of *E. coli* strains to heat intervention treatments has been widely evaluated in the past decades, particularly using strains of *E. coli* O157: H7. Although *E. coli* has been considered as a relatively heat sensitive organisms, the D_{60} values of some strains of *E. coli* are increased to several minutes or even hours by the heat shock response, adaptation to salt or acid stress, acquisition of the LHR, or desiccation. About 2% of *E. coli* including food isolates and pathogens harbor the LHR and exhibit extreme resistance to wet heat. The biochemical function of the LHR links to proteins aggregation and folding as well as thiol- and ion homeostasis, however, the mechanisms of LHR mediated heat resistance are only partially understood. Current pathogen intervention methods or cooking recommendations may not suffice to control these highly heat resistant strains of *E. coli*. Additional hurdles need therefore to be developed to assure the inactivation of highly heat resistant strains. Further evaluations on inactivation of heat resistant strains under improved heat interventions and mechanisms of heat resistance allow us to design more effective applications in food industry.

2.10 References

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

Aertsen, A., Vanoirbeek, K., Spiegeleer, P.D., Sermon, J., Hauben, K., Farewell, A., Nyström, T., and Michiels, C.W. (2004). Heat shock protein-mediated resistance to high hydrostatic pressure in *Escherichia coli*. *Appl. Environ. Microbiol.* 70, 2660-2666.

Ahmed, N.M., Conner, D.E. and Huffman, D.L. (1995) Heat resistance of *Escherichia coli* O157:H7 in meat and poultry as affected by product composition. *J. Food Sci.* 60, 606-610.

Alba, B.M., and Gross, C.A. (2004). Regulation of the *Escherichia coli* sigma-dependent envelope stress response. *Mol. Micribiol*. 52, 613-619.

Aldsworth, T.G., Sharman, R.L., and Dodd, C.E. (1999). Bacterial suicide through stress. *Cell. Mol. Life Sci*. 56, 378-383.

Allen, K.J., D. Lepp, R.C. McKellar, and M.W. Griffiths. (2008). Examination of stress and virulence gene expression in *Escherichia coli* O157:H7 using targeted microarray analysis. *Foodborne Pathog. Dis.* 5, 437-447.

Archer, J., Jervis, E.T., Bird, J., and Gaze, J.E. (1998). Heat resistance of *Salmonella weltevreden* in low-moisture environments. J. Food Prot. 61:969-973.

Arneborg, N., Salskov-Iversen, A.S., and Mathiasen, T.E. (1993). The effect of growth rate and other growth conditions on the lipid composition of *Escherichia coli*. *Appl. Microbiol. Biotech.* 39, 353-357.

Arsène, F., Tomoyasu, T., and Bukau. B. (2000). The heat shock response of *Escherichia coli*. *Int. J. Food. Microbiol*. 55, 3-9.

Bae, Y., Lee, S. (2010). Effect of pre-exposure to sodium chloride on the resistance of pathogens to thermal and acid stresses. *J. Food Safety*. 30, 1016-1025.

Baird-Parker, A. C., Boothroyd, M. and Jones, E. (1970), The effect of water activity on the heat resistance of heat sensitive and heat resistant strains of Salmonellae. *J. Appl. Microbiol.* 33, 515-522.

Beney, L., and Gervais, P. (2001). Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses. *Appl. Microbiol. Biotechnol*. 57, 34-42.

Benov, L., and Fridovich, I. (1995). Superoxide dismutase protects against aerobic heat shock in *Escherichia coli*. *J. Bacteriol*. 177, 3344-3346.

Beuchat, L.R., Komitopoulou, E., Beckers, H., Betts, R.P., Bourdichon, F., Fanning, S., Joosten, H.M., and Kuile, H.T. (2013). Low-water activity foods: increased concern as vehicles of foodborne pathogens. *J. Food Prot. 76*, 150-172.

Beuchat, L.R., and Scouten, A.J. (2002). Combined effects of water activity, temperature and chemical treatments on the survival of Salmonella and *Escherichia coli* O157:H7 on alfalfa seeds. *J. Appl. Microbiol*. 92,382-95.

Bogosian, G., Aardema, N.D., Bourneuf, E.V., Morris, P.J.L., and O'Neil, J.P. (2000). Recovery of hydrogen peroxide-sensitive culturable cells of *Vibrio vulnificus* gives the appearance of resuscitation from a viable but nonculturable state. *J. Bacteriol*. 182, 5070-5075.

Brandl, M.T., Pan, Z., Huynh, S., Zhu, Y., and McHugh, T.H. (2008). Reduction of *Salmonella* Enteritidis population sizes on almond kernels with infrared heat. *J. Food Prot*. 71, 897-902.

Brown, J.L., Ross, T., McMeekin, T.A.,and Nichols, P.D. (1997). Acid habituation of *Escherichia coli* and the potential role of cyclopropane fatty acids in low pH tolerance. *Int. J. Food Microbiol*. 37, 163-173.

Buchanan, R.L., and Edelson, S.G. (1999). Effect of pH-dependent, stationary phase acid resistance on the thermal tolerance of *Escherichia coli* O157:H7. *Food Microbiol*. 16, 447-458.

Bukau, B. (1993). Regulation of the *Escherichia coli* heat-shock response. *Mol. Microbiol.* 9,671-680.

Cabiscol, E., Tamarit, J., and Ros, J. (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int. Microbiol*. 3, 3-8.

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

Chen Y.Y., and Gänzle, M.G. (2016). Influence of cyclopropane fatty acids on heat, high pressure, acid and oxidative resistance in *Escherichia coli*. *Int. J. Food Microbiol*. 222, 16-22.

Cheville, A.M., Arnold, W., Buchrieser, C., Cheng, M.M., and Kaspar, C.W. (1996). rpoS Regulation of acid, heat, and salt tolerance in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol*. 62, 1822-1824.

Choi, S.H., Baumler, D.J., and Kaspar, C.W. (2000). Contribution of *dps* to acid stress tolerance and oxidative stress tolerance in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol*. 66, 3911-3916.

Christ, D., and Chin, J.W. (2008). Engineering *Escherichia coli* heat-resistance by synthetic gene amplification. *Prot. Eng. Design Select*. 21, 121-125.

Chung, H.J., Wang, S. and Tang, J. (2007). Influence of heat transfer with tube methods on measured thermal inactivation parameters for *Escherichia coli*. *J. Food Prot.* 70, 851-859.

Czechowicz, S.M., Santos, O., Zottola, E.A. (1996). Recovery of thermally-stressed *Escherichia coli* O157:H7 by media supplemented with pyruvate. *Int. J. Food Microbiol*. 33, 275-284.

Dartigalongue, C., and Raina, S. (1998). A new heat-shock gene, ppiD, encodes a peptidyl-prolyl isomerase required for folding of outer membrane proteins in *Escherichia coli*. *EMBO J*. 17, 3968-3980.

De Mendoza, D., and Cronan Jr., J.E. (1983). Thermal regulation of membrane lipid fluidity in bacteria. *Trends Biochem. Sci*. 8, 49-52.

Denich, T.J., Beaudette, L.A., Lee, H., and Trevors, J.T. (2003). Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. *J. Microbiol. Methods* 52, 149-182.

Dlusskaya, E.A., McMullen, L.M., and Gänzle, M.G. (2011). Characterization of an extremely heat-resistant *Escherichia coli* obtained from a beef processing facility. *J. Appl. Microbiol*. 110, 840-849.

Doyle, M.P., and Beuchat, L.R. (2013). *Food microbiology: fundamentals and frontiers*. New York: American Society for Microbiology Press.

Du, W., Abd, S.J., McCarthy, K.L., and Harris, L.J. (2010). Reduction of *Salmonella* on inoculated almonds exposed to hot oil. *J. Food Prot*. 73, 1238-1246.

Earnshaw, R.G., Appleyard, J., and Hurst, R.M. (1995). Understanding physical inactivation processes: combined preservation opportunities using heat, ultrasound and pressure. *Int. J. Food Microbiol*. 28, 197-219.

Enache, E., Mathusa, E.C., Elliott, P.H., Black, D.G., Chen, Y., Scott, V.N., et al. (2011). Thermal resistance parameters for shiga toxin-producing *Escherichia coli* in apple juice. *J. Food Prot.* 74, 1231-1237.

Finn, S., Condell, O., McClure, P., Amézquita, A., and Fanning, S. (2013). Mechanisms of survival, responses and sources of *Salmonella* in low-moisture environments. *Front. Microbiol.* 4, 331.

Frenzen, P.D., Drake, A., Angulo, F.J., and the Emerging Infections Program Foodnet Working Group. (2005). Ecomomic cost of illness due to *Escherichia coli* O157 infections in the United States. *J. Food Prot.* 68, 2623-2630.

Gänzle, M.G., and Liu, Y. (2015). Mechanisms of pressure-mediated cell death and injury in *Escherichia coli*: from fundamentals to food applications. *Front. Microbiol*. 6, 599.

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

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

Greig, J.D., Ravel, A. (2009). Analysis of foodborne outbreak data reported internationally for source attribution. *Int. J. Food Microbiol*. 31, 77-87.

Grogan, D.W., and Cronan Jr., J.E. (1997). Cyclopropane ring formation in membrane lipids of bacteria. *Microbiol. Mol. Biol. Rev*. 61, 429-441.

Gupte, A.R., De Rezende, C.L.E., Joseph, S. W. (2003). Induction and resuscitation of viable but nonculturable *Salmonella enterica* serovar Typhimurium DT104. *Appl. Environ. Microbiol.* 69, 6669-6675.

Guyot, S., Pottier, L., Ferret, E., Gal, L., Gervais, P. (2010). Physiological responses of *Escherichia coli* exposed to different heat-stress kinetics. *Arch. Microbiol.* 192, 651-661.

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

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

Hengge-Aronis, R. (2002). Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol. Mol. Biol. Rev.* 66, 373-395.

Hengge-Aronis, R., Klein, W., Lange, R., Rimmele, and M., Boos, W. (1991) Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rpoS* and are involved in stationary-phase thermotolerance in *Escherichia coli*, *J. Bacteriol*. 173, 7918-7924.

Hiramatsu, R., Matsumoto, M., Sakae, K., and Miyazaki, Y. (2005). Ability of shiga toxin-producing *Escherichia coli* and *Salmonella* spp. to survive in a desiccation model system and in dry foods. *Appl. Environ. Microbiol*. 71, 6657-6663.

Hitchener, B.J., and Egan, A.F. (1977). Outer-membrane damage in sublethally heated *Escherichia coli* K-12. *Can. L. Microbiol*. 23, 311-318.

Huang, I.D., Yousef, A.E., Marth, E.H. and Mathew, M.E. (1992). Thermal inactivation of *Listeria monocytogenes* in chicken gravy. *J. Food Prot*. 55, 492-496.

Imlay, J.A. (2013). The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. *Nat. Rev. Microbiol*. 11, 443-454.

Jakob, U., Gaestel, M., Engel, K. and Buchner, J. (1993) Small heat shock proteins are molecular chaperones. *J. Biol. Chem*. 268, 1517-1520.

Jay, J.M. Loessner, M.J., and Golden, D.A. (2005). *Modern Food Microbiology*. New York: Springer Science and Business Media Press.

Jenkins, D.E., Auger, E.A., and Matin, A. (1991) Role of RpoH, a heat shock regulator protein, in *Escherichia coli* carbon starvation protein synthesis and survival. *J. Bacteriol*. 173, 1992-1996.

Jaquette, C.B., Beuchat, L.R., and Mahon, B.E. (1996). Efficacy of chlorine and heat treatment in killing *Salmonella stanley* inoculated onto alfalfa seeds and growth and survival of the pathogen during sprouting and storage. *Appl. Environ. Microbiol*. 62, 2212-2215.

Jay, J.M., Loessner, M.J., and Golden, D.A. (2005). Modern Food Microbiology. New York, NY: Springer Science and Business Media Press.

Jenkins, D.E., Auger, E.A., and Matin, A. (1991). Role of RpoH, a heat shock regulator protein, in *Escherichia coli* carbon starvation protein synthesis and survival. *J. Bacteriol*. 173, 1992-1996.

Jin, T., Zhang, H., Boyd, G. and Tang, J. (2008). Thermal resistance of *Salmonella enteritidis* and *Escherichia coli* K-12 in liquid egg determined by thermal-death-time disks. *J. Food Eng*. 84, 608-614.

Juneja, V.K., Cadavez, V., Gonzales-Barron, U., Mukhopadhyay, S. (2015). Modelling the effect of pH, sodium chloride and sodium pyrophosphate on the thermal resistance of *Escherichia coli* O157:H7 in ground beef. *Food Res. Int.* 69, 289-304.

Juneja, V.K. and Marmer, B.S. (1999) Lethality of heat to *Escherichia coli* O157:H7: D- and z-value determinations in turkey, lamb, and pork. *Food Res. Int*. 32, 23-28.

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

Kato, A., Ohnishi, H., Yamamoto, K., Furuta, E., Tanabe, H., and Utsumi, R. (2000). Transcription of *emrKY* is regulated by the EvgA-EvgS two-component system in *Escherichia coli* K-12. *Biosci. Biotechnol. Biochem*. 64, 1203-1209.

Katsui, N., Tsuchido, T., Takano, M., and Shibasaki, I. (1981). Effect of preincubation temperature on the heat resistance of *Escherichia coli* having different fatty acid compositions. *J. Gen. Microbiol*. 122, 357-361.

Kaur, J., Ledward, D.A., Park, R.W.A., and Robson, R.L. (1998). Factors affecting the heat resistance of *Escherichia coli* O157:H7. *Lett. Appl. Microbiol*. 26, 325-330.

Kempf, B., and Bremmer, E. (1998) Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments, *Arch. Microbiol*. 170, 319-330.

Kennedy, J., Blair, I.S., McDowell, D.A., Bolton, D.J. (2005). An inverstigation of the thermal inactivation of *Staphylococcus aureus* and the potential for increased thermotolerance as a result of chilled storage. *J. Appl. Microbiol*. 99, 1229-1235.

Kim, Y.B., Kim, H.W., Song, M.K., and Rhee, M.S. (2015). Decontamination method using heat and relative humidity for radish seeds achieves a 7-log reduction of *Escherichia coli* O157:H7 without affecting product quality. *Int. J. Food Microbiol.* 201, 42-46.

Kitagawa, M., Matsumura, Y., and Tsuchido, T. (2000). Small heat shock proteins, IbpA and IbpB, are involved in resistances to heat and superoxide stresses in *Escherichia coli*. *FEMS Microbiol. Lett*. 184, 165-171.

Klaiber, R.G., Baur, S., Wolf, G., Hammes, W.P., Carle, R. (2005). Quality of minimally processed carrots as affected by warm water washing and chlorination. *Innov. Food Sci. & Emerg. Technol*. 6, 351-362.

Kotrola, J. and Conner, D.E. (1997). Heat inactivation of *Escherichia coli* O157:H7 in turkey meat as affected by sodium chloride, sodium lactate, polyphosphate, and fat content. *J. Food Prot*, 60, 898-902.

Kuczyńska-Wiśnik, D., Kędzierska, S., Matuszewska, E., Lund, P., Taylor, A., Lipińska, B., and Laskowska, E. (2002). The *Escherichia coli* small heat-shock proteins IbpA and IbpB prevent the aggregation of endogenous proteins denatured in vivo during extreme heat shock. *Microbiol*. 148, 1757-1765.

Lamosa, P., Burke, A., Peist, R., Huber, R., Liu, M.Y., Silva, G., et al. (2000). Thermostabilization of proteins by diglycerol phosphate: a new compatible solute from the hyperthermophile *Archaeoglobus fulgidus*. *Appl. Environ. Microbiol.* 66, 1974-1979.

Landini, P., Egli, T., Wolf, J., and Lacour, S. (2014). SigmaS, a major player in the response to environmental stresses in *Escherichia coli*: role, regulation and mechanisms of promoter recognition. *Environ. Microbiol. Rep*. 6, 1-13.

Lange, R., Hengge-Aronis, R. (1991). Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol. Microbiol*. 5, 49-59.

Laskowska, E., Wawrzynów, A., and Taylor, A. (1996). IbpA and IbpB, the new heat-shock proteins, bind to endogenous *Escherichia coli* proteins aggregated intracellularly by heat shock. *Biochimie*. 78, 117-122.

Lee, C., Wigren, E., Lünsdorf, H., and Römling, U. (2016). Protein homeostasis-more than resisting a hot bath. *Curr. Opin. Microbiol*. 30, 147-154.

Lee, C., Wigren, E., Trcek, J., Peters, V., Kim, J., Hasni, M.S., et al. (2015). A novel protein quality control mechanism contributes to heat shock resistance of worldwide-distributed *Pseudomonas aeruginosa* clone C strains. *Environ. Microbiol*. 17, 4511-4526.

Lee, G.J., Roseman, A.M., Saibil, H.R. and Vierling, E. (1997). A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *EMBO J*. 16, 659-671.

Lee, J., and Kaletunc, G. (2002). Evaluation of the heat inactivation of *Escherichia coli* and *Lactobacillus plantarum* by differential scanning calorimetry. *Appl. Environ. Microbiol*. 68, 5379-86.

Lindner, A.B., Madden, R., Demarez, A., Stewart, E.J., and Taddei, F. (2008). Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. *Proc. Natl. Acad. Sci. U. S. A*. 105, 3076-3081.

Line, J.E., Fain Jr., A.R., Moran, A.B., Martin, L.M., Lechowich, R.V., Carosella, J.M., and Brown, W.L. (1991). Lethality of heat to *Escherichia coli* O157:H7: *D*-value and *Z*-value determinations in ground beef. *J. Food Prot.* 54, 762-766.

Liu, Y. (2015). Heat and pressure resistance of *Escherichia coli* and its inactivation in the presence of antimicrobial compounds. [Doctor's thesis]. [Edmonton(AB)]: University of Alberta.

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

Luchansky, J.B. Porto-Fett, A.C.S., Shoyer, B.A., Call, J.E., Schlosser, W., Shaw, W., et al. (2012). Fate of shiga toxin-producing O157:H7 and non-O157:H7 *Escherichia coli* cells within blade-tenderized beef steaks after cooking on a commercial open-flame gas grill. *J. Food Prot.* 75, 62-70.

Mackey, B.M., Miles, C.A., Parsons, S.E., and Seymour, D.A. (1991). Thermal denaturation of whole cells and cell components of *Escherichia coli* examined by differential scanning calorimetry. *J. Gen. Microbiol*. 137, 2361-2374.

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

Mao, Y., Doyle, M.P., and Chen, J. (2001). Insertion mutagenesis of *wca* reduces acid and heat tolerance of enterohemorrhagic *Escherichia coli* O157:H7. *J. Bacteriol*. 183, 3811-3815.

Masuda, N., and Church, G.M. (2002). *Escherichia coli* gene expression responsive to levels of the response regulator EvgA. *J. Bacteriol*. 184, 6225-6234.

Mazzotta, A.S. (2001). Thermal inactivation of stationary-phase and acid-adapted *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in fruit juices. *J. Food Prot*. 3, 287-429.

Mecsas, J., Rouviere, P.E., Erickson, J.W., Donohue, T.J., and Gross, C.A. (1993). The activity of σ^E, an *Escherichia coli* heat-inducible σ-factor, is modulated by expression of outer membrane proteins. *Genes Dev*. 7, 2618-2628.

Mejía, R., Gómez-Eichelmann, M.C., and Fernández, M.S. (1995). Membrane fluidity of *Escherichia coli* during heat-shock. *Biochim. Biophys. Acta*. 1239, 195-200.

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

Missiakas, D., Betton, J.M., and Raina, S. (1996). New components of protein folding in the extra-cytoplasmic compartments of *Escherichia coli*: SurA, FkpA and Skp/OmpH. *Mol. Microbiol*., 21, 871-884.

Mizunoe, Y., Wai, S.N., Ishikawa, T., Takade, A., Yoshida, S. (2000). Resuscitation of viable but nonculturable cells of *Vibrio parahaemolyticus* induced at low temperature under starvation. *FEMS Microbiol. Lett*. 186, 115-120.

Mogk, A., Deuerling, E., Vorderwulbecke, S., Vierling, E., and Bukau, B. (2003). Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. *Mol. Microbiol*. 50, 585-595.

Mogk, A., Tomoyasu, T., Goloubinoff, P., Ru□diger, S., Röder, D., Langen, H., and Bukau, B. (1999) Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. *EMBO. J.* 18, 6934-6949.

Mohácsi-Farkas, Cs., Farkas, J., Mészáros, L., Reichart, O., and É. Andrássy. (1999). Thermal denaturation of bacterial cells examined by differential scanning calorimetry. *J. Therm. Anal. Calorim*. 57, 409-414.

Morishige, Y., Fujimori, K., Amano, F. (2013). Differential resuscitative effect of pyruvate and its analogues on VBNC (viable but non-culturable) *Salmonella*. *Microbes Environ.* 28, 180-186.

Murphy, R.Y., Marks, B.P., Johnson, E.R., Johnson, M.G., (1999). Inactivation of Salmonella and Listeria in ground chicken breast meat during thermal processing. *J. Food Prot*. 9, 975-1096.

Nair, M.S., Lau, P., Belskie, K., Fancher, S., Chen, C., Karumathil, D.P., et al. (2016). Potentiating the heat inactivation of *Escherichia coli* O157:H7 in ground beef patties by natural antimicrobials. *Front. Microbiol*. 7, 15.

Nakayama, H., Mitsui, T., Nishihara, M., and Kito, M. (1980) Relation between growth temperature of *Escherichia coli* and phase transition temperatures of its cytoplasmic and outer membranes. *Biochim. Biophys. Acta*. 601, 1-10.

Neetoo, H., and Chen, H. (2011). Individual and combined application of dry heat with high hydrostatic pressure to inactivate *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. *Food Microbiol*. 28, 119-127.

Ng, H., Bayne, H.G., Garibaldi, J.A. (1969). Heat resistance of Salmonella: the uniqueness of *Salmonella* Senftenberg 775W. *Appl. Microbiol*. 17, 78-82.

Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol. Mol. Biol. Rev*. 64, 548-572.

Nishino, K., Inazumi, Y., and Yamaguchi, A. (2003). Global analysis of genes regulated by EvgA of the two-component regulatory system in *Escherichia coli*. *J. Bacteriol*. 185, 2667-2672.

Parsell, D.A., and Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet*. 27, 437-496.

Pereira, R., Martins, J., Mateus, C., Teixeira, J.A. and Vicente, A.A. (2006) Death kinetics of *Escherichia coli* in goat milk and *Bacillus licheniformis* in cloudberry jam treated by ohmic heating. *Chem. Papers.* 61, 121-126.

Pleitner, A., Zhai, Y., Winter, R., Ruan, L., McMullen, L.M., and Gänzle, M.G. (2012). Compatible solutes contribute to heat resistance and ribosome stability in *Escherichia coli* AW1.7. *Biochim. Biophys. Acta*. 1824, 1351-1357.

Podolak, R., Enache, E., Stone, W., Black, D.G., and Elliott, P.H. (2010). Sources and risk factors for contamination, survival, persistence, and heat resistance of *Salmonella* in low-moisture foods. *J. Food Prot*. 73, 1919-1936.

Privalle, C.T., and Fridovich, I. (1987). Induction of superoxide dismutase in *Escherichia coli* by heat shock. *Proc. Natl. Acad. Sci. USA.* 84, 2723-2726.

Quinn, P.J. (1981). The fluidity of cell membranes and its regulation. *Prog. Biophys. Mol. Biol*. 38, 1-104.

Rajic, A., Waddell, L.A., Sargeant, J.M., Read, S., Farber, J., Firth, M.J., et al. (2007). An overview of microbial food safety programs in beef, pork, and poultry from farm to processing in Canada. *J. Food Prot.* 70, 1286-1294.

Ramos, A., Raven, N.D.H., Sharp, R.J., Bartolucci, S., Rossi, M., Cannio, R., et al. (1997). Stabilization of enzymes against thermal stress and freeze-drying by mannosylglycerate. *Appl. Environ. Microbiol.* 63, 4020-4025.

Riemann, H. (1968). Effect of water activity on the heat resistance of *Salmonella* in "dry" materials. *Appl. Microbiol*. 16, 1621-1622.

Riordan, D.C.R., Duffy, G., Sheridan, J.J., Whiting, R.C., Blair, I.S. and McDowell, D.A. (2000) Effects of acid adaptation, product pH, and heating on survival of *Escherichia coli* O157:H7 in pepperoni. *Appl. Environ. Microbiol.* 66, 1726-1729.

Ruan, L., Pleitner, A.M., Gänzle, M.G., and McMullen. L.M. (2011). Solute transport proteins and the outer membrane protein NmpC contribute to heat-resistance of *Escherichia coli* AW1.7. *Appl. Environ. Microbiol*. 77, 2961-2967.

Ryu, J., and Beuchat, L.R. (1998). Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. *Int. J. Food Microbiol*. 45, 185-193.

Ryu, J., and Beuchat, L.R. (1999). Changes in heat tolerance of *Escherichia coli* O157:H7 after exposure to acidic environments. *Food Microbiol*. 16, 317-324.

Santillana Farakos, S.M., Schaffner, D.W., and Frank, J.F. (2014). Predicting survival of salmonella in low-water activity foods: an analysis of literature data. *J. Food Prot*. 77, 1448-1461.

Sharma, M., Adler, B.B., Harrison, M.D., and Beuchat, L.R. (2005). Thermal tolerance of acid-adapted and unadapted *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* in cantaloupe juice and watermelon juice. *Lett. Appl. Microbiol*. 41, 448-453.

Sinensky, M. (1974). Homeoviscous adaptation: a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 71, 522-525.

Smith, S.E., Maurer, J.L., Orta-Ramirez, A., Ryser, E.T., and Smith, D.M. (2001). Thermal inactivation of *Salmonella* spp., *Salmonella Typhimurium* DT104 and *Escherichia coli* O157:H7 in ground beef. *J. Food Sci*. 66, 1164-1168.

Studer, P, Heller, W.E, Hummerjohann, J., and Drissner, D. (2013). Evaluation of aerated steam treatment of alfalfa and mung bean seeds to eliminate high levels of *Escherichia coli* O157:H7 and O178:H12, *Salmonella enterica*, and *Listeria monocytogenes*. *Appl. Environ. Microbiol*. 79, 4613-19.

Suutari, M., and Laakso, S. (1994). Microbial fatty acids and thermal adaptation. *Crit. Rev. Microbiol*. 20, 285-328.

Swartz, R.S., Luchansky, J.B., Kulas, M., Shoyer, B.A., Shane, L.E., Strasser, H., et al. (2015). Thermal inactivation of shiga toxin-producing *Escherichia coli* cells within cubed beef steaks following cooking on a griddle. *J. Food Prot.* 78, 1013-1017.

Syamaladevi, R.M., Tang, J., Villa-Rojas, R., Sablani, S., Carter, B., and Campbell, G. (2016). Influence of water activity on thermal resistance of microorganisms in low-moisture foods: a review. *Compr.Rev. Food Sci. Food Saf*. 15, 353-370.

Thomas, J.G., and Baneyx, F. (1998). Roles of the *Escherichia coli* small heat shock proteins IbpA and IbpB in thermal stress management: comparison with ClpA, ClpB, and HtpG in vivo. *J. Bacteriol*. 180, 5165-5172.

Torok, Z., Horvath, I., Goloubinoff, P., Kovacs, E., Glatz, A., Balogh, G., and Vigh, L. (1997) Evidence for a lipochaperonin: association of active protein-folding GroESL oligomers with lipids can stabilize membranes under heat shock conditions. *Proc. Natl. Acad. Sci. USA*. 94, 2192-2197.

Tsuchido, T., Katsui, N., Takeuchi, A., Takano, M., and Shibasaki, I. (1985). Destruction of the outer membrane permeability barrier of *Escherichia coli* by heat treatment. *Appl. Environ. Microbiol*. 50, 298-303.

Vaara, M. (1992). Agents that increase the permeability of the outer-membrane. *Microbiol. Rev.* 56, 395-411.

Vasan, A., Geier, R., Ingham, S.C., and Ingham, B.H. (2014). Thermal tolerance of O157 and non-O157 shiga toxigenic strains of *Escherichia coli*, *Salmonella*, and potential pathogen surrogates, in frankfurter batter and ground beef of varying fat levels. *J. Food Prot*. 77, 1501-1511.

Veinger, L., Diamant, S., Buchner, J., and Goloubinoff, P. (1998). The small heat-shock proteins IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *J. Biol. Chem*. 273, 11032-11037.

Weiss, A., and Hammes, W.P. (2005) Efficacy of heat treatment in the reduction of Salmonella and *Escherichia coli* O157: H^- on alfalfa, mung bean, and radish seeds used for sprout production. *Eur. Food Res. Technol*. 221, 187-191.

Whitfield, C., and Valvano, M.A. (1993). Biosynthesis and expression of cell surface polysaccharides in gram-negative bacteria. *Adv. Microb. Physiol*. 35, 135-246.

Winkler, J., Seybert, A., Konig, L., Pruggnaller, S., Haselmann, U., Sourjik, V., et al. (2010). Quantitative and spatio-temporal features of protein aggregation in *Escherichia coli* and consequences on protein quality control and cellular ageing. *EMBO J*. 29, 910-923.

Woodward, D.L., Clark, C.G., Caldeira, R.A., Ahmed, R. and Rodgers, F.G. (2002). Verotoxigenic *Escherichia coli* (VTEC): a major public health threat in Canada. *Can. J. Infect Dis.* 13, 321-330.

Yang, J., Bingol, G., Pan, Z., Brandl, M.T., McHugh, T.H., and Wang, H. (2010). Infrared heating for dry-roasting and pasteurization of almonds. *J. Food Eng.* 101, 273-280.

Yeni, F., Yavas□, S., Alpas, H., and Soyer, Y. (2015). Most common foodborne pathogens and mycotoxins on fresh produce: a review of recent outbreaks. *Crit. Rev. Food Sci. Nutr*. 56, 1532-1544.

Yoon, Y., Lee, H., Lee, S., Kim, S., and Choi, K.H. (2015). Membrane fluidity-related adaptive response mechanisms of foodborne bacterial pathogens under environmental stresses. *Food Res. Int*. 72, 25-36.

Yuk, H., and Marshall, D.L. (2003). Heat adaptation alters *Escherichia coli* O157:H7 membrane lipid composition and verotoxin production. *Appl. Environ. Microbiol*. 69, 5115-5119.

Zhang, Y., and Griffiths, M.W. (2003). Induced expression of the heat shock protein genes uspA and grpE during starvation at low temperatures and their influence on thermal resistance of *Escherichia coli* O157:H7. *J. Food Prot*. 66, 2045-2050.

Zhang, Y., and Rock, C.O. (2008). Membrane lipid homeostasis in bacteria. *Nat. Rev. Microbiol*. 6, 222-233.

Zhao, G., Ceci, P., Ilari, A., Giangiacomo, L., Laue, T. M., Chiancone, E., et al. (2002). Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of *Escherichia coli*. *J. Biol. Chem*. 277, 27689-22796.

CHAPTER 3 **Heat and pressure resistance relates to protein folding and aggregation**

3.1 Introduction

Some extremely heat resistant strains of *E. coli* such as *E. coli* AW1.7, AW1.3, DM18.3 and GM16.6 are also highly resistant to pressure (Garcia-Hernandez et al., 2015; Liu et al., 2015). This may be related to proteins that contribute against oxidative stress by heat or pressure, including systems for thiol-disulfide redox homeostasis and proteins containing iron-sulfur clusters (Malone et al., 2006; Charonewong et al., 2011; Imlay, 2013; Gänzle and Liu, 2015). Comparative genomic analyses found that a 14.6 kb genomic island named the locus of heat resistance (LHR) played a critical role in highly heat resistant *E. coli* strains including *E. coli* AW1.7, AW1.3, DM18.3 and GM16.6 (Mercer et al., 2015). Putative proteins expressed within LHR include small heat shock proteins, proteins of the YfdX family, heat shock proteases, thioredoxin, and sodium/hydrogen antiporter (Mercer et al., 2015).

A recent study reported that a high fraction of heat resistant *E. coli* (93 of 256 isolates) was found in the raw milk cheese product (Marti et al., 2016). Moreover, some strains of *E. coli* have become naturalized populations in wastewater and survive in a non-host environment, and chlorine tolerant *E. coli* isolates containing the *uspC-IS30-flhDC* locus (a genetic insertion element IS30 located specifically in the *uspC –flhDC* intergenic region) also carry the LHR (Zhi et al., 2016). Thus,

LHR-positive *E. coli* are a major concern among these environmental- or processed-resistant *E. coli* strains.

Protein aggregation within *E. coli* occurs during stress conditions, such as heat shock (Strandberg and Enfors, 1991; Hunke and Betton, 2003). Rosen et al. (2002) found that after heat shock of 42 ^{o}C for 60 min, most of cellular proteins (e.g. metabolic enzymes, protein synthesis) became unfolded or misfolded, and consequently resulted in aggregation if they failed to be refolded or degraded. The genes related to protein folding and turnover such as proteins of the general stress response are generally associated with pressure resistance in *E. coli* (Aertsen et al., 2004; Malone et al., 2006; Govers et al., 2014). Deletion of inclusion body binding proteins IbpA and IbpB reduced the pressure resistance (Charoenwong et al., 2011). Therefore, the orfs encoded within the LHR involved in protein folding and turnover may affect the protein folding and aggregation under pressure, and are involved in pressure resistance of *E. coli* (Mercer et al., 2015; Gänzle and Liu, 2015).

Digital florescence microscopic observation has already been used for the observation of protein aggregation in *E. coli* (Govers et al., 2014, 2015). Cloning of the LHR and proteomic analysis could be applied as complementary tools (Mercer et al., 2015; Jürgen et al., 2001). Based on these methods, the objectives of this study were to determine the expression of proteins within LHR, and the relationship between heat and pressure resistance of *E. coli* and protein folding and aggregation.

3.2 Materials and Methods

3.2.1 Bacterial strains and culture conditions.

The *ibpA-yfp* locus of *E. coli* MGAY was P1 transduced into *E. coli* MG1655 and its pressure-resistant derivative, LMM1010 (Govers et al., 2014), resulting in MG1655 *ibpA-yfp* and LMM1010 *ibpA-yfp*, respectively. Stock cultures of *E. coli* were streaked onto Luria-Bertani (LB, Difco, Sparks, MD, USA) agar, 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. The empty vector pRK767, or pRK767 plasmids carrying fragments or full length LHR were extracted from *E. coli* strains DH5α (pRF1), DH5α (pRF2), DH5α (pRF3), DH5α (pRF1-2) and DH5α (pLHR), respectively (Mercer et al., 2015). The plasmids were transformed into *E. coli* MG1655, LMM1010, and MG1655 *ibpA-yfp*, and PCR was used to confirm the presence of the plasmids (Mercer et al., 2015).

3.2.2 Pressure and heat treatment.

The pressure resistance of *E. coli* strains was assessed by using stationary-phase cells that were prepared as described above. Cell suspensions were packed into sterile 3 cm E3603 tubing (Fisher Scientific, Akron, OH, USA), heat sealed on both sides, and kept on ice before treatment. The samples were placed in a 2.2 mL pressure vessel (Micro-system, Unipress, Warsaw, Poland) filled with bis (2-ethylhexyl) sebacate (Sigma-Aldrich, Germany) as the pressure transmitting fluid. The pressure vessel was submerged in a water bath maintained at 20 °C. *E. coli* MG1655 *ibpA-yfp* and *E. coli* LMM 1010 *ibpA-yfp* cells were treated at 400 MPa for 1 to 10 min. The rates of

compression and decompression were 277.8 MPa/min. The temperature in the pressure vessel was monitored by an internal thermocouple and the temperature changes during compression and decompression did not exceed 4.5 °C. Experiments were performed in triplicate and the viable cell counts were determined in untreated and pressure-treated samples.

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 immediately transfered for microscopic observations.

3.2.3 Isolation of proteins from whole cells and inclusion bodies

Inclusion bodies were isolated according a protocol developed for *E. coli* (Georgious and Valax, 1999). Cells were grown to an OD_{600} of 1.0 in 50 mL cultures and harvested after centrifugation at 5,300 ×g for 10 min at 4 °C and wash in 5 mL buffer at 8,000 ×g for 10 min. The cell pellet is resuspended in 10mM Tris-HC1, pH 7.5, containing 0.75 M sucrose and 0.2 mg/mL lysozyme. After a 10 min incubation at 20 ^{o}C, a 3 mM EDTA solution is added at a 2:1 (v/v) ratio and transferred to ice for approximately 5 min. The cells were lysed by sonication for 3 min, and the lysate was centrifuged at 12,000 ×g for 30 min at 4 °C. The pellet containing the inclusion bodies was resuspended in 10 mM Tris-HC1 buffer, pH 8.0, containing 0.25 M sucrose, 1 mM EDTA. The resuspended pellet was layered on the top of a equal-volume sucrose step gradient [40, 53, and 67% (w/w)] in 1 mM Tris-HC1 buffer, pH 8.0, containing 1 mM EDTA. The sucrose gradient was prepared by carefully layering the sucrose solutions,

with the more dense solution at the bottom of the tube. The total volume was 4.7 mL, with 1.3 mL of 67% sucrose, 1.2 mL of 53% sucrose, 1.2 mL of 40% sucrose solution and ~1.0 mL lysate. Each layer was balanced to 0.001 g. Centrifugation was performed at 108,000 ×g for 90 min at 4 ^{o}C. The inclusion bodies were focused in a band at the interface between the 53% and 67% sucrose layers and were removed from this interphase. After harvesting the cell pellet, it was first washed with Native lysis buffer (100 mM Tris, pH 8, 50 mM NaCl, 1 mM EDTA, 1 mM Dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) for 60 min to remove all the soluble proteins (10,000 ×g, 30 min). Then the pellet was washed with 1XPBS+1% TRITON-100 (30,000 ×g, 30 min). The final pellet was dissolved into LDS buffer (NuPAGE) with 50 mM DTT, heated at 70 ^{o}C for 10 min, and then stored at -20 ^{o}C.

3.2.4 Protein digestion

Samples were reduced and alkylated by using 50 mM DTT and 55 mM chloroacetamide, respectively. Tryptic in-gel digestion was performed according to standard procedures (Cox et al., 2014).

3.2.5 LC-MS/MS analysis

Nanoflow LC-MS/MS was performed by using an Eksigent nanoLC-Ultra 1D+ system (Eksigent, Dublin, CA) coupled online to a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany). Tryptic peptides were dissolved in buffer A_0 [0.1% formic acid (FA) in HPLC grade water] and 1 µg was injected for each measurement. Peptide samples were first loaded on a trap column (75 µm inner

diameter x 2 cm, packed in house with 5 µm, Reprosil ODS-3; Dr. Maisch, Ammerbuch, Germany) in 100% buffer A_0. Peptides were transferred to an analytical column (75 µm x 40 cm, C18 column, Reprosil Gold, 3 µm: Dr. Maisch, Ammerbuch, Germany) and separated using a 110 min gradient from 4-32% solvent B (0.1% FA and 5% DMSO in acetonitrile) in A (0.1% FA and 5% DMSO in HPLC grade water) at a flow rate of 300 nl/min. MS measurement was performed in data-dependent acquisition mode, automatically extracting the ten most prominent precursor ions in the full MS spectra for HCD fragmentation at 30% collision energy. Full MS spectra and MS/MS spectra were acquired at 30,000 resolution and 7,500 resolution, respectively. Dynamic exclusion was set to 60 s. Measurements were carried out by the Bavarian Biomolecular Mass Spectrometry Center in Freising, Germany.

3.2.6 Peptide and protein identification and quantification

Label free quantification was performed using MaxQuant (version 1.5.3.30) by searching MS data against an *E. coli* K12 UniProt reference database (version 31.10.2016, 5970 entries) and a collection of 16 LHR protein sequences (generated in house) using the search engine Andromeda (Cox et al., 2008; 2011). Carbamidomethylated cysteine was used as fixed modification; variable modifications included oxidation of methionine and N-terminal protein acetylation. Trypsin/P was specified as proteolytic enzyme with up to two allowed miscleavage sites. Precursor tolerance was set to 10 ppm and fragment ion tolerance was set to 0.05 Da. Label-free quantification (Cox et al., 2014) and match-between-runs options were enabled and

results were filtered for a minimal length of three amino acids, 1% peptide and protein false discovery rate as well as reverse identifications.

3.2.7 Fluorescence microscopic analysis of protein aggregation

Cell suspensions were transferred to a microscope slide and mounted with a cover slip (Fisher Scientific, Akron, OH, USA). Samples were observed with a digital fluorescence microscope (Carl Zeiss M100, Jena, Germany), and image analysis was performed with AxioVision SE64 (Version 4, Carl Zeiss). The protein aggregation was analyzed by quantifying protein aggregation foci in cells, or the numbers of cells with or without foci. For samples without treatments, the cells were differentiated by the percentages of cells with 0, 1, 2 or >3 protein aggregation foci. For samples treated with pressure or heat, the cells were differentiated by the percentages of cells with or without protein aggregation foci. Each count was performed with no less than 100 cells and experiments were performed in triplicate (>300 cells were included).

3.2.8 Enumeration of *E. coli* before and after pressure treatment

After treatments at 400 MPa and 20 ^{o}C, samples were serially 10-fold diluted using 0.1% peptone water (BD, Mississauga, CA), and cell counts were enumerated by surface plating onto LB agar (Difco, Sparks, MD, USA). The untreated samples were used as control. Experiments were performed in triplicate.

3.2.9 Statistical analysis

Significant differences between two treatments were determined using Student's *t* test; significance was assessed at an error probability of 0.1% ($P<0.001$) for proteomic

analysis, and 5% ($P<0.05$) for data of cell counts. The proteomic analysis was performed in quadruplicate and all other experiments were performed in triplicate.

3.3 Results

3.3.1 Expression of proteins in *E. coli* MG1655 *ibpA-yfp*

The proteome of inclusion bodies in isogenic derivatives of *E. coli* MG1655*ibpA-yfp* expressing the LHR or not expressing the LHR was compared to the whole cell proteome. In strains with pRK767 or pLHR cells, a total of 142 proteins were overrepresented in inclusion bodies (**Table 3-1**). In the LHR positive strain, 128 proteins were found to be overrepresented in inclusion bodies compared to whole cell extraction, while only 52 proteins were overrepresented in the LHR negative strain; 50 proteins were overrepresented in both LHR positive and negative strain. The overlap of proteins in inclusion bodies from both LHR positive and negative strains, and the presence of IbpA (fold overexpression of inclusion bodies was 3.7 times of whole cell extraction in the strain with pRK767 and 4.6 times in the strain with pLHR**)** validated the proper methods of protein extraction.

Table 3-1 Overlap and differential proteins overrepresenting in inclusion bodies rather than in whole cell extraction in untreated *E. coli* MG1655 *ibpA-yfp* (pRK767) and *E. coli* MG1655 *ibpA-yfp* (pLHR).

Protein products	Gene	pLHR	pRK
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	*aceF*	+	+
Multidrug efflux pump subunit AcrA	*acrA*	+	+
Multidrug efflux pump subunit AcrB	*acrB*	+	+
Aldehyde-alcohol dehydrogenase; Pyruvate-formate-lyase deactivase	*adhE*	+	+
HTH-type transcriptional repressor	*allR*	+	+
Aerobic respiration control sensor protein	*arcB*	+	-
Arginine transport ATP-binding protein	*artP*	+	-
ATP synthase subunit alpha	*atpA*	+	+
ATP synthase epsilon chain	*atpC*	+	-
ATP synthase subunit beta	*atpD*	+	+
ATP synthase subunit b	*atpF*	+	
ATP synthase gamma chain	*atpG*	+	+
ATP synthase subunit delta	*atpH*	+	-
Vitamin B12 transporter	*btuB*	+	+
H(+)/Cl(-) exchange transporter	*clcA*	+	+
Adenosylcobinamide-GDP ribazoletransferase	*cobS*	-	+
Sensor protein	*cpxA*	+	-
Carbon starvation protein A	*cstA*	+	+
Cytochrome bd-I ubiquinol oxidase subunit 1	*cydA*	+	+
Cytochrome bd-I ubiquinol oxidase subunit 2	*cydB*	+	-
ATP-binding/permease protein	*cydC*	+	-
Cytochrome bo(3) ubiquinol oxidase subunit 2	*cyoA*	+	+
Cytochrome bo(3) ubiquinol oxidase subunit 1	*cyoB*	+	+
Aerobic C4-dicarboxylate transport protein	*dctA*	+	-
Anaerobic C4-dicarboxylate transporter	*dcuA*	+	-
Anaerobic C4-dicarboxylate transporter	*dcuC*	+	-
PTS-dependent dihydroxyacetone kinase, phosphotransferase subunit	*dhaM*	+	-
DnaJ-like protein DjlA	*djlA*	-	+
Multidrug export protein EmrA	*emrA*	+	-
Acyl-coenzyme A dehydrogenase	*fadE*	+	+
Formate dehydrogenase, nitrate-inducible, major subunit	*fdnG*	+	-
Fe(3+) dicitrate transport protein	*fecA*	+	+
Ferrienterobactin receptor	*fepA*	+	-
Probable iron export permease protein	*fetB*	+	-

Ferrichrome-iron receptor	*fhuA*	+	+
Flagellar hook protein	*flgE*	+	-
Flagellar basal-body rod protein	*flgG*	+	-
Flagellar P-ring protein	*flgI*	+	-
Flagellar biosynthesis protein	*flhA*	+	-
Flagellar M-ring protein	*fliF*	+	-
Flagellar motor switch protein	*fliM*	+	-
Flagellar biosynthetic protein	*fliP*	+	-
Antigen 43 alpha/beta chain	*flu*	+	+
Probable formate transporter 1	*focA*	+	+
PTS system fructose-specific EIIBC component	*fruA*	+	-
ATP-dependent zinc metalloprotease	*ftsH*	+	+
Quinoprotein glucose dehydrogenase	*gcd*	+	-
Glycogen synthase	*glgA*	+	+
Protein HemY	*hemY*	+	-
Modulator of FtsH protease	*hflC*	+	+
Modulator of FtsH protease	*hflK*	+	+
Small heat shock protein	*ibpA*	+	+
Acetate operon repressor	*iclR*	+	+
Apolipoprotein N-acyltransferase	*lnt*	-	+
Inhibitor of vertebrate lysozyme	*ivy*	+	-
Alpha-ketoglutarate permease	*kgtP*	+	-
Lactose operon repressor	*lacI*	+	-
Maltoporin	*lamB*	+	-
Lipopolysaccharide assembly protein A	*lapA*	+	-
Sodium/hydrogen exchanger KefB	*kefB*	+	-
Apolipoprotein N-acyltransferase	*lnt*	+	-
Outer-membrane lipoprotein	*lolB*	+	-
Dihydrolipoyl dehydrogenase	*lpdA*	+	-
Lipopolysaccharide export ATP-binding protein	*lptB*	+	-
LPS-assembly protein LptD	*lptD*	+	+
LPS-assembly lipoprotein LptE	*lptE*	+	-
Leucine-responsive regulatory protein	*lrp*	+	-
Macrolide export protein	*macA*	+	-
Maltose transport system permease protein	*malF*	-	+
Maltose/maltodextrin import ATP-binding protein	*malK*	+	-
Maltodextrin phosphorylase	*malP*	+	-
4-alpha-glucanotransferase	*malQ*	+	-
Probable phospholipid-binding lipoprotein	*mlaA*	+	-
Probable phospholipid ABC transporter-binding protein	*mlaD*	+	-
Protein mlc	*mlc*	+	-
Penicillin-binding protein	*mrcB*	-	+

Mechanosensitive channel MscK	*mscK*	+	-
PTS system mannitol-specific EIICBA	*mtlA*	+	-
Formamidopyrimidine-DNA glycosylase	*mutM*	+	-
PTS system N-acetylglucosamine-specific EIICBA component	*nagE*	+	-
Probable N-acetylneuraminic acid outer membrane channel protein	*nanC*	+	-
Respiratory nitrate reductase 1 beta chain	*narH*	+	-
Nitrate/nitrite sensor protein	*narX*	+	-
NADH-quinone oxidoreductase subunit A	*nuoA*	+	+
NADH-quinone oxidoreductase subunit B	*nuoB*	+	-
NADH-quinone oxidoreductase subunit C	*nuoC*	+	+
Nucleoside permease	*nupC*	+	-
Outer membrane protein A	*ompA*	+	+
Oligopeptide transport ATP-binding protein	*oppF*	+	-
DNA topoisomerase 4 subunit A	*parC*	+	-
NAD(P) transhydrogenase subunit alpha	*pntA*	+	+
NAD(P) transhydrogenase subunit beta	*pntB*	+	+
Polyphosphate kinase	*ppk*	+	-
Paraquat-inducible protein B	*pqiB*	+	-
PTS system glucose-specific EIICB	*ptsG*	+	-
Protein QmcA	*qmcA*	+	-
Outer membrane lipoprotein	*rcsF*	+	-
Recombination-associated protein	*rdgC*	+	-
Ribonuclease E	*rne*	+	-
Ribonuclease R	*rnr*	+	-
Regulator of sigma-E protease	*rseP*	+	-
Serine transporter	*sdaC*	+	-
Protein translocase subunit SecD	*secD*	+	+
Protein translocase subunit SecF	*secF*	+	+
Protein translocase subunit SecY	*secY*	+	-
Negative modulator of initiation of replication	*seqA*	+	-
Outer membrane lipoprotein	*slyB*	+	-
Probable protease	*sohB*	+	-
Glucitol/sorbitol-specific phosphotransferase enzyme IIB	*srlE*	+	-
DNA-binding protein	*stpA*	+	-
Dihydrolipoyllysine-residue succinyltransferase component	*sucB*	+	-
Translocation and assembly module	*tamA*	+	-
Translocation and assembly module	*tamB*	+	+
Outer membrane protein	*tolC*	+	+
Protein TolQ	*tolQ*	+	-
PTS system trehalose-specific EIIBC	*treB*	+	+

Methyl-accepting chemotaxis protein III	*trg*	+	-
UvrABC system protein A	*uvrA*	+	-
Chain length determinant protein	*wzzB*	+	+
Lipopolysaccharide biosynthesis protein	*wzzE*	+	+
Uncharacterized ABC transporter ATP-binding protein	*yadG*	+	+
UPF0092 membrane protein	*yajC*	+	+
Inner membrane protein	*ybaL*	-	+
UPF0194 membrane protein	*ybhG*	-	+
Uncharacterized lipoprotein	*ybjP*	+	-
UPF0755 protein	*yceG*	+	-
Uncharacterized protease	*ydcP*	+	-
UPF0394 inner membrane protein	*yedE*	+	+
Inner membrane protein	*yejM*	+	+
Uncharacterized protein	*yffS*	+	-
Uncharacterized HTH-type transcriptional regulator	*yfhH*	+	-
Uncharacterized lipoprotein	*yfhM*	+	+
Uncharacterized protein	*ygaU*	+	-
Inner membrane protein	*yhcB*	+	-
Uncharacterized protein YhhM	*yhhM*	+	+
Protein YhjK	*yhjK*	+	-
Probable lipoprotein	*yiaD*	+	-
Uncharacterized protein	*yiaF*	-	+
Uncharacterized protein	*yibN*	+	-
Membrane protein insertase	*yidC*	+	-
Putative transport protein	*yidE*	+	-
Inner membrane protein	*yjiY*	+	-

Note: "**+**" means over-representative of protein; "**-**" means not over-representative of protein.

3.3.2 Expression of LHR proteins in untreated *E. coli* MG1655 *ibpA-yfp*

The protein expression was compared between untreated *E. coli* MG1655 *ibpA-yfp* containing pRK767 or pLHR, for whole cell extraction (**Figure 3-1**A) and inclusion bodies (**Figure 3-1**B). Ten proteins encoded within LHR were detected in both whole cell extraction and inclusion bodies. These ORFs encoded genes for proteins including small heat shock proteins (sHSP20), ATPase chaperone ($ClpK_{GI}$), heat shock protein (HSP), function related to thermal, osmotic and desiccation stress

(YfdX 1 and 2), hypothetical protein, thioredoxin (TRX_{GI}), glutathione-dependent potassium-efflux system and methyglyoxal detoxinfication ($KefB_{GI}$), phosphate-starvation-inducible E family protein (PsiE), periplasmic protein with

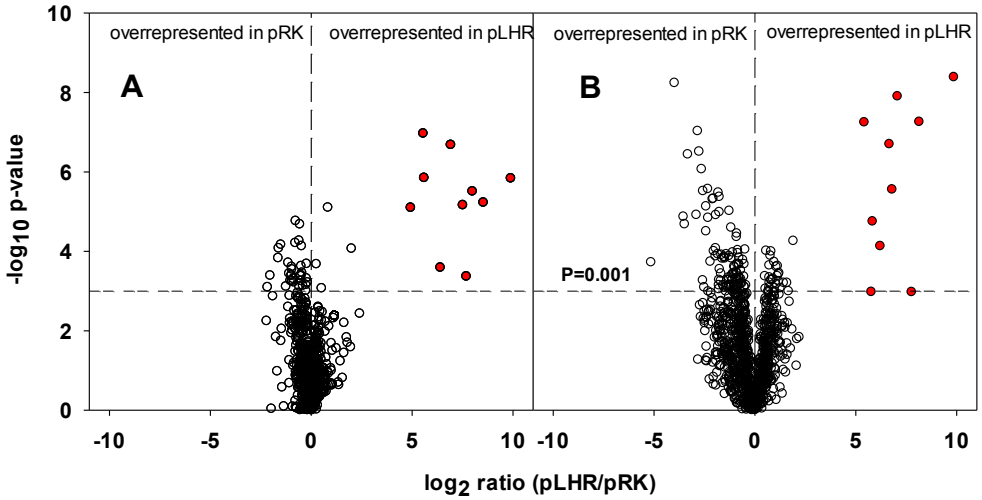

Figure 3-1 The expression ratio of proteins in pLHR and pRK767 strains of *E. coli* MG1655 ibpA-yfp.**Panel A**: whole cell extraction; **Panel B**: inclusion bodies extraction. The ratio was calculated by $\log_2$ values of ratios of Label-Free Quantification (LFQ) intensity. Red dots represent these proteins expressed by the locus of heat resistance (LHR). Experiment was replicated four times.

chaperone and protease activity (DegP) (Mercer et al., 2015; Lee et al., 2016). The expression of these LHR proteins were the main difference between whole cell extracts of strains containing pRK767 or pLHR. However, for inclusion bodies, in addition to the difference of LHR expression, there were 23 proteins that showed higher abundance in the strain containing pRK767 rather than the strain with pLHR strain (**Table 3-2**). In addition to the general metabolism proteins, cell wall hydrolysis and oxidoreductase proteins were found in inclusion bodies of the strain containg pRK767. Among these proteins, three proteins are involved in the general stress regulation, and nine proteins relate to oxidation-reduction reactions. This demonstrated that the presence of the LHR

and the expression of LHR proteins reduced the expression of proteins involved in stress regulation.

Table 3-2 Proteins of inclusion bodies overrepresented in *E. coli* MG1655 *ibpA-yfp* (pRK767) compared to the locus of heat resistance positive strain of untreated cells.

Protein products	**Gene names**	**Razor + unique peptides**	**Fold abundance** (pRK767 /LHR)
Class B acid phosphatase	*aphA*	4	9.97
Probable phospholipid-binding protein MlaC	*mlaC*	7	11.60
Acetyl-coenzyme A synthetase	*acs*	34	4.10
Bifunctional protein FolD;Methylenetetrahydrofolate dehydrogenase;Methenyltetrahydrofolate cyclohydrolase	*folD*	7	5.00
Glucose-6-phosphate isomerase	*pgi*	19	3.80
Putative ABC transporter arginine-binding protein 2	*artI*	6	11.19
Pyridoxine 5-phosphate synthase	*pdxJ*	5	4.96
Transaldolase B	*talB*	25	2.26
Putative ribosome biogenesis GTPase RsgA	*rsgA*	7	3.39
SsrA-binding protein	*smpB*	4	3.51
Universal stress protein F	*uspF*	10	2.40
2,4-dienoyl-CoA reductase [NADPH]	*fadH*	9	3.39
2,5-diketo-D-gluconic acid reductase A	*dkgA*	6	7.43
2-hydroxy-3-oxopropionate reductase	*garR*	8	3.81
3-mercaptopyruvate sulfurtransferase	*sseA*	7	7.12
Aldehyde dehydrogenase B	*aldB*	15	5.89
Glutathione synthetase	*gshB*	13	5.33
Glyoxylate/hydroxypyruvate reductase B	*ghrB*	6	5.33
NADP-dependent 3-hydroxy acid dehydrogenase YdfG	*ydfG*	4	15.79
Peroxiredoxin OsmC	*osmC*	6	6.77
Murein hydrolase activator NlpD	*nlpD*	4	4.17
N-acetylmannosamine kinase	*nanK*	9	6.20
UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	*murE*	6	4.28

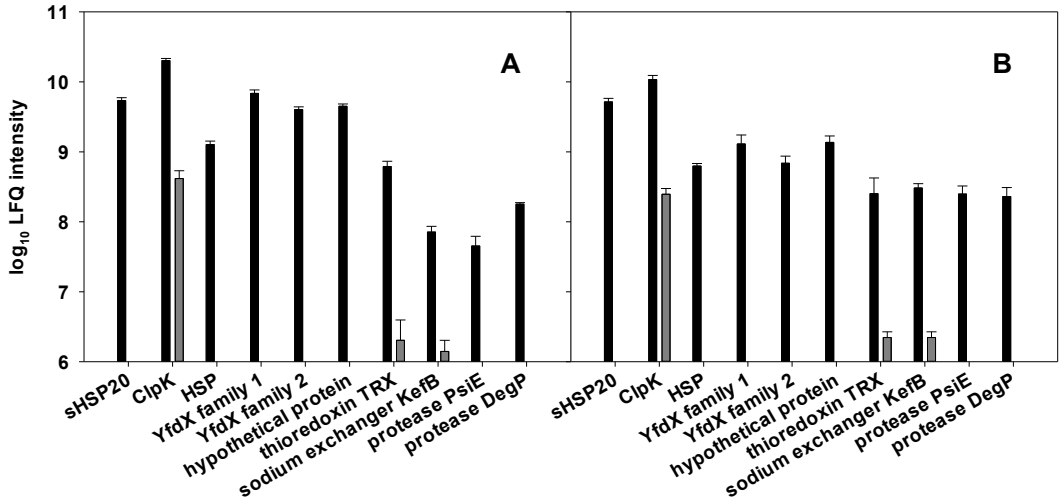

Figure 3-2 Label-Free Quantification of proteins expressed by the locus of heat resistance in *E. coli* MG1655 *ibpA-yfp* (pLHR, black bars) and *E. coli* MG1655 *ibpA-yfp* (pRK767, grey bars). Panel A: whole cell extraction; Panel B: inclusion bodies extraction. Experiment was replicated four times.

To determine the presence of the detected 10 LHR proteins in *E. coli* MG1655, a BLAST search was performed using the corresponding sequences of proteins. With more than 80% coverage, only three LHR proteins were found in *E. coli* MG1655: ATPase chaperone expressed by orf 3, thioredoxin expressed by orf 12 and the sodium/hydrogen exchanger expressed by orf 13. The expression of all three proteins was upregulated by the presence of the LHR (**Figure 3-2**). In addition, in the untreated strain containing pLHR, the sodium hydrogen exchanger and phosphate-starvation-inducible E family protein (expressed by orf 13 and orf 14) showed significantly higher abundance in inclusion bodies, compared to the whole cell extract (4.3 and 5.5 folds, $P<0.001$), which leads to the conclusion that these LHR proteins are specifically associated with inclusion bodies.

3.3.3 Investigation of inclusion bodies before and after treatment

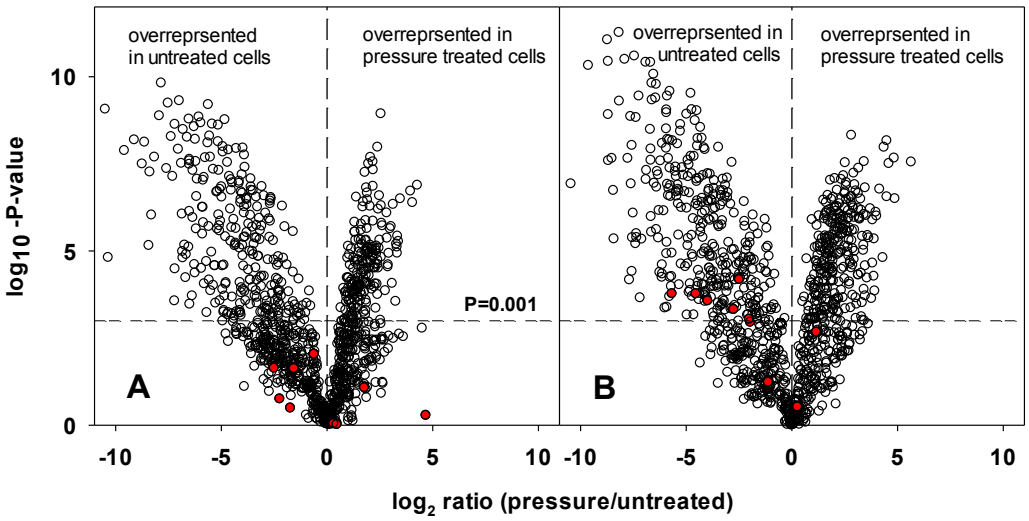

Figure 3-3 The expression ratio of proteins from inclusion bodies extraction in *E. coli* strains before and after pressure treatment at 400 MPa, 20 ^{o}C for 3 min. Panel A: MG1655 *ibpA-yfp* (pRK767); Panel B: MG1655 *ibpA-yfp* (pLHR). The ratio was calculated by $\log_2$ values of Label-Free Quantification (LFQ) intensity. Red dots represent these proteins expressed by the locus of heat resistance. Experiment was replicated four times.

To further explore how the inclusion bodies change under stress, the proteomic analysis of inclusion bodies was performed for MG1655 *ibpA-yfp* (pRK767) and MG1655 *ibpA-yfp* (pLHR) strains before and after pressure treatment at 400 MPa and 20 ^{o}C for 3 min (**Figure 3-3**). In MG1655 *ibpA-yfp* (pLHR), six LHR proteins were overrepresented in untreated cells including two heat shock proteins (orf2 and orf7), general stress protein (orf8), sodium/hydrogen exchanger (orf13), phosphate-starvation-inducible E family protein (orf14), and periplasmic protein with chaperone and protease activity (orf16). The pressure treatment induced significant down-regulation of all of these LHR proteins ($P<0.001$). However, it should be noted that the proteome of pressure treated samples may not be useful because the majority of

the cells were dead and the proteins may behave differently throughout purification protocol.

Table 3-3 Percentages of cells with different foci in *E. coli* MG1655 *ibpA-yfp* with or without the cloning of the locus of heat resistance.

Foci counts	pRK767	pLHR
0	3.0 ± 1.3%	4.8 ± 3.2%
1	92.8 ± 2.3%	73.5 ± 9.4%
2	3.9 ± 2.8%	19.6 ± 10.5%
>3	0.3 ± 0.6%	2.0 ± 0.7%

Table 3-4 Percentages of cells with different foci after heat or pressure treatment in *E.coli* MG1655 *ibpA-yfp* with or without the cloning of the locus of heat resistance.

Foci	pRK767	pLHR
% of cells with or without protein aggregates after treatment at 60 ^{o}C for 5 min		
+	62.9 ± 5.0	29.8 ± 6.6
-	37.1 ± 5.0	70.2 ± 6.6
% of cells with or without protein aggregates after treatment at 400 MPa for 3 min		
+	77.7 ± 2.9	57.9 ± 4.5
-	22.3 ± 2.9	42.1 ± 4.5

For visible observation, microscopic analysis was performed using a digital florescence microscope to observe foci in untreated or pressure/heat treated cells as an indicator of the percentage of inclusion bodies. There were fewer cells with only one foci in the strain with pLHR, but more cells with 2 or more foci in cells with the LHR (**Table 3-3**). Comparing between untreated and treated samples (**Table 3-3** and **Table 3-4**), cells treated with heat or pressure had a lower the percentage of cells with inclusion bodies, which was consistent with the proteomic analysis that showed few proteins of inclusion bodies were overrepresented in the pressure treated strain with

pRK767 or pLHR strains (**Figure 3-3**). After treatment at 60 ^{o}C for 5 min or 400 MPa for 3 min, the protein aggregation was reduced in both *E. coli* MG1655 *ibpA-yfp* and LMM1010 *ibpA-yfp* containing pLHR (**Table 3-4**). For example, the presence of LHR reduced the percentage of cells with protein aggregation from 62.9% to 29.8% in *E. coli* MG1655 *ibpA-yfp*. More profoundly, when cells were treated at 70 ^{o}C for 5 min, no protein aggregation was observed in the strain with pLHR (**Figure 3-4**B), while obvious foci was observed in strain with pRK767 (**Figure 3-4**A), indicating that the presence of the LHR cleared up the inclusion bodies under this treatment (**Table 3-5** and **Figure 3-4**).

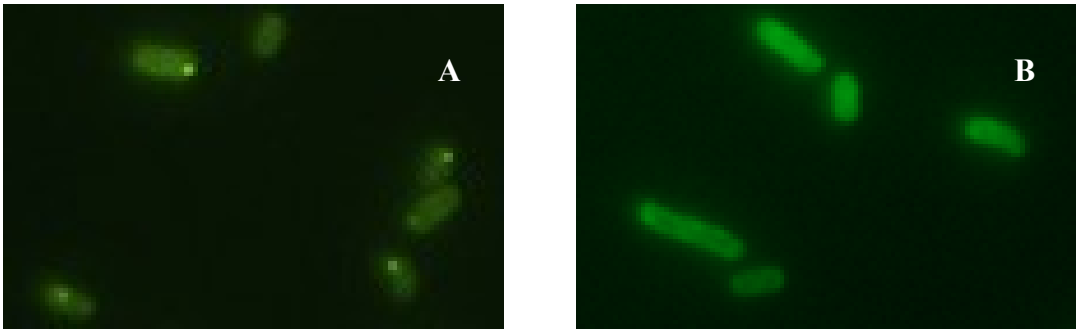

Figure 3-4 Images of MG1655 *ibpA-yfp* cells with empty plasmid pRK767 (A) or with the locus of heat resistance (B) after treatment at 70 ^{o}C for 5 min.

Table 3-5 Numbers of cells differential from foci after heat or pressure treatment in *E. coli* MG1655 *ibpA-yfp* with partial cloning of the locus of heat resistance.

Foci	pRK767	pLHR	pRF1	pRF2	pRF1-2	pRF3
	% of cells with or without protein aggregates after treatment at 70 ^{o}C for 5 min					
+	44.7±1.3	0.0	0.0	43.9±3.2	0.0	38.8±11.5
-	55.3 ± 1.3	100.0	100.0	56.1±3.2	100.0	61.2±11.5
	% of cells with or without protein aggregates after treatment at 400 MPa for 3 min					
+	77.7±2.9	57.9±4.5	52.0±3.9	63.5± 6.2	36.7±10.3	66.6±3.3
-	22.3±2.9	42.1±4.5	48.0±3.9	36.5± 6.2	63.3±10.3	33.4±3.3

3.3.4 Contribution of LHR to pressure resistance of *E. coli* MG1655 *ibpA-yfp*

Based on the proteins encoded in the LHR, it was speculated that LHR might contribute not only to heat resistance, but also to pressure resistance of *E. coli*. To determine whether the LHR affects the pressure resistance of *E. coli*, and which part is responsible for the potential increased pressure resistance, the full length or partial of LHR on a plasmid was transformed into *E. coli* MG1655 *ibpA-yfp*, and the derivative strains were then treated by high pressure at 400 MPa at 20 ^{o}C for 1 to 10 min (**Figure 3-5**). For each holding time of the pressure treatment, the cell counts of the strain *E. coli* MG1655 *ibpA-yfp* (pRF1) and MG1655 *ibpA-yfp* (pRF3) were significantly higher than that of the control strain *E. coli* MG1655 *ibpA-yfp* (pRK767) ($P<0.05$), indicating that the addition of individual fragments 1 and 3 of LHR increased the pressure resistance of *E. coli* significantly. The cell counts of the strain *E. coli* MG1655 *ibpA-yfp* (pLHR) were the highest among all strains, meaning that the full length LHR contributed the most to the pressure resistance of *E. coli*. Cell counts of *E. coli* MG1655 *ibpA-yfp* (pRF2) and the control strain MG1655 *ibpA-yfp* (pRK767) were significantly different after 5 min of treatment but not for other treatment times, demonstrating that the addition of fragment 2 of LHR did not make a major contribution to the pressure resistance of *E. coli*, unless combined with other fragment(s) of LHR.

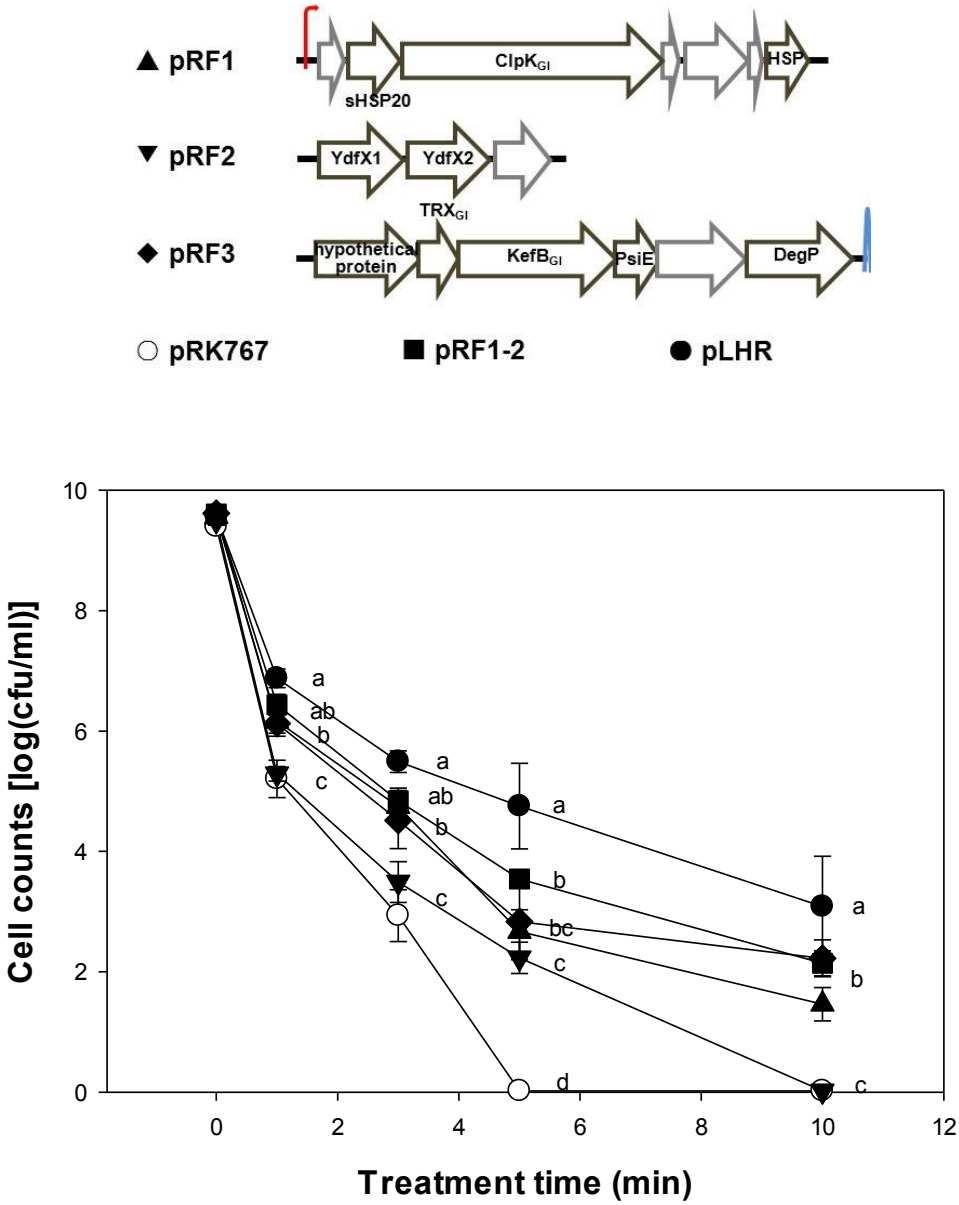

Figure 3-5 Contribution of partial or full of locus of heat resistance (LHR) to pressure resistance of *E. coli* 1655 *ibpA-yfp*. The strains carrying the vector of pRK767 (○) or the derivatives of this vector with the full length LHR (●) or the LHR fragments F1 (▲), F2 (▼), F3 (◆), or F1-2 (■) were treated at 400 MPa at 20 ^{o}C for 1 to 10 min. Statistical analysis was performed for each treatment time: samples labeled with different letters are significantly different ($P<0.05$). All treatments were performed in triplicate.

3.3.5 Contribution of partial cloning of LHR to protein aggregation

Knowing that fragment 1 and 3 of LHR contributed to the pressure resistance of *E. coli* MG1655 *ibpA-yfp*, the effect of the LHR fragments on protein aggregation in cells

was further investigated. After heat treatment at 70 °C for 5 min, no protein aggregation was found in *E. coli* MG1655 *ibpA-yfp* strains containing pLHR or pRF1 (**Table 3-5**), meaning that fragment 1 of the LHR was sufficient to contribute to the protein aggregation phenotype as the full length LHR did. The data for pressure treatment at 400 MPa for 3 min was also provided (**Table 3-5**), however, it is difficult to draw a conclusion because the percentages were too close to be differentiated.

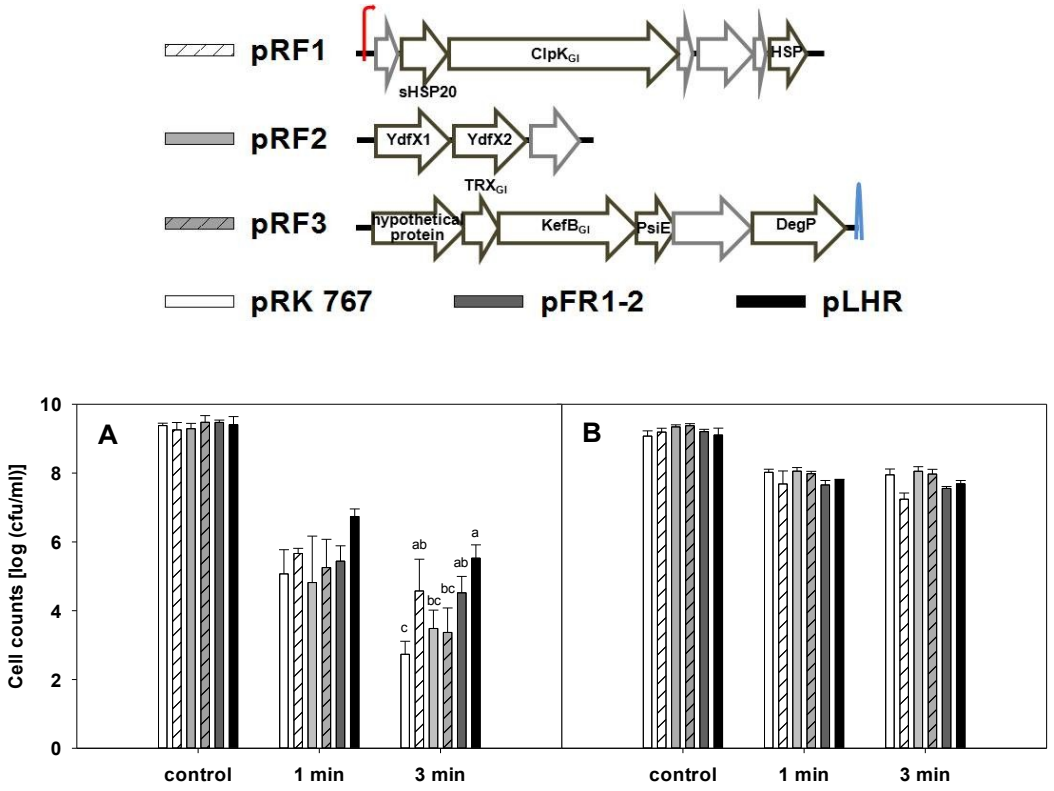

Figure 3-6 Contribution of partial or full of the locus of heat resistance (LHR) to pressure resistance of the wild type strain *E. coli* MG1655 (Panel A) and its derivative strain *E. coli* LMM1010 (Panel B). The strains carrying the vector pRK767 or the derivatives of this vector with the full length LHR or the LHR fragments F1, F2, F3, or F1-2 were treated at 400 MPa and 20 °C for 1 or 3 min. Statistical analysis was performed for each treatment time: samples labeled with different letters are significantly different ($P<0.05$). All treatments were performed in triplicate.

3.3.6 Cloning of LHR to pressure resistance of *E. coli* LMM1010

To determine whether the pressure resistance of strains with the *ibpA-yfp* fusion differs from their wild type strains, the partial or full length LHR was also cloned into *E. coli* MG1655 and LMM1010. For *E. coli* MG1655, fragment 1 and 3 contributed to the pressure resistance as it did in *E. coli* MG1655 *ibpA-yfp* when treated by pressure at 400 MPa for 3 min (**Figure 3-6**A). However, for *E. coli* LMM1010, all strains with cloning of partial or full length LHR showed much higher pressure resistance compared to *E. coli* MG1655 strains (**Figure 3-6**). It is noteworthy that the cell counts of *E. coli* LMM1010 were reduced by only about 1 log (cfu/mL) after treatment at 400 MPa for 3 min, however, the cell counts of *E. coli* LMM1010 *ibpA-yfp* were reduced by about 6 log (cfu/mL) after treatment, providing further evidence that pressure resistance is linked to protein folding.

Table 3-6 Numbers of cells differential from foci in the locus of heat resistance (LHR) negative and positive *E. coli* LMM1010 *ibpA-yfp*.

Foci counts	pRK767	pLHR
0	0.6 ± 0.5%	10.9 ± 7.2%
1	97.6 ± 1.5%	70.1 ± 5.7%
2	1.7 ± 1.5%	6.3 ± 0.9%
>3	0.1 ± 0.2%	12.6 ± 4.1%

Aertsen et al. (2004) found that the induction of heat shock proteins protect against the damage of high pressure and increased the pressure resistance of *E. coli*. Remarkably, the LHR did not do anything in the pressure resistant strain *E. coli* LMM1010, indicating that there are other mechanisms of pressure resistance in *E. coli*

LMM1010. Moreover, this unchanged pressure resistance after cloning of the LHR also occurred in *E. coli* LMM1010 *ibpA-yfp* (data were not shown). Even though the presence of the LHR did not change the pressure resistance of *E. coli* LMM1010 *ibpA-yfp*, it altered protein aggregation in the untreated or heat/pressure treated cells (**Table 3-6** and **Table 3-7**), showing to reduce protein aggregation after treatment (**Table 3-7**).

Table 3-7 Numbers of cells differential from foci after heat or pressure treatment in the locus of heat resistance (LHR) negative and positive *E. coli* LMM1010 *ibpA-yfp*.

Foci	pRK767	pLHR
% of cells with or without protein aggregates after treatment at 60 ^{o}C for 5 min		
+	69.9 ± 11.3	37.4 ± 9.4
-	30.1 ± 11.3	62.6 ± 9.4
% of cells with or without protein aggregates after treatment at 400 MPa for 3 min		
+	83.7 ± 2.2	50.7 ± 2.8
-	16.3 ± 2.2	49.3 ± 2.8

3.4 Discussion

The two methods used in this study were microscopic observation of inclusion bodies and proteomic analysis of inclusion bodies and whole cell extracts. Microscopic observation of inclusion bodies has been successfully used for determining the relationship between pressure resistance and protein folding in previous studies (Govers et al., 2014, 2015). The genetic deteminants of the LHR allows the further study using the cloning of LHR into targeted *E. coli* strains as a complementary tool (Mercer et al., 2015). Proteomic analysis using MS is a new method for protein folding and aggregation, but the fundamentals of this method have been validated by previous

studies (Jürgen et al., 2001; LeThanh et al., 2005). The introduction of *yfp* may have affected the protein aggregation (Govers et al., 2014, 2016); however, this should not be a problem for this study because strains for comparison all included *ibpA-yfp* fusion.

Proteomic analysis showed that a total of 142 proteins were overrepresented in inclusion bodies, 50 proteins were overrepresented in strains with pRK767 and pLHR. To avoid false discoveries, protein overexpression was evaluated with an error probability of 0.1% ($P<0.001$). An overlap of 50 proteins between these two strains is thus highly significant ($P<10^{-50}$). To be noticed, the preparation of inclusion bodies was probably contaminated with membrane proteins, as the comparison of whole cells and inclusion bodies includes many membrane proteins, indicating that membrane vesicles were also enriched by the ultracentrifugation (**Table 3-1**). However, the main conclusions are based on the comparison of proteins in inclusion bodies in strains with pRK767 and pLHR, and this comparison largely excludes membrane proteins.

Proteomic analysis confirmed that the expressed LHR proteins include two small heat shock proteins, two heat shock proteases, proteins of the YfdX family, thioredoxin, and sodium/hydrogen antiporter (Mercer et al., 2015). These proteins are predicted to function against stresses including heat shock, osmotic stress and oxidative stress (Mercer et al., 2015). The contribution of HSP20 to protein folding and turnover has also been confirmed in the homologous gene cluster PACGI-1 in *P. aeruginosa* (Lee et al., 2015). The small heat shock protein sHSP20 and ATP-dependent protease ClpG has been confirmed to contribute to heat resistance in *P. aeruginosa* through their function

as holdases and disaggregating chaperones (Lee et al., 2015, 2016). Thus pressure resistance from LHR was successfully confirmed using the proteomic analysis. Moreover, the expression of 10 proteins of the LHR demonstrated that LHR tended to overexpress especially during heat shock, and oxidative stress, to re-fold protein aggregates and to maintain the ion homeostasis.

The genes related to protein folding and turnover are generally associated with pressure resistance in *E. coli* (Malone et al., 2006; Govers et al., 2014). Govers et al., (2015) found that compared to the average cells, *E. coli* cells with lower degree of protein aggregation had a significantly lower chance to survive after high pressure treatment, indicating that there is a connection between the pressure resistance and protein folding or aggregation. In this study, treatment of pressure reduced the percentage of cells with inclusion bodies (**Table 3-3** and **Table 3-4**), which was consistent with the previous result of pressure dispersing protein aggregation (Aertsen et al., 2014).

Orf 2, orf 3 and orf 7 in the fragment 1 encoded for expressed proteins of sHSP20, $ClpK_{GI}$ and HSP, respectively and the five ORFs out of the six in the fragment 3, expressing proteins of thioredoxin and glutathione-dependent redox system, sodium/hydrogen antiporter and heat shock proteases are responsible for the increased pressure resistance. Many studies have shown that small heat shock proteins prevent protein aggregation by heat (Jakob et al., 1993; Lee et al., 1997; Kitagawa et al., 2000; Mogk et al., 2003). ClpG is an ATP-dependent HSP100 family protein, and it is similar

to ClpB, which interacts with DnaK, or cooperatively supports with small heat shock proteins IbpA/B in reversing protein aggregation in *E. coli* (Mogk et al., 1999; Zolkiewski 1999; Mogk et al., 2003; Lee et al., 2016). However, a zinc finger motif formed from three conserver cysteines and a single histidine in the N-terminal region functions uniquely and differently from ClpB (Lee et al., 2016). The protein of sHSP20 was found to form a sphere-like 24-meric oligomeric structure and exhibits holding chaperone activity, functioning like a bacterial class B small heat shock protein (Lee et al., 2015). Mercer et al. (2015) showed that the fragment 1 of LHR is not sufficient for increasing the heat resistance of *E. coli*, thus proteins expression of sHSP20, $ClpK_{GI}$ and HSP contribute to pressure resistance but not to the heat resistance of *E. coli*. The mutant of *shsp20* was proposed to modestly decrease the heat resistance in *P. aeruginosa* in a previous report (Lee et al., 2015); however, the percentages instead of log_{10} values used for the evaluation of cell counts might lead to a different conclusion. The reason that the fragment 3 also contributes to the pressure resistance should be related to the reductases and the heat shock protease. It has been confirmed oxidative stress induced by high pressure in *E. coli* is one of the major reasons for cell lethality, and proteins including thioredoxin, catalase and superoxide dismutase that function against peroxide and superoxide could increase the pressure resistance of *E. coli* (Aertsen et al., 2005; Malone et al., 2006; Charoenwong et al., 2011; Gänzle and Liu, 2016). Moreover, due to the oxidative stress under pressure, misfolded proteins accumulate in *E. coli* and the periplamic protease DegP expressed by orf 16 functions to

eliminate these misfolded or damaged proteins through the transformation of the structure of itself (Aertsen et al., 2005; Baneyx and Mujacic, 2004; Krojer et al., 2008). Noticeably, the presence of the LHR increased the pressure resistance of the wild type *E. coli* MG1655 but not in its pressure resistant derivative strain *E. coli* LMM1010, indicates that there are some other mechanisms of pressure resistance of *E. coli*.

In conclusion, the LHR confers pressure resistance to *E. coli*, and this phenotype was successfully confirmed using microscopic observation of inclusion bodies and proteomic analysis. The study provided strong evidence that pressure resistance of LHR is related to protein folding and aggregation through the functions for mitigation of protein aggregation.

3.5 References

Aertsen, A., Vanoirbeek, K., Spiegeleer, P.D., Sermon, J., Hauben, K., Farewell, A., Nyström, T., and Michiels, C.W. (2004). Heat shock protein-mediated resistance to high hydrostatic pressure in *Escherichia coli*. *Appl. Environ. Microbiol.* 70, 2660-2666.

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

Baneyx, F., and Mujacic, M. (2004). Recombinant protein folding and misfolding in *Escherichia coli*. *Nature Biotechnol*. 22, 1399-1408.

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

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367-1372.

Cox, J., Neuhauser, N., Michalski, A., Scheltema, R.A., Olsen, J.V., Mann, M. (2011). Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* 10, 1794-1805.

Cox, J., Hein, M.Y., Luber, C.A., Paron, I., Nagaraj, N., Mann, M. (2014). Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol. Cell Proteomics.* 13, 2513-2526.

Croxen, M., Law, R., Scholz, R., Keeney, K., Wlodarska, M., and Finlay, B. (2013). Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clin. Microbiology Rev.*, 26, 822-880.

Frenzen, P.D., Drake, A., Angulo, F.J., and the Emerging Infections Program Foodnet Working Group. (2005). Ecomomic cost of illness due to *Escherichia coli* O157 infections in the United States. *J. Food Prot.* 68, 2623-2630.

Gänzle, M.G., and Liu, Y. (2015). Mechanisms of pressure-mediated cell death and injury in *Escherichia coli*: from fundamentals to food applications. *Front. Microbiol*. 6, 599.

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

Georgious G, Valax P. (1999). Isolating inclusion bodies from bacteria. *Methods Enzymol*, 309, 48-58.

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

Govers, S.K., and Aertsen, A. (2015). Impact of high hydrostatic pressure processing on individual cellular resuscitation times and protein aggregates in *Escherichia coli*. *Int. J. Food Microbiol*. 213, 17-23.

Govers, S.K., Gayan, E., and Aertsen, A. (2016). Intracellular movement of protein aggregates reveals heterogeneous inactivation and resuscitation dynamics in stressed populations of *Escherichia coli*. *Environ. Microbiol*. doi: 10.1111/1462-2920.13460.

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

Hunke, S., and Betton, J. (2003). Temperature effect on inclusion body formation and stress response in the periplasm of *Escherichia coli*. *Mol. Microbiol*. 50, 1579-1589.

Imlay, J.A. (2013). The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. *Nat. Rev. Microbiol*. 11, 443-454.

Jakob, U., Gaestel, M., Engel, K. and Buchner, J. (1993) Small heat shock proteins are molecular chaperones. *J. Biol. Chem*. 268, 1517-1520.

Jürgen, B., Hanschke, R., Sarvas, M., Hecker, M., and Schweder, T. (2001). Proteome and transcriptome based analysis of *Bacillus subtilis* cells overproducing and insoluble heterologous protein. *Appl. Microbiol. Biotechnol*. 55, 326-332.

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

Kitagawa, M., Matsumura, Y., and Tsuchido, T. (2000). Small heat shock proteins, IbpA and IbpB, are involved in resistances to heat and superoxide stresses in *Escherichia coli*. *FEMS Microbiol. Lett*. 184, 165-171.

Krojer, T., Sawa, J., Schäfer, E., Saibil, H.R., Ehrmann, M., and Clausen, T. (2008). Structural basis for the regulated protease and chaperone function of DegP. *Nature*. 453, 885-890.

Lee, G.J., Roseman, A.M., Saibil, H.R. and Vierling, E. (1997). A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *EMBO J*. 16, 659-671.

Lee, C., Wigren, E., Lünsdorf, H., and Römling, U. (2016). Protein homeostasis-more than resisting a hot bath. *Curr. Opin. Microbiol*. 30, 147-154.

Lee, C., Wigren, E., Trcek, J., Peters, V., Kim, J., Hasni, M.S., et al. (2015). A novel protein quality control mechanism contributes to heat shock resistance of worldwide-distributed *Pseudomonas aeruginosa* clone C strains. *Environ. Microbiol*. 17, 4511-4526.

LeThanh, H., Neubauer, P., and Hoffmann, F. (2005). The small heat-shock proteins IbpA and IbpB reduce the stress load of recombinant *Escherichia coli* and delay degradation of inclusion bodies. *Microb. Cell Fact*. 4, 6.

Lindner AB, Madden R, Demarez A, Stewart EJ, Taddei F. (2008). Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. *Proc. Natl. Acad. Sci. U.S.A*. 105, 3076-3081.

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

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

Marti, R., Muniesa, M., Schmid, M., Ahrens, C.H., Naskova, J., and Hummerjohann, H. (2016). *Short communication*: heat-resistant *Escherichia coli* as potential persistent reservoir of extended-spectrum β-lactamases and shiga-toxin-encoding phages in dairy. *J. Dairy Sci*. 99, 8622-8632.

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

Mogk, A., Deuerling, E., Vorderwulbecke, S., Vierling, E., and Bukau, B. (2003). Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. *Mol. Microbiol*. 50, 585-595.

Mogk, A., Tomoyasu, T., Goloubinoff, P., Rüdiger, S., Röder, D., Langen, H., and Bukau, B. (1999). Identification of thermolabile Escherichia coli proteins: prevention and reversion of aggregation by DnaK and ClpB. *EMBO. J.* 18, 6934-6949.

Rosen, R., Biran, D., Gur, E., Becher, D., Hecker, M., Ron, E.Z. (2002). Protein aggregation in *Escherichia coli*: role of proteases. *FEMS Microbiol. Lett*. 207, 9-12.

Strandberg, L., and Enfors, S. (1991). Factors influencing inclusion body formation in the production of a fused protein in *Escherichia coli*. *Appl. Environ. Microbiol*. 57, 1669-1674.

Zhi, S., Banting, G., Li, Q., Edge, T.A., Topp, E., Sokurenko, M., Scott, C., Braithwaite, S., Ruecker, N.J., Yasui, Y., McAllisterm T., Chui, L., and Neumann, N.F. (2016). Evidence of naturalized stress-tolerant strains of *Escherichia coli* in municipal wastewater treatment plants. *Appl. Environ. Microbiol*. 82, 5505-5518.

Zolkiewski, M. (1999). ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. *J. Biol. Chem.* 274, 28083-28086.

CHAPTER 4 **Effect of the food matrix on pressure resistance of Shiga-toxin producing *Escherichia coli***

4.1 Introduction

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

(Garcia-Graells et al., 1998; Lavinas et al., 2008; Liu et al., 2012 and 2015; Reineke et al., 2015).

As pressure processing alone does not sufficiently inactivate STEC, the use of additional antimicrobial hurdles is necessary. The targeted design of improved pressure processes necessitates an improved understanding of the role of matrix constituents on pressure resistance. Multiple pressure-sensitive targets have been described in *E. coli*. Pressure permeabilises the outer membrane of Gram-negative bacteria (Gänzle and Vogel, 2001; Ritz et al., 2000). Pressure also induces a phase transition in the cytoplasmic membrane (Casadei et al., 2002), resulting in 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 also are pressure-sensitive targets in *E. coli* (Niven et al, 1999; Aertsen et al., 2004; Govers et al., 2014). Moreover, pressure induces 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 a strong synergistic bactericidal activity with pressure (Feyaerts et al., 2015).

The use of hurdle technology in food included combinations of pressure with high (40 – 60°C) temperature (Liu et al., 2012, Reineke et al., 2015). However, even elevated temperatures in the range of 40 – 60°C may alter food quality when combined with high pressure (Omama et al., 2011). The pressure treatment at low pH also

eliminates *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 antimicrobial compounds, including thiol-reactive antimicrobials and bacteriocins, was demonstrated in model systems but rarely in food. This study therefore aimed to compare the pressure resistance of *E. coli* in foods and to assess the matrix effect on pressure resistance. Experiments were performed with a cocktail of 5 pathogenic *E. coli* and a cocktail of non-pathogenic strains (Garcia-Hernandez et al., 2015). Moreover, model studies were carried out in buffer systems with the heat- and pressure resistant *E. coli* AW1.7 (Dlusskaya et al., 2011; Liu et al., 2012).

4.2 Materials and Methods

4.2.1 Bacterial strains and culture conditions.

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

subcultured in LB broth and incubated at 37 °C and 200 rpm for 16-18 h. Equal volumes of each of the five single cultures were mixed to form the respective strain cocktails.

4.2.2 Preparation of samples for pressure treatment.

Bruschetta (pH 4.1) and tzatziki (pH 4.0) were obtained from the Food Processing and Development Centre, Leduc of AB, Canada. The formulation of the products is shown in **Table 4-1**. Plain low-fat yogurt (pH 4.0, Astro, Canada) and ground beef (20% fat) were purchased from a local supermarket. Products were used as obtained, or after adjusting the pH to 5.5 or 7.5. Cell counts of each batch of each food product were quantified by surface plating on LB agar; all cell counts were less than 2.6 log(cfu/g). Strain cocktails or the pressure resistant strain *E. coli* AW1.7 (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. 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, USA) filled with 10% bleach.

4.2.3 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 yogurt were enumerated by surface plating on modified de Man Rogosa Sharpe medium. Samples were stored at 4 °C over 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.

Table 4-1 Product composition of bruschetta and tzatziki.

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

4.2.4 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, ON, Canada) buffer at pH 5.5. The food constituents were used at the following concentration: 10 mmol/L calcium chloride (Sigma, Morris plains, NJ, USA), 10 mmol/L magnesium sulfate heptahydrate (Sigma, Morris plains, NJ, USA), 10 mmol/L L-glutamic acid

monosodium salt hydrate (Sigma, Morris plains, NJ, USA), 1 g/L caffeic acid (Sigma, St. Louis, MO, 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.

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

4.2.6 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 4.2.5. Experiments were performed in triplicate.

4.2.7 Effect 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 at 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, ON, 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.

4.2.8 Statistical analysis.

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

4.3 Results

4.3.1 The effects of food matrix on pressure resistance.

The initial interest was on the survival of two pressure resistant strain cocktails of *E. coli* in bruschetta, a tomato-based sauce, and tzatziki, a sauce containing yogurt, cucumbers, and garlic. The products were inoculated with two strain cocktails and treated at conditions matching current industrial practice. Cell counts of both *E. coli* cocktails in bruschetta and tzatziki after pressure treatment were reduced by more than 5 log(cfu/mL) and remained below the detection limit during storage (**Figure 4-1**). Similar cell counts were observed in products inoculated with the cocktail composed of pathogenic strains and the cocktail composed of surrogate strains. Cell counts after pressure treatment were not different from that of the uninoculated control. Moreover, the colony morphology of cells cultured after pressure treatment demonstrated that these counts originated from background microbiota rather than surviving *E. coli* (**Figure 4-1**).

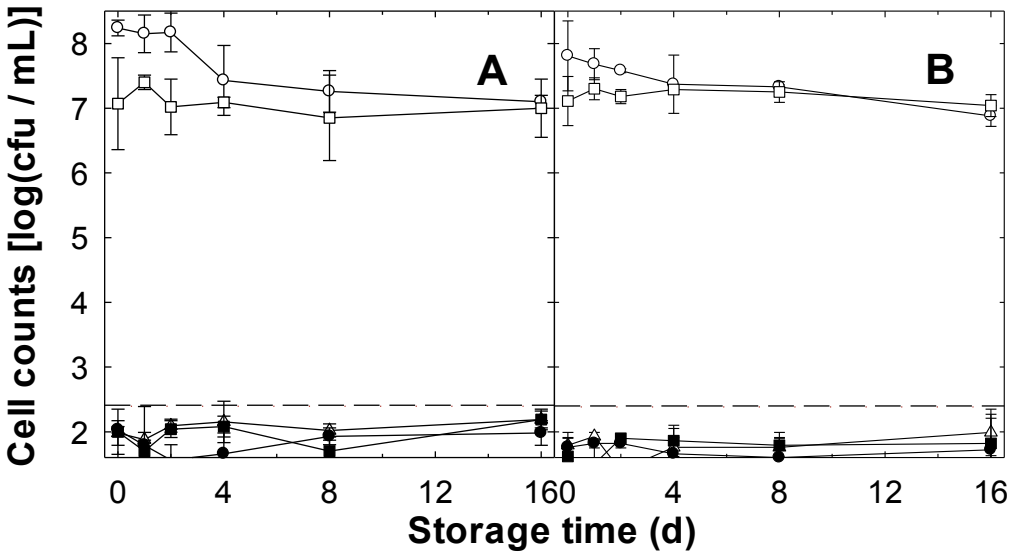

Figure 4-1 Cell counts of bruschetta (Panel A) and tzatziki (Panel B) during storage at 4 °C.The products were inoculated with a surrogate cocktail consisting of 5 non-pathogenic strains of *E. coli* (●) or a pathogenic cocktail consisting of 4 strains of STEC and one EPEC (■). Uninoculated product was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

The sensitivity to pressure of the two strain cocktails in bruschetta and tzatziki was greater when compared to the survival of the same cocktails in beef (Garcia-Hernandez et al., 2015). To determine whether the low pH accounts for this difference, the pH of bruschetta and tzatziki was adjusted to 5.5, equivalent to the pH of ground beef. Bruschetta was inoculated with the two strain cocktails; tzatziki was inoculated only with the non-VTEC cocktail. Products were subjected to pressure treatment, followed by refrigerated storage (**Figure 4-2**). Treatments in ground beef served as comparison

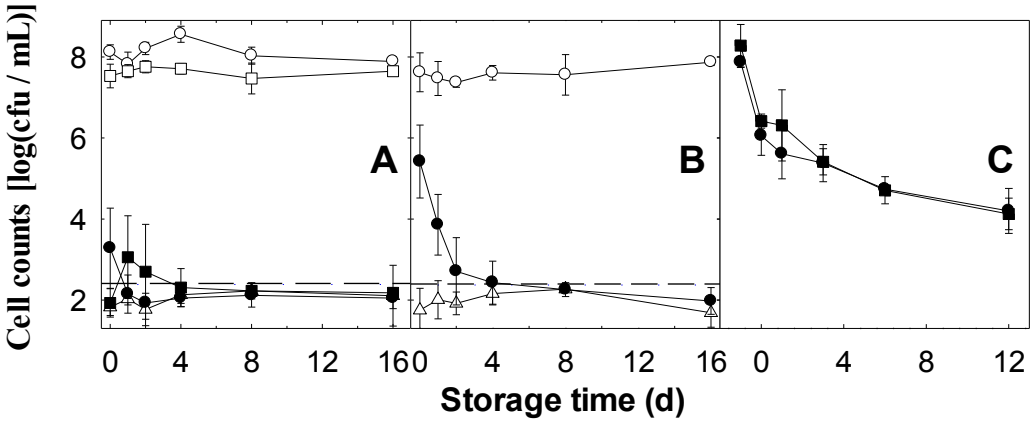

Figure 4-2 Cell counts of bruschetta (**Panel A**), tzatziki (**Panel B**) and ground beef (**Panel C**) during storage at 4°C.The pH of bruschetta and tzatziki was adjusted to 5.5 prior to inoculation and treatment to match the pH of ground beef. The products were inoculated with a surrogate cocktail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

(**Figure 4-2**C). Increasing the pH increased pressure resistance of *E. coli* slightly (bruschetta, **Figure 4-2**A) or substantially (tzatziki, **Figure 4-2**B). 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 storage while cell counts of *E. coli* in ground beef were reduced by less than 90%. These results demonstrate that the food matrix differentially affects

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

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, and with plain yogurt after adjustment to pH 4.0 (unadjusted), 5.5, and 7.5. The two strain cocktails composed of pathogenic and non-pathogenic strains exhibited similar survival during and after pressure treatment in previous experiments, and among the surrogate strains, *E. coli* AW 1.7 is a typical pressure resistant strain. Therefore, subsequent experiments were carried out only with *E. coli* AW 1.7. Adjusting the pH of bruschetta and tzatziki to 7.5 did not substantially alter the lethality of pressure treatment (**Figure 4-2** and data not shown). Pressure treatments in yogurt demonstrated the effect of pH on the lethality of pressure and post-pressure refrigerated storage (**Figure 4-3**). At pH 4.0, pressure treatment reduced cell counts of *E. coli* by more than 5 log(cfu/mL). At pH 5.5, the resistance of *E. coli* to pressure was substantially increased but cell counts were reduced to less than 2 log(cfu/mL) after 4 d of refrigerated storage. Treatments in yogurt at pH 7.5 did not change the lethality of pressure when compared to treatments at pH 5.5, however, cell counts remained unchanged during storage (**Figure 4-3**). 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*.

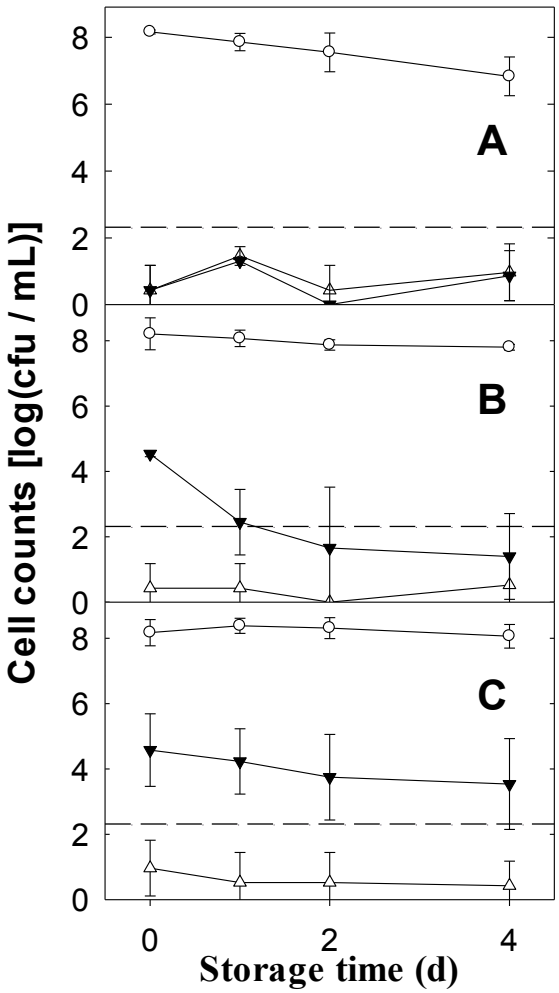

Figure 4-3 Cell counts of yoghourt during storage at 4°C. The initial pH of yogurt was 4.0 **(Panel A)**; the pH was also adjusted to 5.5 **(Panel B)** or 7.5 **(Panel C)** prior to inoculation and treatment. Products were inoculated with *E. coli* AW 1.7. Uninoculated product was used as control (△).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

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

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, model experiments were designed with or without addition of individual compounds. Calcium, magnesium, and glutamate were chosen because they were suggested to exert protective effects (Niven et al., 1999; Kilimann et al., 2005) and occur in meat or dairy products but at a lower concentration in plant foods. Acetic and caffeic acids were selected as antimicrobial organic acids with a potential synergistic effect (Sanchez-Maldonado et al., 2011) that are present in plant foods. Experiments in buffer systems were carried out with *E. coli* AW1.7 as pressure-resistant model organism. Addition of magnesium, or glutamate protected *E. coli* AW 1.7 against pressure-induced inactivation (**Figure 4-4**A). Surprisingly, caffeic acid and acetic acid also protected *E. coli* after 16 min of treatment when compared to the control without additives (**Figure 4-4**A). 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* (**Figure 4-4**B).

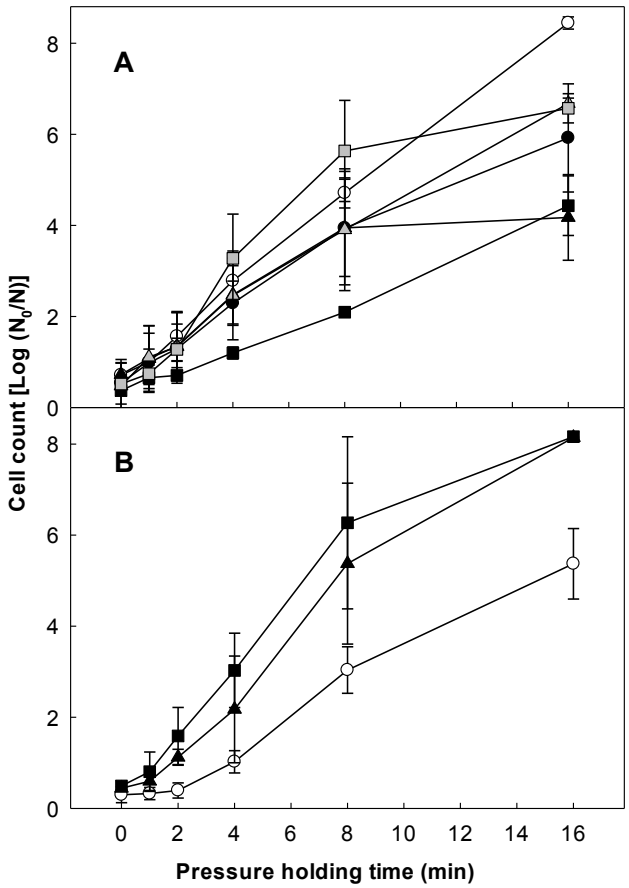

Figure 4-4 Cell counts of *E. coli* AW1.7 after pressure treatment in buffer (pH 5.5) with or without additions of food constituents. For Panel A, the following compounds were added to the MES buffer: 10 mmol/L calcium (●), 10 mmol/L magnesium (▲), 10 mmol/L glutamate (▲), 1 g/L acetic acid (■) or 1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

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

Because individual food products differentially affected the resistance of *E. coli* during pressure treatment and post-pressure refrigerated storage, the role of selected food constituents were additionally explored 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. None of these compounds affected survival of *E. coli* after 3 min at 600 MPa (**Figure 4-5**). However, cell counts of *E. coli* in buffer at pH 5.5 were reduced by more than 5 log(cfu/mL) over 12 days of post-pressure refrigerated storage (**Figure 4-5**A and B). Survival was improved by the addition of calcium or magnesium (**Figure 4-5**A); other compounds had no effect on survival of *E. coli* after pressure treatment (**Figure 4-5**B).

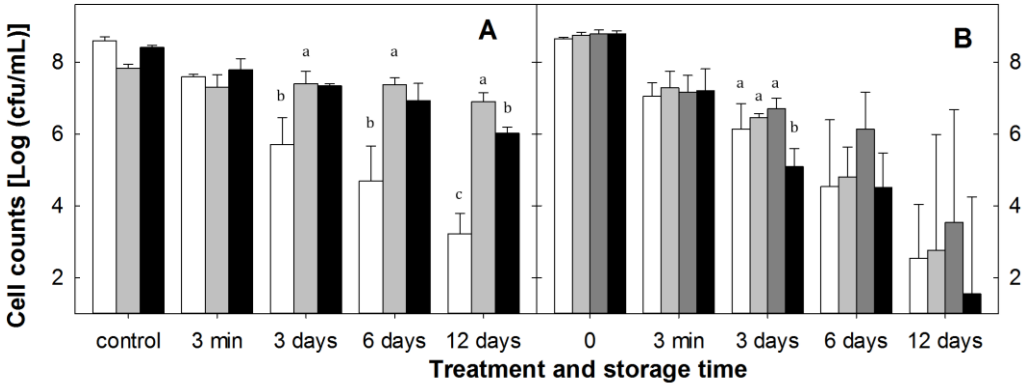

Figure 4-5 Cell counts of *E. coli* AW1.7 after treatment at 600 MPa for 3 min at 20°C in imidazole buffer at a pH of 5.5.Treatments were performed in buffer without additives (white bars) or with addition of 10 mM calcium (grey bars), magnesium (black bars) **(Panel A)**, or with addition of glutamine (light grey), glutamate (dark grey), or glutathione (black) (**Panel B**). Viable cell counts were enumerated on LB agar before treatment, after 3 min pressure treatment, and after pressure treatment and 3, 6, or 12 days of refrigerated storage. Without pressure treatment, cell counts of *E. coli* were not significantly reduced during refrigerated storage; survival of *E. coli* in controls shown in Panel A and B was not significantly different. 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 ($P<0.05$). *Note: Experiments were performed by Rigoberto Garcia-Hernandez.

4.3.4 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, NPN was used to probe the integrity of the outer membrane of *E. coli* AW1.7 that was pressure treated in the presence or absence of 10 mmol/L calcium (**Table 4-2**). Pressure fully permeabilised outer membrane of *E. coli* after treatment with 300 MPa or higher (**Table 4-2**). The addition of calcium did not influence the permeability of the outer membrane of pressure treated cells.

Table 4-2 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. *Note: Experiments were performed by Rigoberto Garcia-Hernandez.

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.

4.4 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; Garcia-Hernandez et al., 2015; Reineke, et al., 2015; Gänzle and Liu, 2015). 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 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). The strain cocktail composed of surrogate non-pathogenic strain is thus useful for validation of pressure processes in a wider range of products. However, it was also demonstrated that the lethality of the same pressure treatment on the same strains differs by up to 4 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 and thus impairs the ability to maintain a transmembrane pH gradient (ΔpH) (Wouters et al., 1998, Kilimann et al., 2005). The pressure mediated loss of pH gradients and acid resistance eliminates *E. coli* in acidic food products after pressure treatment (Garcia-Graells et al., 1998; Jordan et al., 2001, Pagán et al., 2001). It was 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. The 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 & Storz, 2000), and may thus counteract the pressure mediated "oxidative suicide" of *E. coli*

(Aertsen et al., 2005, Malone et al., 2006). Meat but not dairy products or tomatoes are rich in low-molecular weight thiols. However, glutathione did not change the pressure resistance or the post-pressure survival of *E. coli*. Caffeic acid, used as a representative of antimicrobial plant phenolic compounds, and acetic acid, a food preservative, exhibited a modest protective effect on pressure resistance of *E. coli*. Organic acids alter 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 concentration which exceeds the minimum inhibitory concentration (MIC) against *E. coli* AW1.7 (Sánchez-Maldonado et al., 2011). Acidification of the cytoplasm by undissociated caffeic acid (Choi and Gu, 2001; Cueva et al., 2010; Sánchez-Maldonado et al., 2011) may 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*. Glutamate decarboxylation consumes intracellular protons, exports negative charges and thus contributes to the generation of the pmf (Foster 2004;

Feehily and Karatzas, 2012; Teixeira et al., 2014). Glutamate mediated acid resistance 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 also consumes an intracellular proton (Lu et al., 2013). Surprisingly, glutamate addition did not affect post-pressure survival of *E. coli*. Refrigerated storage of *E. coli* may have reduced the rate of glutamate decarboxylation; prior studies incubated *E. coli* at a temperature permitting growth and metabolism (Kilimann et al., 2005).

The accumulation of cyclopropane fatty acids in the membrane of *E. coli* increases its pressure resistance (Casadei et al., 2002; Charoenwong et al., 2011). Ethanol and phenylethanol fluidize the membrane and thus antagonize pressure effects on bacterial membranes (Welch and Bartlett, 1998; Huffer et al, 2011); however, membrane-bound proteins are more sensitive to pressure-mediated denaturation when embedded in a liquid crystalline membrane (Ulmer et al., 2002).

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 data in this study conform with Hauben et al., (1998) who concluded that the protective effect of Ca^{2+} is not related to the stabilization of the outer membrane. Prior data was extended 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. 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).

Ground beef has much higher fat content (20%), which was much higher than fat content in bruschetta and tzatziki. However, fat content has little or no influence on the pressure resistance of *E. coli*. For example, ovine milk with 6 and 50% of fat did not improve the pressure resistance of all microorganisms including *E. coli*, *Pseudomonas fluorescens*, *Listeria innocua*, *Staphylococcus aureus* and *Lactobacillus helveticus* (Gervilla et al., 1999). Therefore, evaluation of fat content was not performed in this work.

In conclusion, the food matrix strongly influenced the pressure-mediated inactivation of STEC and EPEC. The product pH influenced both the survival of *E. coli* during pressure treatment and the survival after pressure treatment. However, differences in the product pH did not explain 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 the 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.

4.5 References

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

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

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

Carmel-Harel, O., Storz, G. (2000). Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Ann. Rev. Microbiol.* 54, 439-461.

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

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

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

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

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

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

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

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

Frenzen, P.D., Drake, A., Angulo, F.J., and the Emerging Infections Program Foodnet Working Group. (2005). Ecomomic cost of illness due to *Escherichia coli* O157 infections in the United States. *J. Food Prot*. 68, 2623-2630.

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

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

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

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. Int. J. Food Microbiol*. 205, 16-22.

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

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

Gervilla, R., Ferragut, V., and Guamis, B. (1999). High pressure inactivation of microorganisms inoculated into ovine milk of different fat contents. *J. Dairy Sci*. 83, 674-682.

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

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

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

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

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

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

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

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

Keweloh, H., Diefenbach, R., Rehm, H. (1991). Increase of phenol tolerance of *Escherichia coli* by alternations of the fatty acid composition of the membrane lipids. *Arch. Microbiol*. 157, 49-53.

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

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

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

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

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

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

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

Molina-Gutierrez, A., Stippl, V., Delgado, A., Gänzle, M.G., Vogel, R.F. (2002). In situ determination of the intracellular pH of *Lactococcus lactis* and *Lactobacillus plantarum* during pressure treatment. *Appl. Environ. Microbiol*. 68, 4399-4406.

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

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

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

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

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

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

Sánchez-Maldonado, A.F., Schieber, A., Gänzle, M.G. (2011). Structure-function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. *J. Appl. Microbiol*. 111, 1176-1184.

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

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

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

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

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

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

CHAPTER 5 **Effect of hydrostatic pressure and antimicrobials on survival of *Listeria monocytogenes* and enterohaemorrhagic *Escherichia coli* in beef**

5.1 Introduction

Meat marination is used to improve the taste and tenderness of meat products by immersing them in solutions containing sodium chloride, polyphosphates, sugars and other ingredients (Verbeke et al., 2010; Vlahova-Vangelova & Dragoev, 2014). Effects of marinades on the microbiota of meat depend on the ingredients. Marination with salt, phosphates and spices alone has little effect on the survival of pathogens on meat but shifts spoilage microbiota towards growth of some psychrotrophic lactic acid bacteria (Björkroth 2005). Extension of the shelf life of marinated meats and the reduction of pathogen levels thus necessitates the combination of marination and antimicrobial ingredients or pressure processing (Wang et al., 2015). Marination mitigates pressure effects on meat quality, discoloration and lipid oxidation (Buckow et al., 2013), when the marinade is formulated with coloring and anti-oxidant ingredients. Marination in combination with treatment at 450 MPa lowered the expressible moisture of beef steaks, and extended their shelf life to 85 days without adverse effect on meat quality. Treatment at 500 or 600 MPa negatively affected meat texture and color with no further increase in shelf life (Wang et al., 2015).

Treatment at 600 MPa was suggested to control risks associated with *E. coli* and *L. monocytogenes* in marinated beef loins (Hugas et al., 2002; Jofré et al., 2009); however,

treatment of meat does not eliminate pressure resistant strains of *E. coli* or *L. monocytogenes* (Liu et al., 2012 and 2015; Marcos et al., 2008). Marinating may allow enhancing the pressure inactivation of microorganisms by adding antimicrobial compounds in meat. Among antimicrobial compounds used in meat preservation, essential oils have received increased interest owing to their antimicrobial activity, their synergistic activity with pressure, and because they allow marketing of "clean label" meat products (Feyaerts et al., 2015; Gayán et al., 2012). Synergistic effects of antimicrobial compounds depend on the environment and treatment conditions (Karatzas et al., 2001; Espina et al., 2013, Hofstetter et al., 2013). Pressure induces endogenous oxidative stress in bacteria (Aertsen et al., 2005; Gänzle & Liu, 2015; Malone et al., 2006) which contributes to cell death during and after pressure treatment. Synergistic interaction of antimicrobial compounds with pressure was suggested to depend on the reactivity of the antimicrobial compound with thiols (Feyaerts et al., 2015). The synergistic activity of thiol reactive antimicrobials and pressure, however, has not been described in food applications.

Shiga-toxigenic *Escherichia coli* (STEC) are associated with beef (Frenzen et al., 2005; Karch et al., 2005) and causes severe disease with an infectious dose of less than 10 cells (Paton et al., 1996; Tilden et al., 1996). *L. monocytogenes* also occurs in fresh meat and meat products (Frenzen et al., 2005; Sofos, 2008). The objective of this study was to determine the effect of meat marination on the lethality of pressure on pathogenic *E. coli* and *L. monocytogenes*, and to assess the combined effect of

antimicrobials and pressure. Pressure was applied at a level of 450 MPa, providing optimal quality of marinated beef steaks (Wang et al., 2015), or at 600 MPa, the current upper limit of equipment used in food processing.

5.2 Methods and Materials

5.2.1 Bacterial strains and culture conditions.

The *L. monocytogenes* strain cocktail was composed of strains FSL J1-177, FSL C1-056, FSL N3-013, FSL R2-499, FSL N1-227 (Fugett et al. 2006). *E. coli* strains were selected according to Garcia-Hernandez et al. (2015). The cocktail of pathogenic strains was composed the *eae*-positive STEC strains 05-6544 (O26:H11), 03-2832 (O121:H19), 03-6430 (O145:NM) and C0283 (O157:H7), and the *stx*-negative enteropathogenic *E. coli* (EPEC) PARC 449. These strains were selected from more than 100 *E. coli* strains to represent the most pressure resistant strains (Liu et al., 2015). For reasons pertaining to laboratory safety, and to expand the study to a larger number of strains, experiments with pathogenic *E. coli* were complemented with experiments using a strain cocktail of non-pathogenic surrogate strains that was composed of *E. coli* AW1.7, AW1.3, GM16.6, DM18.3 and MG1655 and had a comparable resistance to pressure as the cocktail composed of pathogenic strains (Garcia Hernandez et al., 2015; Chapter 4). The pressure resistant *E. coli* strain AW 1.7 was also used singly as a model organism.

Stock cultures of *Listeria monocytogenes* were streaked onto PALCAM agar (Oxoid, Basingstoke, Hants, England) at 35 °C, and subcultured at 35 °C for 20-24 h

with 200 rpm agitation in Tryptone Soy broth (BD, Sparks, MD, USA). *E. coli* were streaked onto Luria-Bertani (LB, Difco, Sparks, MD, USA) agar at 37 °C, and subcultured at 37 °C for 16-18 h with 200 rpm agitation in LB broth. Equal volumes of single cultures were mixed to form the respective strain cocktails.

5.2.2 Meat products, marinades and chemicals.

Lean ground beef (15% fat) and beef steaks were provided by a federally inspected meat processing facility. Beef steaks were surface-decontaminated by flaming with ethanol and removal of the denatured surface with a sterile knife. Steaks were then cored perpendicular to the muscle fibres with a sterile corer with a diameter of 4.8 mm to obtain aseptic cuts with a thickness of 2 cm. Ground beef was obtained on the day of processing and stored frozen at -20°C. Cell counts of uninoculated ground beef were determined by plating on LB agar; the cell counts were below the detection limit of 200 cfu/g. Two marinades (honey garlic and teriyaki) were provided in powder form by the Food Processing Development Centre, Leduc, AB, Canada. The ingredients of the marinade mix are listed in **Table 5-1**; marination according to the supplier's suggestions increases the NaCl concentration to 1%. Carvacrol, thymol and allyl isothiocyanate (AITC) were purchased from Fisher Scientific (Mullica Hill, NJ, USA); cinnamaldehyde was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Table 5-1 Ingredients of honey garlic and teriyaki marinades.

Marinade	Ingredients
Honey Garlic	Sugar, salt, fructose, honey powder (honey, wheat starch, soy flour, soy lecithin), granulated garlic, sodium phosphates, soy sauce powder (soy sauce from wheat and soybeans, corn maltodextrin), garlic powder, caramel, calcium silicate, spices, monounsaturated vegetable oil, artificial flavor.
Teriyaki	Sugar, salt, soy sauce powder (soy sauce from wheat and soybeans, corn maltodextrin), sodium phosphates, flavor, caramel, garlic powder, onion powder, spices, xanthan gum, monounsaturated vegetable oil, sulphites.

5.2.3 Preparation of marinated meat for pressure treatment.

Meat was inoculated with *L. monocytogenes* or *E. coli* by dipping beef steaks into cell suspensions for 15 seconds, or by mixing 1 mL of cell suspensions with 10 g of ground beef thoroughly. The initial population of *L. monocytogenes* or *E. coli* ranged from 10^7 to 10^8 cfu/mL. Inoculated meat was mixed with the marinade mix (honey garlic or teriyaki) and water at a proportion of 83.3%, 5.7% and 11.0% (w/w), packed into 3-cm R3603 tygon tubes (Akron, PA, USA), and heat-sealed. To prevent contamination of the pressure equipment with pathogens, the packaged samples were inserted into 2-mL cryovials (Wheaton, Millville, NJ, USA) filled with 10% bleach.

5.2.4 Pressure treatment.

Pressure treatments were carried out in a Multivessel Apparatus U111 (Unipress Equipment, Warsaw, Poland) as described previously (Liu et al., 2012). Beef steaks were treated with 450 MPa for 3 min; ground beef and buffer were treated at 600 MPa for 3-15 min. The time of compression was 45-60 s; the pressure transmission fluid was glycol. The temperature of the pressure vessels was maintained at 20 °C by a thermostated jacket, and monitored by an internal thermocouple. Temperature changes

during compression and decompression were 2 °C or less. After pressure treatment, samples were immediately taken for microbial analysis, or removed to refrigerated storage at 4 °C over 16 days. Experiments were performed in triplicate.

5.2.5 Enumeration of *Listeria monocytogenes* and *E. coli* in pressure treated steaks.

After treatments at 450 MPa, 20 ^{o}C for 3 min, cell counts were enumerated by surface plating onto non-selective agar to allow recovery of injured cells, and selective agar to suppress the recovery of injured cells. *L. monocytogenes* was enumerated by plating onto Tryptone Soy agar and PALCAM agar with selective supplement; *E. coli* was enumerated by plating onto LB and Violet Red Bile agar (Difco, Sparks, MD, USA). No contaminating microbiota were observed in uninoculated control steaks, and the colony morphology of samples matched the colony morphology of the inoculum in all samples before treatment, after treatment, and after treatment and storage.

5.2.6 Selection of antimicrobials and determination of concentrations.

Compounds with two different mechanisms of antibacterial activity were selected to investigate their combined effect with pressure on inactivation of *E. coli*. Carvacrol and thymol were selected as membrane-active compounds. AITC and cinnamaldehyde were selected as thiol reactive compounds. Stock solutions of the four compounds were prepared by mixing with ethanol in a ratio of 1:1 (v/v). The concentrations for their application were determined in 100 mM MES (Fisher, Ottawa, ON, Canada) buffer at pH 5.5. At the ambient temperature, stock solution of each compound was added into

the buffer to achieve the following concentrations: 0.01, 0.025, 0.04, 0.06, 0.08, 0.10, 0.15, 0.20, 0.25 and 0.30% (v:w). Each dilution was inoculated with cells from an overnight culture of *E. coli* AW1.7 to an initial cell count of around 10^8 cfu/mL, and incubated at 20 °C for 4 h. After incubation, cell counts in each sample were obtained by surface plating of appropriate dilutions on LB agar. Sample with inoculation of *E. coli* but without addition of antimicrobials was used as a control. For further applications, the highest concentration of antimicrobial compounds causing inactivation of less than 1 log (cfu/mL) was chosen. These concentrations were as 0.04%, 0.025%, 0.15%, and 0.10% (v:w) for carvacrol, thymol, AITC, and cinnamaldehyde, respectively.

5.2.7 Effects of antimicrobials with pressure on *E. coli* in buffer and raw ground beef.

Experiments were carried out in 100 mM MES (Fisher, Ottawa, ON, Canada) buffer, pH 5.5, or with ground beef. Carvacrol, thymol, AITC and cinnamaldehyde stock solutions were added into MES buffer (pH 5.5) or ground beef to a final concentration of 0.04%, 0.025%, 0.15%, and 0.10% (v:w), respectively. Samples with ethanol but without essential oils were used as a control. Samples were inoculated with *E. coli* and treated with 600 MPa at 20^{o}C for 3 or 6 min. Cell counts were obtained after surface plating of appropriate dilutions on LB agar. Data are shown as mean ± standard deviation based on the three independent experiments.

5.2.8 Effects of antimicrobials with HHP on *E. coli* in marinated meat.

Experiments were carried out with marinated ground beef and marinated beef steaks. For investigation of essential oils, the marinade of honey garlic was prepared at the ratio of 5.7: 11 by weight (powder: water). Carvacrol, AITC and cinnamaldehyde were dissolved in ethanol and added to the honey garlic marinade. Meat was inoculated with *E. coli* to initial cell counts of about 10^7-10^8 cfu/mL. Honey garlic marinade supplemented with essential oils was then mixed with meat at a ratio of 16.7: 83.3 (marinade: meat) by weight. The resulting final concentrations of carvacrol, AITC and cinnamaldehyde were 0.04 or 0.10, 0.06 or 0.15, and 0.10% (v:w), respectively. Marinated meat was treated by high pressure at 450 MPa, 20^{o}C for 3 min. Cell counts were obtained after surface plating of appropriate dilutions on LB agar. Data are shown as mean ± standard deviation based on the three independent experiments.

5.2.9 Statistical analysis

Significant differences between two treatments were determined using Student's *t* test; significant differences between more than two treatments were determined using one way ANOVA with the Holm-Sidak method for pairwise multiple comparison. Significance was assessed at an error probability of 5 % ($P<0.05$).

5.3 Results

5.3.1 Effect of marination on survival of *L. monocytogenes* and *E. coli* during pressure treatment of beef steaks.

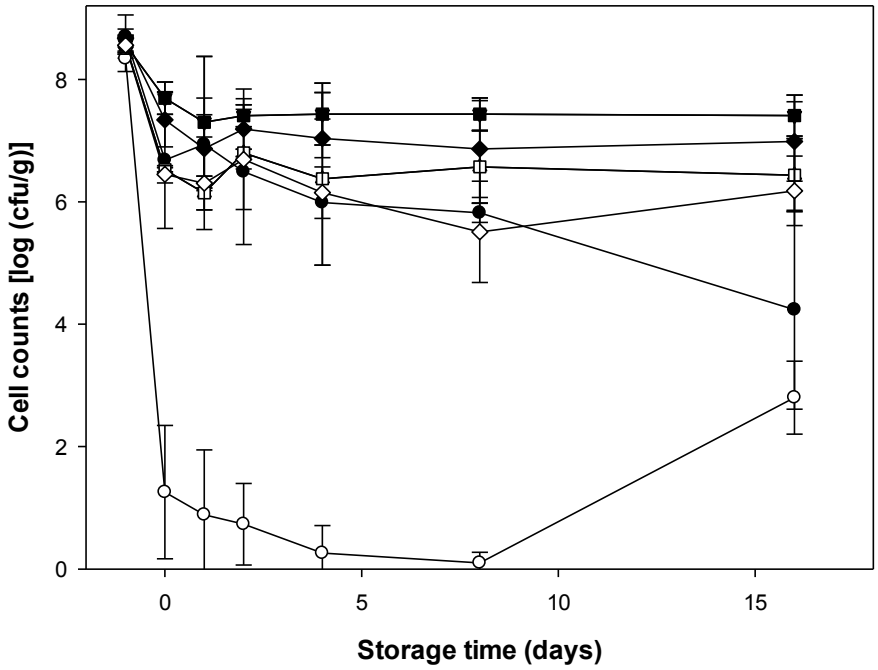

Figure 5-1 The effect of meat marination on survival of a 5 strain *Listeria monocytogenes* cocktail in beef steaks after pressure treatment.Samples were treated at 450 MPa and 20^{o}C for 3 min, and stored at 4^{o}C for 16 days after treatment. Prior to treatment, steaks were marinated with honey garlic (■) or teriyaki (◆) marinades. Un-marinated steaks were used as control (●). Cell counts were obtained on Tryptone Soy agar (closed symbols) or PALCAM agar (open symbols), respectively. Data are shown as mean ± standard deviation based on three independent experiments.

To determine the effect of meat marination on the lethality of pressure, cell counts of *L. monocytogenes* and *E. coli* cocktails were determined in beef steaks that were marinated with two different marinades. Beef steaks were treated by pressure at 450 MPa and 20°C for 3 min, conditions which significantly extend the shelf life without adverse effect on meat quality (Wang et al., 2015). Pressure treatment reduced cell

counts of *L. monocytogenes* on marinated beef steaks by 90% (**Figure 5-1**). Marinationdid not influence the survival of *L. monocytogenes* during pressure treatment but improved survival during storage. Cell counts of un-injured cells obtained on selective agar were lower in control steaks when compared to marinated steaks. During storage, cell counts of *L. monocytogenes* in marinated steaks remained stable. In control steaks, total cell counts tended to decrease while counts of un-injured cells increased, indicating that sublethally injured cells died or recovered during storage (**Figure 5-1**).

Pressure treatment at 450 MPa reduced cell counts of the cocktail of pathogenic *E. coli* by about 99% (**Figure 5-2**). Meat marination did not influence survival of *E. coli* during or after pressure treatment and cell counts remained essentially unchanged over 16 days of refrigerated storage.

5.3.2 Effects of antimicrobials on *E. coli* AW1.7 in buffer.

Both *L. monocytogenes* and *E. coli* cocktails showed high resistance to pressure in marinated beef steaks. Subsequent studies explored the use of clean label antimicrobial compounds to enhance the lethal effect of pressure. These experiments were carried out in MES buffer with the pressure resistant model organism *E. coli* AW 1.7. Pressure treatments were carried out at 600 MPa and 20°C to match current industrial practice for pressure treated food, and to allow sensitive detection on synergistic or antagonistic activity (**Figure 5-3**). The four antimicrobial compounds

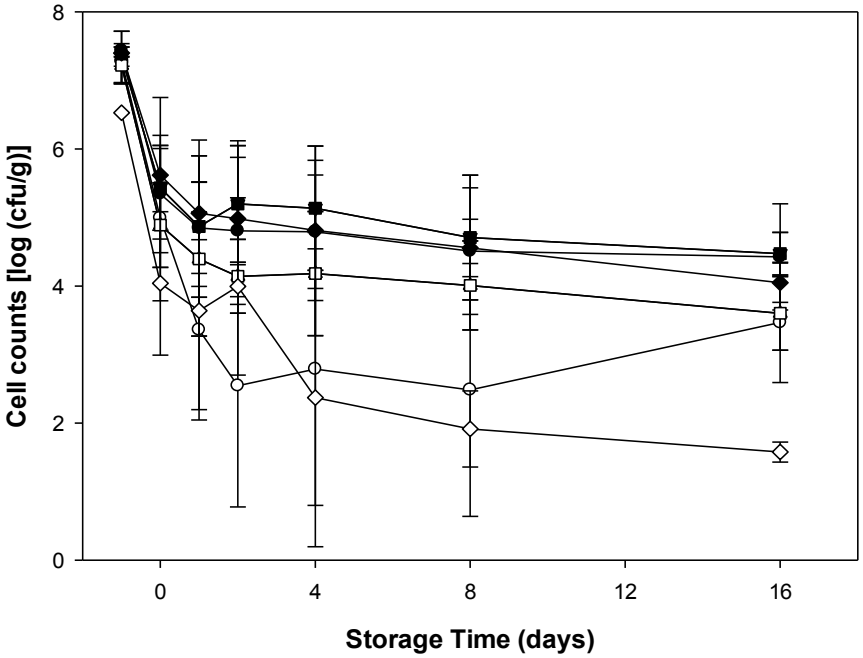

Figure 5-2 The effect of meat marination on survival of a cocktail of 5 pathogenic strains of *E. coli* on beef steaks after pressure treatment.Samples were treated at 450 MPa, 20^{o}C for 3 min, and stored at 4^{o}C for 16 days after treatment. Prior to treatment, steaks were marinated with honey garlic (■) or teriyaki (◆) marinades. Un-marinated steaks were used as control (●). Cell counts were obtained on Luria-Bertani agar (closed symbols) or Violet Red Bile agar (open symbols). Data are shown as mean ± standard deviation of three independent experiments.

were applied at the level of their respective minimum bactericidal concentrations. Both AITC and cinnamaldehyde showed strong synergistic activity with pressure (**Figure 5-3**). For example, treatments in the presence of AITC or cinnamaldehyde increased the lethality of pressure by about 5 and 3 log(cfu/mL), respectively (**Figure 5-3**). Thymol addition at 0.025% had no effect on the survival of *E. coli*. Carvacrol at a concentration of 0.025% reduced the cell counts of *E. coli* by around 1 log, however, cell counts after combined application of carvacrol and 600 MPa were not different from those obtained after treatment with 600 MPa without addition of carvacrol (data not shown). Addition of 0.04% carvacrol reduced cell counts of *E. coli* to levels below the detection limit in

untreated samples (**Figure 5-3**). In pressure treated samples with the addition of 0.04% carvacrol, however, *E. coli* was reduced by less than 5 log (cfu/mL).

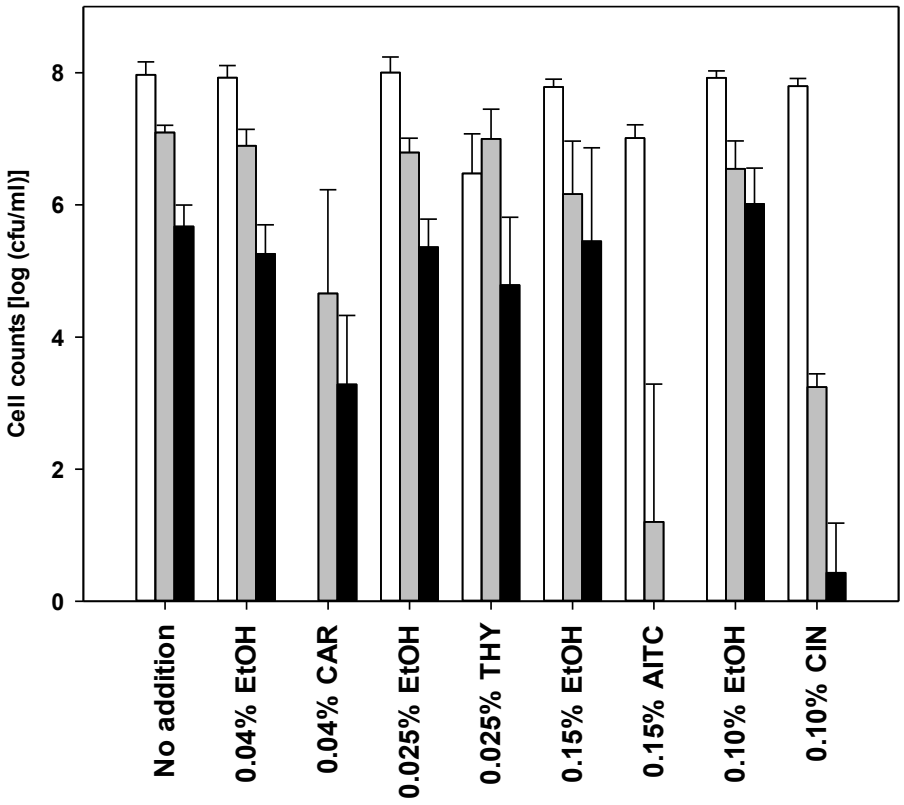

Figure 5-3 Effects of carvacrol (CAR), thymol (THY), allyl isothiocyanate (AITC), and cinnamaldehyde (CIN) on the pressure resistance of *E. coli* AW 1.7 in MES buffer (pH 5.5).The four compounds were dissolved in ethanol (1:1 v/v), and added into MES buffer to a final concentration of 0.025-0.15% before inoculation. Samples with only ethanol (EtOH) but without essential oils were used as controls. Samples were treated at 600 MPa and 20^{o}C for 3 (gray bars) or 6 (black bars) min. Cell counts of untreated controls are shown as white bars. Surviving cells were enumerated on Luria-Bertani agar. Data are shown as mean ± standard deviation based on three independent experiments.

5.3.3 Effects of antimicrobials on *E. coli* AW1.7 in ground beef.

To determine whether the synergistic activity of AITC and cinnamaldehyde is also observed in a meat matrix, these antimicrobials were added to ground beef inoculated with *E. coli* AW1.7. Samples were treated at 600 MPa for 3 or 6 min (**Figure 5-4**).

AITC (0.15%) showed a synergistic effect on pressure inactivation of *E. coli* at the treatment for 6 min, however, the effect was less pronounced when compared to the effect observed in buffer (**Figure 5-3** and **Figure 5-4**). Addition of 0.1% cinnamaldehyde did not affect the pressure inactivation of *E. coli* in ground beef.

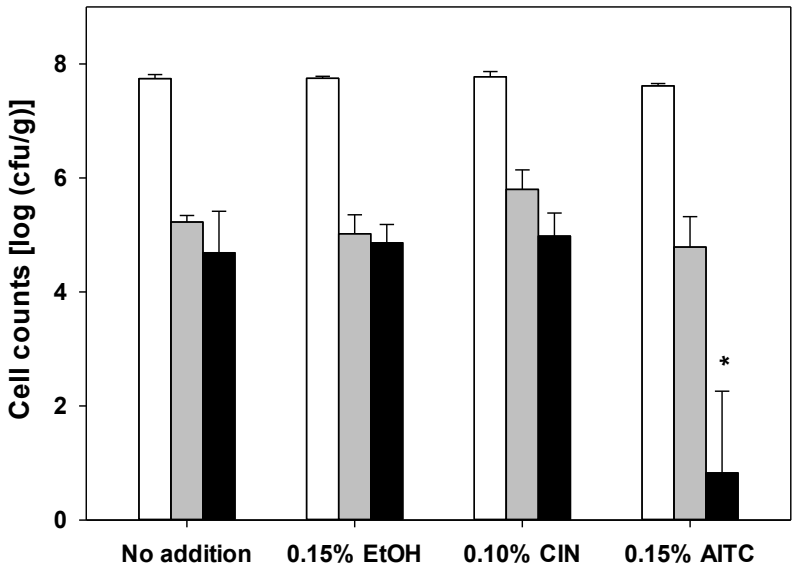

Figure 5-4 Effects of cinnamaldehyde and allyl isothiocyanate (AITC) on cell counts of *E. coli* AW 1.7 in ground beef after pressure treatments. Before inoculation, the essential oils were diluted with ethanol (1:1 v/v), and added into ground beef to a final concentration of 0.10% and 0.15%, respectively. Samples with addition of ethanol (EtOH) served as control. Samples were treated at 600 MPa and 20^{o}C for 3 min (gray bars) or 6 min (black bars). Untreated samples are shown as white bars. Surviving cells were enumerated on Luria-Bertani agar. Data are shown as mean ± standard deviation of three independent experiments. Significant differences (p<0.05) between treatments and the corresponding controls performed with addition of 0.15% ethanol is indicated by an asterisk.

Effects of carvacrol and thymol were also investigated in ground beef. The addition of thymol and carvacrol to ground beef at concentrations ranging from 0.04 – 0.1% did not influence survival of *E. coli* when compared to control treatments containing ethanol only (data not shown).

5.3.4 Effects of antimicrobials on survival of *E. coli* AW1.7 in marinated beef steaks and marinated ground beef.

The effect of antimicrobial compounds was also evaluated at 450 MPa, i.e. conditions that allow shelf life extension without compromising quality (Wang et al., 2015). Treatment of marinated steaks was compared to an equivalent treatment of marinated ground beef (**Figure 5-5**). None of the antimicrobial compounds increased the bactericidal effect of treatment at 450 MPa when supplemented to marinated ground beef (**Figure 5-5**). In beef steaks with honey garlic marinade, however, addition of 0.10 % carvacrol or 0.15% AITC enhanced the pressure inactivation of *E. coli*. Reduced concentrations of 0.04% and 0.06% carvacrol and AITC, respectively, did not influence inactivation of *E. coli* at 450 MPa; likewise, 0.10% cinnamaldehyde had no effect on pressure inactivation of *E. coli* in beef steaks (**Figure 5-5**).

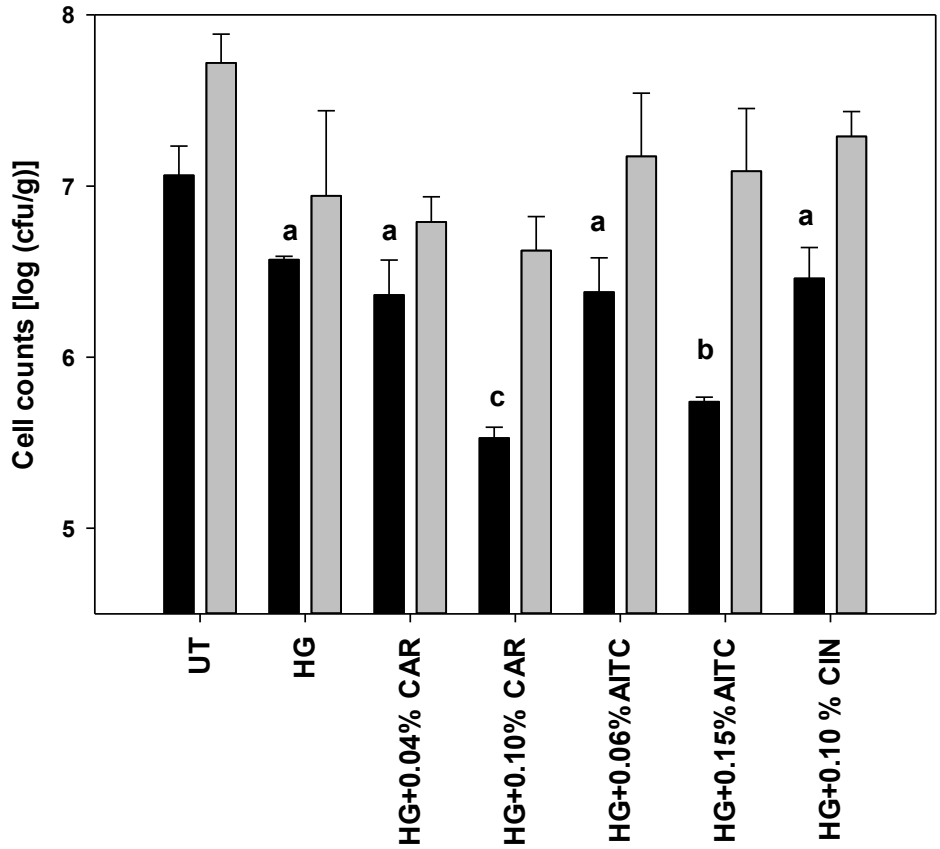

Figure 5-5 Effect of antimicrobial compounds on pressure resistance of *E. coli* AW1.7 in marinated beef steaks (black bars) and marinated ground beef (gray bars). Samples were treated at 450 MPa 20^{o}C for 3 min. Prior to pressure treatment, meat was marinated with honey garlic (HG) marinade supplemented with carvacrol (CAR), allyl isothiocyanate (AITC) or cinnamaldehyde (CIN) at a final concentration of 0.04/0.1%, 0.06/0.15% or 0.10%. UT (untreated) represents marinated meat without pressure treatment. Marinated meat with no antimicrobial supplement (HG) was also used as control. Cell counts were enumerated on Luria-Bertani agar. Different letters above the bars indicate significant differences (p<0.05) to the marinated and pressure treated control without addition of antimicrobial compounds. Data are shown as mean ± standard deviation based on three independent experiments.

5.3.5 Effects of antimicrobials on survival of the surrogate cocktail of *E. coli* in marinated beef steaks supplemented with clean label antimicrobials.

To validate the combined activity of antimicrobials with a strain cocktail, and to assess their influence on the survival of *E. coli* during post-treatment storage, carvacrol and AITC were added to marinade and the survival of a 5 strain surrogate cocktail of *E.*

coli in marinated beef steaks was observed after pressure treatment and during post-pressure storage (**Figure 5-6**). Survival of the surrogate cocktail of *E. coli* in marinated beef steaks was comparable to the survival of the STEC and EPEC cocktails (compare **Figure 5-2** and **Figure 5-6**). Carvacrol and AITC reduced cell counts of *E. coli* by more than 1 log (cfu/mL) when compared to marinated beef steaks without the addition of antimicrobials (**Figure 5-6**). The effect of carvacrol was already observed in untreated samples (**Figure 5-6**); however, the effect of AITC was observed only after pressure treatment. Cell counts of the *E. coli* cocktail remained essentially unchanged on pressure-treated marinated beef steaks, and in the corresponding samples with the addition of carvacrol (**Figure 5-6**). Cell counts of *E. coli* decreased by more than 1 log (cfu/mL) during storage of pressure treated beef steaks supplemented with AITC.

5.4 Discussion

Marination improves the sensory quality of meats; marinades may additionally include antimicrobials to enhance the shelf life and the safety of meat products (Björkroth 2005). Synergistic activity of pressure with antimicrobial compounds added to the marinade potentially eliminates pressure resistant pathogens. Plant essential oils are used as antimicrobial preservatives in meat and meat products (Jayasena & Jo, 2013) and allow "clean label" meat preservation. For example, AITC and mustard powder reduced cell counts of *E. coli* in beef and in fermented sausages (Chacon et al., 2006; Luciano et al., 2011; Nadarajah et al., 2005). The activity of AITC is related to its reactivity with thiols (Luciano & Holley, 2009), while other essential oils destabilize

the cytoplasmic membrane (Gharsallaoui et al., 2015). Synergistic or antagonistic activities of antimicrobial compounds with pressure applications relate to their mode of action (Feyaerts et al., 2015; Hofstetter et al., 2013). This study compared the effect of meat marination and antimicrobial compounds differing in their mode of action. Applications in whole muscle meat were compared to applications in ground beef.

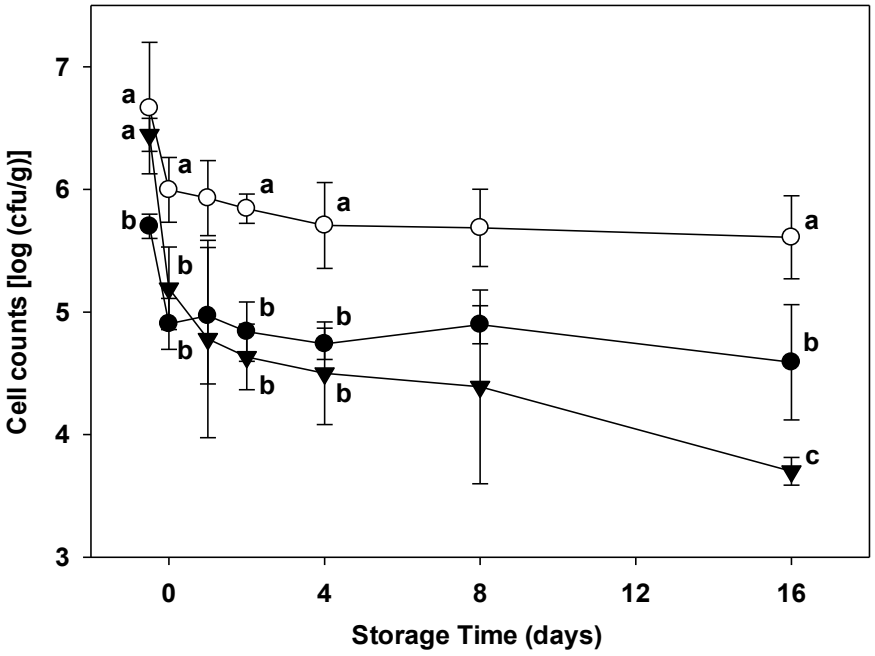

Figure 5-6 Effect of essential oils on survival of a 5-strain surrogate cocktail of *E. coli* in marinated beef steaks after pressure treatment and refrigerated storage.Samples were treated at 450 MPa and 20°C for 3 min, and stored at 4°C after treatment. A storage time of -0.5 days represents untreated controls; a storage time of 0 days represents cell counts taken immediately after pressure treatment. Prior to pressure treatment, steaks were marinated with marinades of honey garlic (HG) supplemented with 0.1% carvacrol (CAR, ●) or 0.15% allyl isothiocyanate (AITC, ▼). Marinated steaks without antimicrobial supplement were used as control (○). Cell counts were enumerated on Luria-Bertani agar. Data are shown as mean ± standard deviation of three independent experiments. Data obtained at the same storage time are significantly different (p<0.05) if they do not share a common superscript.

Treatment with 450 MPa reduced cell counts of *L. monocytogenes* in meat only by 90 to 99%, in keeping with prior reports on the pressure resistance of *L. mon*cy*togenes*

(Ates et al., 2016; Balamurugan et al., 2016; Teixeira et al., 2016). Meat marination increased the pressure resistance of *L. monocytogenes* and prevented pressure-induced sub-lethal injury. Salt addition to cooked ham exerted a comparable effect on pressure resistance and pressure-induced sublethal injury of *L. monocytogenes* (Teixeira et al., 2016) and the protective effect of marinades is thus likely attributable to the presence of salt in marinades. An increased osmotic pressure generally enhances the tolerance of microorganisms to pressure (Georget et al., 2015; Molina-Höppner et al., 2004; Van Opstal et al., 2003). In *L. monocytogenes*, baroprotective effects of NaCl are attributed to the accumulation of glycine betaine and carnitine (Smiddy et al., 2004).

This study describes combined effect of clean label antimicrobials with pressure on *E. coli* in beef. Feyaerts et al. (2015) proposed that thiol reactive antimicrobial compounds act synergistically with pressure because they enhance pressure-induced oxidative stress. Two membrane-active antimicrobials, carvacrol and thymol (Jayasena, et al., 2013; Sikkema et al., 1994), and two thiol reactive antimicrobials, cinnamaldehyde and AITC were evaluated. To allow comparison of the different compounds, all four compounds were applied at the level of their minimum bactericidal concentration. Treatments of *E. coli* in buffer confirmed that the thiol reactive antimicrobials AITC and cinnamaldehyde but not the membrane-active carvacrol and thymol show synergistic activity with pressure (Feyaerts et al., 2015; this study). Likewise, the combination of carvacrol and pressure did not exert synergism on the inactivation of *L. monocytogenes* in milk (Karatzas et al., 2001). Carvacrol suppressed

inactivation of *E. coli* after pressure treatment in buffer (Feyaerts et al., 2015), and reduced inactivation of *Bacillus cereus* spores at a temperature of ≤65^{o}C (Luu-Thi et al. 2015).

Synergisms of AITC or cinnamaldehyde with pressure were previously reported in buffer (Feyaerts et al., 2015; Ogawa et al., 2000). Their synergistic antimicrobial effect with pressure likely relate to the effect of these antimicrobials on the bacterial oxidative stress resistance (Feyaerts et al., 2015). AITC reacts with proteins (Luciano & Holley, 2009), cysteine and glutathione (Hanschen et al., 2012; Luciano et al. 2008) and thus disturbs redox homeostasis. AITC also reduces oxidative stress resistance of *E. coli* by inhibition of thiol-containing enzymes such as thioredoxin reductase and glutathione reductases (Carmel-Harel et al., 2000; Luciano & Holley 2009). Cinnamaldehyde also reacts with thiol group of proteins or cysteine (Weibel & Hansen, 1989), and decreases glutathione levels in bacteria (Cocchiara et al., 2005).

AITC and cinnamaldehyde are also reactive towards amino groups and thiol groups that are present in the food matrix (Hanschen et al., 2012; LoPachin, et al., 2009; Nakamura et al., 2009). The antioxidant capacity of the food matrix thus provides protection to bacterial cells. Accordingly, thiol-mediated compounds exhibited strong synergistic effects with pressure in buffer, but this activity was diminished or abolished when antimicrobials and pressure were applied to *E. coli* on meat (**Figure 5-4** & **Figure 5-5**). The amount of AITC that can be applied to foods is limited by its effect on sensory properties. The use of 0.05% AITC in dry fermented sausages resulted in an acceptable

level of spiciness while a level of 0.75% or 0.1% AITC resulted in an unacceptable level of spiciness (Chacon et al. 2006).

Substantial differences were observed in the efficacy of antimicrobials when applied on ground beef, or for marination of whole muscle meat. During marination of whole muscle meat, the antimicrobial compounds are initially concentrated on the surface of the meat and equilibrate only slowly during storage. In contrast, marination of ground beef rapidly distributes antimicrobial compounds throughout the meat matrix. The initial concentration of essential oils on the surface of beef steaks is thus substantially higher than in ground beef and carvacrol and AITC accelerated pressure inactivation of *E. coli* in marinated steaks but not in marinated ground beef (**Figure 5-4**, **Figure 5-5**, and **Figure 5-6**).

In conclusion, the use of clean label antimicrobial additives to meat marinades can contribute to the elimination of pathogens during pressure processing, or during post-process refrigerated storage. The addition of antimicrobial additives to marinade thus complements the use of marinade to improve the quality and to extend the shelf life pressure-treated meat (Wang et al., 2015). Depending on their mode of action, antimicrobial compounds exert synergistic and antagonistic activities with pressure. The antioxidant capacity of the meat matrix diminishes the activity of thiol-reactive antimicrobials. The application of antimicrobials in marination of whole muscle meat, however, can take advantage of an initial high concentration in the marinade, which is

effective against microorganisms on the surface until their concentration has equilibrated throughout the meat matrix.

5.5 References

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

Ates, M.B., Rode1, T.M., Skipnes, D., & Lekang, O-I. (2016). Modeling of *Listeria monocytogenes* inactivation by combined high‐pressure and mild‐temperature treatments in model soup. *Eur. Food Res. Technol. 242*, 279–287.

Balamurugan, S., Ahmed, R., Chibeu, A., Gao, A., Koutchma, T. & Strange, P. (2016). Effect of salt types and concentrations on the high-pressure inactivation of *Listeria monocytogenes* in ground chicken. *Int. J. Food Microbiol.* 218, 51-56.

Björkroth, J. (2005) Microbiological ecology of marinated meat products. *Meat Sci. 70*, 477-480.

Buckow, R., Sikes, A., & Tume, R. (2013). Effect of high pressure on physicochemical properties of meat. *Crit. Rev. Food Sci. Nutr. 53*, 770-786.

Carmel-Harel, O., & Storz, G. (2000). Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annu. Rev. Microbiol. 54*, 439-461.

Chacon, P.A., Buffo, R.A., & Holley, R.A. (2006). Inhibitory effects of microencapsulated allyl isothiocyanate (AIT) against *Escherichia coli* O157:H7 in refrigerated, nitrogen packed, finely chopped beef. *Int. J. Food Microbiol. 107*, 231-237.

Cocchiara, J., Letizia, C.S., Lalko, J., Lapczynski, A., & Api, A.M. (2005). Fragrance material review on cinnamaldehyde. *Food Chem. Toxicol. 43*, 867-923.

Espina, L., Garcia-Gonzalo, D., Laglaoui, A., Mackey, B.M., & Pagan, R. (2013). Synergistic combinations of high hydrostatic pressure and essential oils or their constituents and their use in preservation of fruit juices. *Int. J. Food Microbiol. 161*, 23-30.

Feyaerts, J., Rogiers, G., Corthouts, J., & Michiels, C. (2015). Thiol-reactive natural antimicrobials and high pressure treatment synergistically enhance bacterial inactivation. *Innov. Food Sci. Emerg. Technol. 27*, 26-34.

Frenzen, P.D., Drake, A., Angulo, F.J., and the Emerging Infections Program Foodnet Working Group. (2005). Ecomomic cost of illness due to *Escherichia coli* O157 infections in the United States. *J. Food Prot. 68*, 2623-2630.

Fugett, E., Fortes, E., Nnoka, C. & Wiedmann, M. (2006). International Life Sciences Institute North America *Listeria monocytogenes* strain collection: development of standard *Listeria monocytogenes* strain sets for research and validation studies. *J. Food Prot. 69*, 2929-2938.

Gänzle, M.G., & Liu, Y. (2015). Mechanisms of pressure-mediated cell death and injury in *Escherichia coli*: from fundamentals to food applications. *Front. Microbiol. 6*, 599.

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. Int. Food Microbiol. 205*, 16-22.

Gayán, E., Antonio Torres, J., & Paredes-Sabja, D. (2012). Hurdle approach to increase the microbial inactivation by high pressure processing: effect of essential oils. *Food Eng. Rev. 4*, 141-148.

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

Gharsallaoui, A., Oulahal, N., Joly, C., & Degraeve, P. (2015). Nisin as a Food Preservative: Part 1: Physicochemical Properties, Antimicrobial Activity, and Main Uses. *Crit. Rev. Food Sci. Nutr. DOI*:10.1080/10408398.2013.763765.

Hanschen, F.S., Bruggemann, N., Brodehl, A., Mewis, I., Schreiner, M., Rohn, S. & Kroh, L.W. (2012). Characterization of products from the reaction of glucosinolate-derived isothiocyanates with cysteine and lysine derivatives formed in either model systems or broccoli sprouts. *J. Agric. Food Chem. 60*, 7735-7745.

Hofstetter, S., Gebhardt, D., Ho, L., Gänzle, M., & McMullen, L.M. (2013). Effects of nisin and reutericyclin on resistance of endospores of *Clostridium* spp. to heat and high pressure. *Food Microbiol. 34,* 46-51.

Hugas, M., Garriga, M., & Monfort, J. (2002). New mild technologies in meat processing: high pressure as a model technology. *Meat Sci. 62*, 359-371.

Jayasena, D.D., & Jo, C. (2013). Essential oils as potential antimicrobial agents in meat and meat products: A review. *Trends Food Sci. Tech. 34*, 96-108.

Jofré, A., Aymerich, T., Grèbol, N., & Garriga, M. (2009). Efficiency of high hydrostatic pressure at 600 MPa against food-borne microorganisms by challenge tests on convenience meat products. *LWT-Food Sci. Tech. 42*, 924-928.

Karatzas, A.K., Kets, E.P.W., Smid, E.J., & Bennik, M.H.J. (2001). The combined action of carvacrol and high hydrostatic pressure on *Listeria monocytogenes* Scott A. *J. Appl. Microbiol. 90*, 463–469.

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

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

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

LoPachin, R.M., Gavin, T., Petersen, D.R., & Barber, D.S. (2009). Molecular mechanisms of 4-hydroxy-2-nonenal and acrolein toxicity: Nucleophilic targets and adduct formation. *Chem. Res. Toxicol. 22*, 1499-1508.

Luciano, F.B., Hosseinian, F.S., Beta, T., & Holley, R.A. (2008). Effect of free-SH containing compounds on allyl isothiocyanate antimicrobial activity against *Escherichia coli* O157:H7. *J. Food Sci. 73*, 214-220.

Luciano, F.B., & Holley, R.A. (2009). Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7. *Int. J. Food Microbiol. 131*, 240–245.

Luciano, F.B., Belland, J., & Holley, R.A. (2011). Microbial and chemical origins of the bactericidal activity of thermally treated yellow mustard powder toward *Escherichia coli* O157:H7 during dry sausage ripening. *Int. J. Food Microbiol 145*, 69-76.

Luu-Thi, H., Corthouts, J., Passaris, I., Grauwet, T., Aertsen, A., Hendrickx, M., & Michiels, C.W. (2015). Carvacrol suppresses high pressure high temperature inactivation of *Bacillus cereus* spores. *Int. J. Food Microbiol. 197*, 45-52.

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

Marcos, B., Aymerich, T., Monfort, J.M., & Garriga, M. (2008). High-pressure processing and antimicrobial biodegradable packaging to control *Listeria monocytogenes* during storage of cooked ham. *Food Microbiol. 25,* 177-182.

Molina-Höppner, A., Doster,W., Vogel, R.F., & Gänzle, M.G. (2004). Protective effect of sucrose and sodium chloride for *Lactococcus lactis* during sublethal and lethal highpressure treatments. *Appl. Environ. Microbiol. 70*, 2013-2020.

Nadarajah, D., Han, J.H., & Holley, R. A. (2005). Inactivation of *Escherichia coli* O157:H7 in packaged ground beef by allyl isothiocyanate. *Int. J. Food Microbiol. 99*, 269-279.

Nakamura, T., Kawai, Y., Kitamoto, N., Osawa, T., & Kato, Y. (2009). Covalent modification of lysine residues by allyl isothiocyanate in physiological conditions: Plausible transformation of isothiocyanate from thiol to amine. *Chem. Res. Toxicol. 22*, 536-542.

Ogawa, T., Nakatani, A., Matsuzaki, H., Isobe, S., & Isshiki, K. (2000). Combined effects of hydrostatic pressure, temperature, and the addition of allyl isothiocyanate on inactivation of *Escherichia coli*. *J. Food Prot. 63*, 884-888.

Paton, A.W., Ratcliff, R.M., Doyle, R.M., Seymour-Murray, J., Davos, D., Lanser, J.A., & Paton, J.C. (1996). Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli*. *J. Clin. Microbiol. 34*, 1622-1627.

Sikkema, J., Debont, J.A.M., & Poolman, B. (1994). Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem. 269*, 8022-8028.

Smiddy, M., Sleator, R.D., Patterson, M.F., Hill, C. & Kelly, A.L. (2004). Role for compatible solutes glycine betaine and L-carnitine in listerial barotolerance. *Appl. Environ. Microbiol. 70,* 7555-7557.

Sofos, J. (2008). Challenges to meat safety in the 21st century. *Meat Sci. 78*, 3-13.

Tilden, J., Young, W., McNamara, A.M., Custer, C., Boesel, B., Lambert-Fair, M.A., Majkowski, J., Vugia, D., Werner, S.B., Hollingsworth, J., & Morris, J.G. (1996).

A new route of transmission for *Escherichia coli*: infection from dry fermented salami. *Am. J. Public Health*. *86*, 1142-1145.

Teixeira, J.S., Maier, M.B., Miller, P., Gänzle, M.G., McMullen, L.M. (2016). The effect of growth temperature, process temperature, and sodium chloride on the high pressure inactivation of *Listeria monocytogenes* on ham. *Eur. Food Res. Tech.* 242, 2021-2029.

Van Opstal, I., Vanmuysen, S.C., & Michiels, C.W. (2003). High sucrose concentration protects *E. coli* against high pressure inactivation but not against high pressure sensitization to the lactoperoxidase system. *Int. J. Food Microbiol. 88*, 1-9.

Verbeke, W., Pérez-Cueto, F., de Barcellos, M., Krystallis, A., & Grunert, K. (2010). European citizen and consumer attitudes and preferences regarding beef and pork. *Meat Sci. 84*, 284-292.

Vlahova-Vangelova, D., & Dragoev, S. (2014). Marination: effect on meat safety and human health. A review. *Bulg. J. Agric. Sci. 20*, 503-509.

Wang, H., Yao, J., Erin, K., & Gänzle, M. (2015). Effect of pressure on quality and shelf life of marinated beef semitendinosus steaks. *Meat Sci. 99*, 148.

Weibel, H., & Hansen, J. (1989). Interaction of cinnamaldehyde (a sensitizer in fragrance) with protein. *Contact Derm. 20*, 161-166.

CHAPTER 6 **General Discussion and Conclusion**

Genome plasticity and mobile elements allow the acquisition of genes such as virulence factors through lateral gene transfer and promotes the evolution and diversity of pathogens (Kuhnert et al., 2006). *E. coli* strains can survive in an environment of low pH, or even during the heat or pressure processing, and may potentially have the ability to cause severe human diseases when they acquire specific virulence genes (Haberbeck et al., 2015; Mercer et al., 2015; Liu et al., 2015; Zhi et al., 2016; Croxen et al., 2013). The 140 out of 188 (~75%) *E. coli* strains exhibited a high probability of growth in low pH conditions, as low as 3.9 (Haberbeck et al., 2015). Liu et al. (2015) subjected 100 *E. coli* strains to heat or pressure, and observed most of the *E. coli* strains were heat or pressure resistant with less than 5 log (cfu/mL) reduction. A high fraction of heat resistant *E. coli* (93 of 256 isolates) was recently reported in a raw milk cheese product (Marti et al., 2016).

The mechanism of heat resistance in *E. coli* has gained more understanding recently. A genomic island named the locus of heat resistance (LHR) is an important genetic determinant for heat resistance, and was estimated to be present in 2% of all sequenced *E. coli* genomes (Mercer et al., 2015). Chlorine tolerant *E. coli* strains isolated from wastewater were also found to carry the LHR (Zhi et al., 2016). The introduction of the LHR cloned on a plasmid into heat sensitive *E. coli* strains has been confirmed to restore heat resistance (Mercer et al., 2015; Pleitner et al., 2012). The LHR contains 16 open reading frames (ORFs), and proteins expressed in LHR were

predicted to include small heat shock proteins, heat shock proteases, YfdX family with unknown function, thioredoxin, and sodium/hydrogen antiporter (Mercer et al., 2015). These proteins function to re-fold the protein aggregates, combat against the osmotic stress response, and mitigate oxidative stress, and therefore may not only increase heat resistance, but also contribute to pressure resistance.

Heat treatment/cooking and high hydrostatic pressure are currently two of the major interventions for reducing the numbers of vegetative cells and eliminating pathogenic microorganisms in food (Balasubramaniam et al., 2015; Georget et al., 2015). However, extremely heat (71 ^{o}C) and pressure (600 MPa) resistant pathogenic *E. coli* strains question the ability of inactivation by current heat interventions or novel technology of using high pressure in beef processing (Hauben et al., 1997; Dlusskaya et al., 2011; Liu et al., 2015; Gänzle and Liu, 2015; Chapter 4). To optimize the conditions of heat or pressure treatments for effective decontamination of pathogenic *E. coli* strains, it is necessary to fully understand the mechanisms of heat and pressure resistance in *E. coli*. Therefore, this thesis aimed to evaluate the genetic determinants of the LHR on pressure resistance of *E. coli*, as well as its mechanisms relating to protein folding and aggregation through proteomic analysis and microscopic observations on inclusion bodies. Moreover, the factors that affect heat and pressure resistance in pathogens were also determined, and more effective interventions were explored, including the addition of antimicrobials to products undergoing high pressure treatment in the food industry.

6.1 Heat/pressure resistance of *E. coli* relates to protein folding and aggregation

Highly heat resistant *E. coli* strains commonly survive in foods such as raw cheese product, and were also identified in wastewater (Dlusskaya et al., 2011; Liu et al., 2015; Marti et al., 2016; Zhi et al., 2016). Some heat resistant strains of *E. coli* such as *E. coli* AW1.7, AW1.3, DM18.3 and GM16.6 are also highly resistant to pressure (Garcia-Hernandez et al., 2015; Liu et al., 2015). A genomic island termed the locus of heat resistance (LHR) was present in only highly heat resistant strains including the previously mentioned strains (Mercer et al., 2015). The LHR confers high heat resistance to *E. coli*, and the loss of the LHR reduced the pressure resistance of *E. coli* AW1.7 (Garcia-Hernandez et al., 2015; Liu et al., 2015; Mercer et al., 2015). Chapter 3 of this thesis determined the expression of proteins within LHR through proteomic analysis using mass spectrometry, and explored the relationship between the heat and pressure resistance in *E. coli* and protein folding and aggregation. In this study, plasmid pRK767 carrying the full length or fragments of LHR was tranformed into two *E. coli* strains MG1655 *ibpA-yfp* and LMM1010 *ibpA-yfp*, which expressed a fluorescent fusion of *ibpA-yfp*. In the untreated strain *E. coli* MG1655 *ibpA-yfp* (pLHR), 10 out of the 16 ORFs of the LHR, which were orf 2, orf3, orf7, orf8, orf9, orf11, orf12, orf13, orf14, and orf16, expressed proteins including small heat shock protein (sHSP20), ATPase chaperone ($ClpK_{GI}$), heat shock protein (HSP), function related to thermal, osmotic and desiccation stress (YfdX 1 and 2), hypothetical protein, thioredoxin (TRX_{GI}), glutathione-dependent potassium-efflux system and methyglyoxal

detoxinfication ($KefB_{GI}$), phosphate-starvation-inducible E family protein (PsiE), periplasmic protein with chaperone and protease activity (DegP), respectively (Mercer et al., 2015; Lee et al., 2016). The result in Chapter 3 suggests the grouping of the orfs could be modified with only the 10 expressed proteins (**Figure 6-1**).

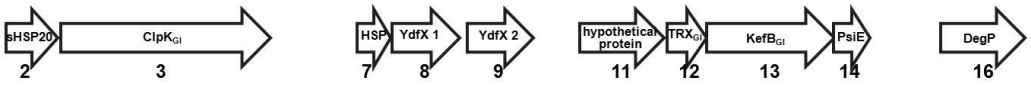

Figure 6-1 Expression of proteins in the locus of heat resistance.Modified according to Chapter 5 of this thesis, Mercer et al. (2015) and Lee et al. (2016).

For better understanding of protein aggregation under stress, the inclusion bodies of untreated and pressure or heat treated cells were observed under the fluorescence microscope. Inclusion bodies were observed to be eliminated after pressure treatment, which confirmed the previous report of Govers et al. (2014). Moreover, the presence of the LHR reduced the inclusion bodies after heat treatment at 60 ^{o}C for 5 min or the pressure treatment at 400 MPa for 3 min, indicating that proteins expressed within LHR function to re-fold and turnover aggregated proteins. The individual fragments 1 and 3 were found to contribute to pressure resistance of *E. coli*, but did not contribute to heat resistance (Mercer et al., 2015). Combined with the proteome analysis, the heat shock proteins sHSP20 and HSP, heat shock proteases $ClpK_{GI}$ and $DegP_{GI}$, reductases of thioredoxin and glutathione-dependent redox system, should be responsible for the increased pressure resistance through stress regulation, mitigation of protein aggregation and reduction of oxidative stress (Lee et al., 2016; Aertsen et al., 2005; Gänzle and Liu, 2016). However, the presence of the LHR increased the pressure

resistance of the wild type *E. coli* MG1655 but not in its pressure resistant derivative strain *E. coli* LMM1010, which indicated that there are some other mechanisms of pressure resistance of *E. coli*.

6.2 Effect of the food matrix on pressure resistance of STEC

Some strains of foodborne pathogens including STEC are highly pressure resistant, and pressure alone does not sufficiently inactivate these resistant strains. The pressure resistance of *E. coli* is variable, and may be affected by the food matrix, process temperature and pH values of food (Liu et al., 2015; Gänzle and Liu, 2015). Chapter 4 compared the resistance of two five-strain *E. coli* cocktails and the typical pressure resistant strain AW1.7, to the applications of high pressure in bruschetta, tzatziki, yogurt and ground beef and during post-pressure survival at 4 °C. Pressure of 600 MPa at 20 °C reduced STEC in plant and dairy products by more than 5 logs (cfu/mL), but this reduction did not occur in ground beef. The pH partially affected pressure resistance of STEC as well as the post-pressure survival. Elimination of *E. coli* after pressure was demonstrated at a pH of 5.5 instead of neutral pH. There are differences in food composition and pressure resistance of *E. coli* was evaluated with food constituents including calcium, magnesium, glutamate, caffeic acid and acetic acid at 600 MPa and 20 °C. All compounds were shown to exhibit a protective effect on *E. coli*. The protective effect of Ca^{2+} and Mg^{2+} partially explained the higher resistance of *E. coli* in meat and dairy products than bruschetta because of their richness of calcium and magnesium, but this effect is not related to the stabilization of the outer membrane

based on the evaluation using fluorescence probe 1-N-phenylnaphthylamine. Glutamate also showed a protective effect on *E. coli* likely through its decarboxylation, by consuming an intracellular proton and exporting negative charges (Foster 2004; Feehily and Karatzas, 2012; Teixeira et al., 2014). The antimicrobial compounds ethanol and phenylethanol enhanced inactivation by pressure, which may be related to the decreased membrane fluidity (Casadei et al., 2002; Charoenwong et al., 2011). The improved understanding of the role of the food matrix and its constituents are meaningful to improve pressure processes.

6.3 Effect of antimicrobials on pressure resistance of *E. coli* and *Listeria monocytogenes* in beef

STEC and *L. monocytogenes* are both associated with fresh meat and meat products (Frenzen et al., 2005; Karch et al., 2005; Sofos, 2008). Pressure treatment at 600 MPa was suggested to control risks associated with *E. coli* and *L. monocytogenes* in marinated beef loins (Hugas et al., 2002; Jofré et al., 2009), but pressure does not eliminate pressure resistant strains (Liu et al., 2012, 2015; Marcos et al., 2008). Meat marination is used to improve the taste and tenderness of meat (Verbeke et al., 2010; Vlahova-Vangelova & Dragoev, 2014), and can be used to enhance pressure inactivation of microorganisms by adding antimicrobial compounds in meat (Björkroth 2005). Chapter 5 investigated the effects of marinades on *L. monocytogenes* and enterohaemorrhagic *E. coli* in beef steaks treated by pressure at 600 or 450 MPa. The pressure resistance of *E. coli* in beef steaks was not reduced by marinades as expected,

and the sublethal injury induced by pressure was prevented in *L. monocytogenes*. Potential synergistic activity of clean label antimicrobials including membrane-active essential oils carvacrol and thymol, and thiol-reactive allyl-isothiocyanate (AITC) and cinnamaldehyde were selected to investigate with pressure. Carvacrol enhanced the pressure inactivation of *E. coli* in beef steaks; however, it suppressed the pressure inactivation of *E. coli* in buffer, and had no effect on inactivation of *E. coli* in ground beef. Thymol had no effect in either buffer or meat. AITC and cinnamaldehyde exhibited synergistic activity with pressure on *E. coli* in buffer; however, cinnamaldehyde did not affect survival of *E. coli* after pressure treatment of meat. In this study, treatments of *E. coli* in buffer confirmed a previous report that the thiol reactive antimicrobials (AITC and cinnamaldehyde) but not the membrane-active (carvacrol and thymol) show synergistic activity with pressure (Feyaerts et al., 2015). Moreover, several studies reported that the combination of carvacrol and pressure did not enhance or suppress inactivation of pathogens or spores (Karatzas et al., 2001; Feyaerts et al., 2015; Luu-Thi et al. 2015). The synergistic antimicrobial effects of AITC or cinnamaldehyde with pressure are related to the effect of these antimicrobials on the pressure induced oxidative stress (Aertsen et al., 2005; Feyaerts et al., 2015). AITC and cinnamaldehyde have been found to reduce oxidative stress resistance of *E. coli*, through the reaction with amino acids or peptides such as cysteine and glutathione (Luciano & Holley, 2009, Hanschen et al., 2012; Luciano et al. 2008; Weibel & Hansen, 1989), and through the inhibition of thiol-containing enzymes such as reductases of

thioredoxin and glutathione (Carmel-Harel et al., 2000; Luciano & Holley 2009; Cocchiara et al., 2005). According to this study, the antimicrobial compounds differ in their mode of action with pressure, exerting synergistic or antagonistic activities. The meat matrix diminishes activity of thiol-reactive antimicrobials because of the antioxidant capacity. Therefore, AITC and carvacrol may be practically applied for enhancing the bacterial inactivation and extending the shelf life of beef steaks.

In conclusion, the LHR was found to reduce the protein aggregation in *E. coli*, as well as contribute to pressure resistance. The contribution of pressure resistance was found to be from the fragment 1 and fragment 3 of the LHR. The two heat shock proteins sHSP20 and HSP, the two heat shock proteases $ClpK_{GI}$ and DegP, and the two reductases are responsible for the increased pressure resistance through stress regulation, mitigation of protein aggregation and reduction of oxidative stress. Thus this thesis research has determined that heat and pressure resistance relates to protein folding and aggregation. Moreover, the pressure resistance of STEC depends on the food matrix, and the antimicrobials AITC and cinnamaldehyde showed synergistic effect with high pressure on the inactivation of STEC.

6.4 Recommendations for future work

The current work illuminated the proteins expressed by the LHR, and made use of novel methods for elucidating mechanisms for heat and pressure resistance of *E. coli*.

Current work showed that carvacrol suppressed inactivation of *E. coli* after pressure treatment in buffer, drawing a consideration that the mechanism of carvacrol

on *E. coli* may be related to protein folding and aggregation. In addition, this study correlated the heat or pressure resistance to protein folding and aggregation in a model system, but further research would be necessary to investigate whether there is any difference in food, such as products of fruit juice, dairy or meat.

6.5 References

Aertsen, A., Spiegeleer, P.D., Vanoirbeek, K., Lavilla, M., & Michiels, C.W. (2005). Induction of oxidative stress by high hydrostatic pressure in *Escherichia coli. Applied Microbiology and Biotechnology*, *71*, 2226-2231.

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

Björkroth, J. (2005) Microbiological ecology of marinated meat products. *Meat Science*, *70*, 477-480.

Carmel-Harel, O., & Storz, G. (2000). Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annual Review of Microbiology*, *54*, 439-461.

Cocchiara, J., Letizia, C.S., Lalko, J., Lapczynski, A., & Api, A.M. (2005). Fragrance material review on cinnamaldehyde. *Food and Chemical Toxicology*, *43*, 867-923.

Croxen, M., Law, R., Scholz, R., Keeney, K., Wlodarska, M., and Finlay, B. (2013). Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clin. Microbiology Rev.*, 26, 822-880.

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

Feyaerts, J., Rogiers, G., Corthouts, J., & Michiels, C. (2015). Thiol-reactive natural antimicrobials and high pressure treatment synergistically enhance bacterial inactivation. *Innovative Food Science and Emerging Technology*, *27*, 26-34.

Frenzen, P.D., Drake, A., Angulo, F.J., and the Emerging Infections Program Foodnet Working Group. (2005). Ecomomic cost of illness due to *Escherichia coli* O157 infections in the United States. J. Food Prot. 68, 2623-2630.

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

Gänzle, M.G., and Liu, Y. (2015). Mechanisms of pressure-mediated cell death and injury in *Escherichia coli*: from fundamentals to food applications. *Front. Microbiol*. 6, 599.

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

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

Haberbeck, L.U., Oliveira, R.C., Vivijs, B., Wenseleers, T., Aertsen, A., Michiels, C., and Geeraerd, A.H. (2015). Variability in growth/no growth boundaries of 188 different *Escherichia coli* strains reveals that approximately 75% have a higher growth probability under low pH conditions than *E. coli* O157:H7 strains ATCC43888. *Food Microbiol*. 45, 222-230.

Hanschen, F.S., Bruggemann, N., Brodehl, A., Mewis, I., Schreiner, M., Rohn, S. & Kroh, L.W. (2012). Characterization of products from the reaction of glucosinolate-derived isothiocyanates with cysteine and lysine derivatives formed in either model systems or broccoli sprouts. *Journal of Agricultural and Food Chemistry*, *60*, 7735–7745.

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

Hugas, M., Garriga, M., & Monfort, J. (2002). New mild technologies in meat processing: high pressure as a model technology. *Meat Science*, *62*, 359-371.

Jofré, A., Aymerich, T., Grèbol, N., & Garriga, M. (2009). Efficiency of high hydrostatic pressure at 600 MPa against food-borne microorganisms by challenge tests on convenience meat products. *LWT-Food Science and Technology*, *42*, 924-928.

Karatzas, A.K., Kets, E.P.W., Smid, E.J., & Bennik, M.H.J. (2001). The combined action of carvacrol and high hydrostatic pressure on *Listeria monocytogenes* Scott A. *Jounal of Applied Microbiology. 90*, 463–469.

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

Kuhnert, P., Boerlin, P., Frey, J. (2006). Target genes for virulence assessment of *Escherichia coli* isolates from water, food and the environment. *Microbiol. Rev.* 24, 107-117.

Lee, C., Wigren, E., Lünsdorf, H., and Römling, U. (2016). Protein homeostasis-more than resisting a hot bath. *Curr. Opin. Microbiol*. 30, 147-154.

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

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

Luciano, F.B., Hosseinian, F.S., Beta, T., & Holley, R.A. (2008). Effect of free-SH containing compounds on allyl isothiocyanate antimicrobial activity against *Escherichia coli* O157:H7. *Journal of Food Science*, *73*, 214–220.

Luciano, F.B., & Holley, R.A. (2009). Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7. *International Journal of Food Microbiology*, *131*, 240–245.

Luu-Thi, H., Corthouts, J., Passaris, I., Grauwet, T., Aertsen, A., Hendrickx, M., & Michiels, C.W. (2015). Carvacrol suppresses high pressure high temperature inactivation of *Bacillus cereus* spores. *International Journal of Food Microbiology*, *197*, 45-52.

Marcos, B., Aymerich, T., Monfort, J.M., & Garriga, M. (2008). High-pressure processing and antimicrobial biodegradable packaging to control *Listeria monocytogenes* during storage of cooked ham. *Food Microbiology 25,* 177-182.

Marti, R., Muniesa, M., Schmid, M., Ahrens, C.H., Naskova, J., and Hummerjohann, H. (2016). *Short communication*: heat-resistant *Escherichia coli* as potential persistent reservoir of extended-spectrum β-lactamases and shiga-toxin-encoding phages in dairy. *J. Dairy Sci*. 99, 8622-8632.

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

Sofos, J. (2008). Challenges to meat safety in the 21st century. *Meat Science*, *78*, 3-13.

Verbeke, W., Pérez-Cueto, F., de Barcellos, M., Krystallis, A., & Grunert, K. (2010). European citizen and consumer attitudes and preferences regarding beef and pork. *Meat Science*, *84*, 284-292.

Vlahova-Vangelova, D., & Dragoev, S. (2014). Marination: effect on meat safety and human health. A review. *Bulgarian Journal of Agricultural Science*, *20*, 503-509.

Weibel, H., & Hansen, J. (1989). Interaction of cinnamaldehyde (a sensitizer in fragrance) with protein. *Contact Dermatitis*, *20*, 161-166.

Zhi, S., Banting, G., Li, Q., Edge, T.A., Topp, E., Sokurenko, M., Scott, C., Braithwaite, S., Ruecker, N.J., Yasui, Y., McAllisterm T., Chui, L., and Neumann, N.F. (2016). Evidence of naturalized stress-tolerant strains of *Escherichia coli* in municipal wastewater treatment plants. *Appl. Environ. Microbiol*. 82, 5505-5518.

BIBLIOGRAPHY

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

Aertsen, A., Vanoirbeek, K., Spiegeleer, P.D., Sermon, J., Hauben, K., Farewell, A., Nyström, T., and Michiels, C.W. (2004). Heat shock protein-mediated resistance to high hydrostatic pressure in *Escherichia coli*. *Appl. Environ. Microbiol.* 70, 2660-2666.

Ahmed, N.M., Conner, D.E. and Huffman, D.L. (1995) Heat resistance of *Escherichia coli* O157:H7 in meat and poultry as affected by product composition. *J. Food Sci.* 60, 606-610.

Alba, B.M., and Gross, C.A. (2004). Regulation of the *Escherichia coli* sigma-dependent envelope stress response. *Mol. Micribiol*. 52, 613-619.

Aldsworth, T.G., Sharman, R.L., and Dodd, C.E. (1999). Bacterial suicide through stress. *Cell. Mol. Life Sci*. 56, 378-383.

Allen, K.J., D. Lepp, R.C. McKellar, and M.W. Griffiths. (2008). Examination of stress and virulence gene expression in *Escherichia coli* O157:H7 using targeted microarray analysis. *Foodborne Pathog. Dis.* 5, 437-447.

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

Archer, J., Jervis, E.T., Bird, J., and Gaze, J.E. (1998). Heat resistance of *Salmonella weltevreden* in low-moisture environments. J. Food Prot. 61:969-973.

Arneborg, N., Salskov-Iversen, A.S., and Mathiasen, T.E. (1993). The effect of growth rate and other growth conditions on the lipid composition of *Escherichia coli*. *Appl. Microbiol. Biotech.* 39, 353-357.

Arsène, F., Tomoyasu, T., and Bukau. B. (2000). The heat shock response of *Escherichia coli*. *Int. J. Food. Microbiol*. 55, 3-9.

Ates, M.B., Rode1, T.M., Skipnes, D., & Lekang, O-I. (2016). Modeling of *Listeria monocytogenes* inactivation by combined high‐pressure and mild‐temperature treatments in model soup. *Eur. Food Res. Technol. 242*, 279–287.

Bae, Y., Lee, S. (2010). Effect of pre-exposure to sodium chloride on the resistance of pathogens to thermal and acid stresses. *J. Food Safety*. 30, 1016-1025.

Baird-Parker, A. C., Boothroyd, M. and Jones, E. (1970), The effect of water activity on the heat resistance of heat sensitive and heat resistant strains of Salmonellae. *J. Appl. Microbiol.* 33, 515-522.

Balamurugan, S., Ahmed, R., Chibeu, A., Gao, A., Koutchma, T. & Strange, P. (2016). Effect of salt types and concentrations on the high-pressure inactivation of *Listeria monocytogenes* in ground chicken. *Int. J. Food Microbiol.* 218, 51-56.

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

Beney, L., and Gervais, P. (2001). Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses. *Appl. Microbiol. Biotechnol*. 57, 34-42.

Baneyx, F., and Mujacic, M. (2004). Recombinant protein folding and misfolding in *Escherichia coli*. *Nature Biotechnol*. 22, 1399-1408.

Benov, L., and Fridovich, I. (1995). Superoxide dismutase protects against aerobic heat shock in *Escherichia coli*. *J. Bacteriol*. 177, 3344-3346.

Beuchat, L.R., Komitopoulou, E., Beckers, H., Betts, R.P., Bourdichon, F., Fanning, S., Joosten, H.M., and Kuile, H.T. (2013). Low-water activity foods: increased concern as vehicles of foodborne pathogens. *J. Food Prot. 76*, 150-172.

Beuchat, L.R., and Scouten, A.J. (2002). Combined effects of water activity, temperature and chemical treatments on the survival of Salmonella and *Escherichia coli* O157:H7 on alfalfa seeds. *J. Appl. Microbiol*. 92,382-95.

Björkroth, J. (2005) Microbiological ecology of marinated meat products. *Meat Sci. 70*, 477-480.

Bogosian, G., Aardema, N.D., Bourneuf, E.V., Morris, P.J.L., and O'Neil, J.P. (2000). Recovery of hydrogen peroxide-sensitive culturable cells of *Vibrio vulnificus*

gives the appearance of resuscitation from a viable but nonculturable state. *J. Bacteriol*. 182, 5070-5075.

Brandl, M.T., Pan, Z., Huynh, S., Zhu, Y., and McHugh, T.H. (2008). Reduction of *Salmonella* Enteritidis population sizes on almond kernels with infrared heat. *J. Food Prot*. 71, 897-902.

Brown, J.L., Ross, T., McMeekin, T.A.,and Nichols, P.D. (1997). Acid habituation of *Escherichia coli* and the potential role of cyclopropane fatty acids in low pH tolerance. *Int. J. Food Microbiol*. 37, 163-173.

Buchanan, R.L., and Edelson, S.G. (1999). Effect of pH-dependent, stationary phase acid resistance on the thermal tolerance of *Escherichia coli* O157:H7. *Food Microbiol*. 16, 447-458.

Buckow, R., Sikes, A., & Tume, R. (2013). Effect of high pressure on physicochemical properties of meat. *Crit. Rev. Food Sci. Nutr. 53*, 770-786.

Bukau, B. (1993). Regulation of the *Escherichia coli* heat-shock response. *Mol. Microbiol.* 9,671-680.

Cabiscol, E., Tamarit, J., and Ros, J. (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int. Microbiol*. 3, 3-8.

Carmel-Harel, O., Storz, G. (2000). Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Ann. Rev. Microbiol.* 54, 439-461.

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

Chacon, P.A., Buffo, R.A., & Holley, R.A. (2006). Inhibitory effects of microencapsulated allyl isothiocyanate (AIT) against *Escherichia coli* O157:H7 in refrigerated, nitrogen packed, finely chopped beef. *Int. J. Food Microbiol. 107*, 231-237.

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

Chen Y.Y., and Gänzle, M.G. (2016). Influence of cyclopropane fatty acids on heat, high pressure, acid and oxidative resistance in *Escherichia coli*. *Int. J. Food Microbiol*. 222, 16-22.

Cheville, A.M., Arnold, W., Buchrieser, C., Cheng, M.M., and Kaspar, C.W. (1996). rpoS Regulation of acid, heat, and salt tolerance in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol*. 62, 1822-1824.

Choi, S.H., Baumler, D.J., and Kaspar, C.W. (2000). Contribution of *dps* to acid stress tolerance and oxidative stress tolerance in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol*. 66, 3911-3916.

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

Christ, D., and Chin, J.W. (2008). Engineering *Escherichia coli* heat-resistance by synthetic gene amplification. *Prot. Eng. Design Select*. 21, 121-125.

Chung, H.J., Wang, S. and Tang, J. (2007). Influence of heat transfer with tube methods on measured thermal inactivation parameters for *Escherichia coli*. *J. Food Prot.* 70, 851-859.

Cocchiara, J., Letizia, C.S., Lalko, J., Lapczynski, A., & Api, A.M. (2005). Fragrance material review on cinnamaldehyde. *Food Chem. Toxicol. 43*, 867-923.

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367-1372.

Cox, J., Neuhauser, N., Michalski, A., Scheltema, R.A., Olsen, J.V., Mann, M. (2011). Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* 10, 1794-1805.

Cox, J., Hein, M.Y., Luber, C.A., Paron, I., Nagaraj, N., Mann, M. (2014). Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol. Cell Proteomics.* 13, 2513-2526.

Croxen, M., Law, R., Scholz, R., Keeney, K., Wlodarska, M., and Finlay, B. (2013). Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clin. Microbiology Rev.*, 26, 822-880.

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

Czechowicz, S.M., Santos, O., Zottola, E.A. (1996). Recovery of thermally-stressed *Escherichia coli* O157:H7 by media supplemented with pyruvate. *Int. J. Food Microbiol*. 33, 275-284.

Dartigalongue, C., and Raina, S. (1998). A new heat-shock gene, ppiD, encodes a peptidyl-prolyl isomerase required for folding of outer membrane proteins in *Escherichia coli*. *EMBO J*. 17, 3968-3980.

De Mendoza, D., and Cronan Jr., J.E. (1983). Thermal regulation of membrane lipid fluidity in bacteria. *Trends Biochem. Sci*. 8, 49-52.

Denich, T.J., Beaudette, L.A., Lee, H., and Trevors, J.T. (2003). Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. *J. Microbiol. Methods* 52, 149-182.

Dlusskaya, E.A., McMullen, L.M., and Gänzle, M.G. (2011). Characterization of an extremely heat-resistant *Escherichia coli* obtained from a beef processing facility. *J. Appl. Microbiol*. 110, 840-849.

Doyle, M.P., and Beuchat, L.R. (2013). *Food microbiology: fundamentals and frontiers*. New York: American Society for Microbiology Press.

Du, W., Abd, S.J., McCarthy, K.L., and Harris, L.J. (2010). Reduction of *Salmonella* on inoculated almonds exposed to hot oil. *J. Food Prot*. 73, 1238-1246.

Earnshaw, R.G., Appleyard, J., and Hurst, R.M. (1995). Understanding physical inactivation processes: combined preservation opportunities using heat, ultrasound and pressure. *Int. J. Food Microbiol*. 28, 197-219.

Enache, E., Mathusa, E.C., Elliott, P.H., Black, D.G., Chen, Y., Scott, V.N., et al. (2011). Thermal resistance parameters for shiga toxin-producing *Escherichia coli* in apple juice. *J. Food Prot.* 74, 1231-1237.

Espina, L., Garcia-Gonzalo, D., Laglaoui, A., Mackey, B.M., & Pagan, R. (2013). Synergistic combinations of high hydrostatic pressure and essential oils or their constituents and their use in preservation of fruit juices. *Int. J. Food Microbiol. 161*, 23-30.

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

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

Finn, S., Condell, O., McClure, P., Amézquita, A., and Fanning, S. (2013). Mechanisms of survival, responses and sources of *Salmonella* in low-moisture environments. *Front. Microbiol.* 4, 331.

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

Frenzen, P.D., Drake, A., Angulo, F.J., and the Emerging Infections Program Foodnet Working Group. (2005). Ecomomic cost of illness due to *Escherichia coli* O157 infections in the United States. *J. Food Prot.* 68, 2623-2630.

Fugett, E., Fortes, E., Nnoka, C. & Wiedmann, M. (2006). International Life Sciences Institute North America *Listeria monocytogenes* strain collection: development of standard *Listeria monocytogenes* strain sets for research and validation studies. *J. Food Prot. 69*, 2929-2938.

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

Gänzle, M.G., and Liu, Y. (2015). Mechanisms of pressure-mediated cell death and injury in *Escherichia coli*: from fundamentals to food applications. *Front. Microbiol*. 6, 599.

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

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

Gayán, E., Antonio Torres, J., & Paredes-Sabja, D. (2012). Hurdle approach to increase the microbial inactivation by high pressure processing: effect of essential oils. *Food Eng. Rev. 4*, 141-148.

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

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

Gervilla, R., Ferragut, V., and Guamis, B. (1999). High pressure inactivation of microorganisms inoculated into ovine milk of different fat contents. *J. Dairy Sci*. 83, 674-682.

Georgious G, Valax P. (1999). Isolating inclusion bodies from bacteria. *Methods Enzymol*, 309, 48-58.

Gharsallaoui, A., Oulahal, N., Joly, C., & Degraeve, P. (2015). Nisin as a Food Preservative: Part 1: Physicochemical Properties, Antimicrobial Activity, and Main Uses. *Crit. Rev. Food Sci. Nutr. DOI*:10.1080/10408398.2013.763765.

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

Govers, S.K., and Aertsen, A. (2015). Impact of high hydrostatic pressure processing on individual cellular resuscitation times and protein aggregates in *Escherichia coli*. *Int. J. Food Microbiol*. 213, 17-23.

Govers, S.K., Gayan, E., and Aertsen, A. (2016). Intracellular movement of protein aggregates reveals heterogeneous inactivation and resuscitation dynamics in stressed populations of *Escherichia coli*. *Environ. Microbiol*. doi: 10.1111/1462-2920.13460.

Greig, J.D., Ravel, A. (2009). Analysis of foodborne outbreak data reported internationally for source attribution. *Int. J. Food Microbiol*. 31, 77-87.

Grogan, D.W., and Cronan Jr., J.E. (1997). Cyclopropane ring formation in membrane lipids of bacteria. *Microbiol. Mol. Biol. Rev*. 61, 429-441.

Gupte, A.R., De Rezende, C.L.E., Joseph, S. W. (2003). Induction and resuscitation of viable but nonculturable *Salmonella enterica* serovar Typhimurium DT104. *Appl. Environ. Microbiol.* 69, 6669-6675.

Guyot, S., Pottier, L., Ferret, E., Gal, L., Gervais, P. (2010). Physiological responses of *Escherichia coli* exposed to different heat-stress kinetics. *Arch. Microbiol.* 192, 651-661.

Haberbeck, L.U., Oliveira, R.C., Vivijs, B., Wenseleers, T., Aertsen, A., Michiels, C., and Geeraerd, A.H. (2015). Variability in growth/no growth boundaries of 188 different *Escherichia coli* strains reveals that approximately 75% have a higher growth probability under low pH conditions than *E. coli* O157:H7 strains ATCC43888. *Food Microbiol*. 45, 222-230.

Hanschen, F.S., Bruggemann, N., Brodehl, A., Mewis, I., Schreiner, M., Rohn, S. & Kroh, L.W. (2012). Characterization of products from the reaction of glucosinolate-derived isothiocyanates with cysteine and lysine derivatives formed in either model systems or broccoli sprouts. *J. Agric. Food Chem. 60*, 7735-7745.

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

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

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

Hengge-Aronis, R. (2002). Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol. Mol. Biol. Rev.* 66, 373-395.

Hengge-Aronis, R., Klein, W., Lange, R., Rimmele, and M., Boos, W. (1991) Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rpoS* and are involved in stationary-phase thermotolerance in *Escherichia coli*, *J. Bacteriol*. 173, 7918-7924.

Hiramatsu, R., Matsumoto, M., Sakae, K., and Miyazaki, Y. (2005). Ability of shiga toxin-producing *Escherichia coli* and *Salmonella* spp. to survive in a desiccation model system and in dry foods. *Appl. Environ. Microbiol*. 71, 6657-6663.

Hitchener, B.J., and Egan, A.F. (1977). Outer-membrane damage in sublethally heated *Escherichia coli* K-12. *Can. L. Microbiol*. 23, 311-318.

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

Huang, I.D., Yousef, A.E., Marth, E.H. and Mathew, M.E. (1992). Thermal inactivation of *Listeria monocytogenes* in chicken gravy. *J. Food Prot*. 55, 492-496.

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

Hugas, M., Garriga, M., & Monfort, J. (2002). New mild technologies in meat processing: high pressure as a model technology. *Meat Sci. 62*, 359-371.

Hunke, S., and Betton, J. (2003). Temperature effect on inclusion body formation and stress response in the periplasm of *Escherichia coli*. *Mol. Microbiol*. 50, 1579-1589.

Imlay, J.A. (2013). The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. *Nat. Rev. Microbiol*. 11, 443-454.

Jakob, U., Gaestel, M., Engel, K. and Buchner, J. (1993) Small heat shock proteins are molecular chaperones. *J. Biol. Chem*. 268, 1517-1520.

Jay, J.M. Loessner, M.J., and Golden, D.A. (2005). *Modern Food Microbiology*. New York: Springer Science and Business Media Press.

Jayasena, D.D., & Jo, C. (2013). Essential oils as potential antimicrobial agents in meat and meat products: A review. *Trends Food Sci. Tech. 34*, 96-108.

Jenkins, D.E., Auger, E.A., and Matin, A. (1991) Role of RpoH, a heat shock regulator protein, in *Escherichia coli* carbon starvation protein synthesis and survival. *J. Bacteriol*. 173, 1992-1996.

Jaquette, C.B., Beuchat, L.R., and Mahon, B.E. (1996). Efficacy of chlorine and heat treatment in killing *Salmonella stanley* inoculated onto alfalfa seeds and growth and survival of the pathogen during sprouting and storage. *Appl. Environ. Microbiol*. 62, 2212-2215.

Jay, J.M., Loessner, M.J., and Golden, D.A. (2005). Modern Food Microbiology. New York, NY: Springer Science and Business Media Press.

Jenkins, D.E., Auger, E.A., and Matin, A. (1991). Role of RpoH, a heat shock regulator protein, in *Escherichia coli* carbon starvation protein synthesis and survival. *J. Bacteriol*. 173, 1992-1996.

Jin, T., Zhang, H., Boyd, G. and Tang, J. (2008). Thermal resistance of *Salmonella enteritidis* and *Escherichia coli* K-12 in liquid egg determined by thermal-death-time disks. *J. Food Eng*. 84, 608-614.

Jofré, A., Aymerich, T., Grèbol, N., & Garriga, M. (2009). Efficiency of high hydrostatic pressure at 600 MPa against food-borne microorganisms by challenge tests on convenience meat products. *LWT-Food Sci. Tech. 42*, 924-928.

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

Juneja, V.K., Cadavez, V., Gonzales-Barron, U., Mukhopadhyay, S. (2015). Modelling the effect of pH, sodium chloride and sodium pyrophosphate on the thermal resistance of *Escherichia coli* O157:H7 in ground beef. *Food Res. Int.* 69, 289-304.

Juneja, V.K. and Marmer, B.S. (1999) Lethality of heat to *Escherichia coli* O157:H7: D- and z-value determinations in turkey, lamb, and pork. *Food Res. Int*. 32, 23-28.

Jürgen, B., Hanschke, R., Sarvas, M., Hecker, M., and Schweder, T. (2001). Proteome and transcriptome based analysis of *Bacillus subtilis* cells overproducing and insoluble heterologous protein. *Appl. Microbiol. Biotechnol*. 55, 326-332.

Karatzas, A.K., Kets, E.P.W., Smid, E.J., & Bennik, M.H.J. (2001). The combined action of carvacrol and high hydrostatic pressure on *Listeria monocytogenes* Scott A. *J. Appl. Microbiol. 90*, 463–469.

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

Kato, A., Ohnishi, H., Yamamoto, K., Furuta, E., Tanabe, H., and Utsumi, R. (2000). Transcription of *emrKY* is regulated by the EvgA-EvgS two-component system in *Escherichia coli* K-12. *Biosci. Biotechnol. Biochem*. 64, 1203-1209.

Katsui, N., Tsuchido, T., Takano, M., and Shibasaki, I. (1981). Effect of preincubation temperature on the heat resistance of *Escherichia coli* having different fatty acid compositions. *J. Gen. Microbiol*. 122, 357-361.

Kaur, J., Ledward, D.A., Park, R.W.A., and Robson, R.L. (1998). Factors affecting the heat resistance of *Escherichia coli* O157:H7. *Lett. Appl. Microbiol*. 26, 325-330.

Kempf, B., and Bremmer, E. (1998) Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments, *Arch. Microbiol*. 170, 319-330.

Kennedy, J., Blair, I.S., McDowell, D.A., Bolton, D.J. (2005). An inverstigation of the thermal inactivation of *Staphylococcus aureus* and the potential for increased thermotolerance as a result of chilled storage. *J. Appl. Microbiol*. 99, 1229-1235.

Keweloh, H., Diefenbach, R., Rehm, H. (1991). Increase of phenol tolerance of *Escherichia coli* by alternations of the fatty acid composition of the membrane lipids. *Arch. Microbiol*. 157, 49-53.

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

Kim, Y.B., Kim, H.W., Song, M.K., and Rhee, M.S. (2015). Decontamination method using heat and relative humidity for radish seeds achieves a 7-log reduction of *Escherichia coli* O157:H7 without affecting product quality. *Int. J. Food Microbiol.* 201, 42-46.

Kitagawa, M., Matsumura, Y., and Tsuchido, T. (2000). Small heat shock proteins, IbpA and IbpB, are involved in resistances to heat and superoxide stresses in *Escherichia coli*. *FEMS Microbiol. Lett*. 184, 165-171.

Klaiber, R.G., Baur, S., Wolf, G., Hammes, W.P., Carle, R. (2005). Quality of minimally processed carrots as affected by warm water washing and chlorination. *Innov. Food Sci. & Emerg. Technol*. 6, 351-362.

Kotrola, J. and Conner, D.E. (1997). Heat inactivation of *Escherichia coli* O157:H7 in turkey meat as affected by sodium chloride, sodium lactate, polyphosphate, and fat content. *J. Food Prot*, 60, 898-902.

Krojer, T., Sawa, J., Schäfer, E., Saibil, H.R., Ehrmann, M., and Clausen, T. (2008). Structural basis for the regulated protease and chaperone function of DegP. *Nature*. 453, 885-890.

Kuczyńska-Wiśnik, D., Kędzierska, S., Matuszewska, E., Lund, P., Taylor, A., Lipińska, B., and Laskowska, E. (2002). The *Escherichia coli* small heat-shock proteins IbpA and IbpB prevent the aggregation of endogenous proteins denatured in vivo during extreme heat shock. *Microbiol*. 148, 1757-1765.

Kuhnert, P., Boerlin, P., Frey, J. (2006). Target genes for virulence assessment of *Escherichia coli* isolates from water, food and the environment. *Microbiol. Rev.* 24, 107-117.

Lamosa, P., Burke, A., Peist, R., Huber, R., Liu, M.Y., Silva, G., et al. (2000). Thermostabilization of proteins by diglycerol phosphate: a new compatible solute from the hyperthermophile *Archaeoglobus fulgidus*. *Appl. Environ. Microbiol.* 66, 1974-1979.

Landini, P., Egli, T., Wolf, J., and Lacour, S. (2014). SigmaS, a major player in the response to environmental stresses in *Escherichia coli*: role, regulation and mechanisms of promoter recognition. *Environ. Microbiol. Rep*. 6, 1-13.

Lange, R., Hengge-Aronis, R. (1991). Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol. Microbiol*. 5, 49-59.

Laskowska, E., Wawrzynów, A., and Taylor, A. (1996). IbpA and IbpB, the new heat-shock proteins, bind to endogenous *Escherichia coli* proteins aggregated intracellularly by heat shock. *Biochimie*. 78, 117-122.

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

Lee, G.J., Roseman, A.M., Saibil, H.R. and Vierling, E. (1997). A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *EMBO J*. 16, 659-671.

Lee, C., Wigren, E., Lünsdorf, H., and Römling, U. (2016). Protein homeostasis-more than resisting a hot bath. *Curr. Opin. Microbiol*. 30, 147-154.

Lee, C., Wigren, E., Trcek, J., Peters, V., Kim, J., Hasni, M.S., et al. (2015). A novel protein quality control mechanism contributes to heat shock resistance of

worldwide-distributed *Pseudomonas aeruginosa* clone C strains. *Environ. Microbiol*. 17, 4511-4526.

Lee, G.J., Roseman, A.M., Saibil, H.R. and Vierling, E. (1997). A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *EMBO J*. 16, 659-671.

Lee, J., and Kaletunc, G. (2002). Evaluation of the heat inactivation of *Escherichia coli* and *Lactobacillus plantarum* by differential scanning calorimetry. *Appl. Environ. Microbiol*. 68, 5379-86.

LeThanh, H., Neubauer, P., and Hoffmann, F. (2005). The small heat-shock proteins IbpA and IbpB reduce the stress load of recombinant *Escherichia coli* and delay degradation of inclusion bodies. *Microb. Cell Fact*. 4, 6.

Lindner, A.B., Madden, R., Demarez, A., Stewart, E.J., and Taddei, F. (2008). Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. *Proc. Natl. Acad. Sci. U. S. A*. 105, 3076-3081.

Line, J.E., Fain Jr., A.R., Moran, A.B., Martin, L.M., Lechowich, R.V., Carosella, J.M., and Brown, W.L. (1991). Lethality of heat to *Escherichia coli* O157:H7: *D*-value and *Z*-value determinations in ground beef. *J. Food Prot.* 54, 762-766.

Liu, Y. (2015). Heat and pressure resistance of *Escherichia coli* and its inactivation in the presence of antimicrobial compounds. [Doctor's thesis]. [Edmonton(AB)]: University of Alberta.

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

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

LoPachin, R.M., Gavin, T., Petersen, D.R., & Barber, D.S. (2009). Molecular mechanisms of 4-hydroxy-2-nonenal and acrolein toxicity: Nucleophilic targets and adduct formation. *Chem. Res. Toxicol. 22*, 1499-1508.

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

Luchansky, J.B. Porto-Fett, A.C.S., Shoyer, B.A., Call, J.E., Schlosser, W., Shaw, W., et al. (2012). Fate of shiga toxin-producing O157:H7 and non-O157:H7 *Escherichia coli* cells within blade-tenderized beef steaks after cooking on a commercial open-flame gas grill. *J. Food Prot.* 75, 62-70.

Luciano, F.B., Hosseinian, F.S., Beta, T., & Holley, R.A. (2008). Effect of free-SH containing compounds on allyl isothiocyanate antimicrobial activity against *Escherichia coli* O157:H7. *J. Food Sci. 73*, 214-220.

Luciano, F.B., & Holley, R.A. (2009). Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7. *Int. J. Food Microbiol. 131*, 240–245.

Luciano, F.B., Belland, J., & Holley, R.A. (2011). Microbial and chemical origins of the bactericidal activity of thermally treated yellow mustard powder toward *Escherichia coli* O157:H7 during dry sausage ripening. *Int. J. Food Microbiol 145*, 69-76.

Luu-Thi, H., Corthouts, J., Passaris, I., Grauwet, T., Aertsen, A., Hendrickx, M., & Michiels, C.W. (2015). Carvacrol suppresses high pressure high temperature inactivation of *Bacillus cereus* spores. *Int. J. Food Microbiol. 197*, 45-52.

Mackey, B.M., Miles, C.A., Parsons, S.E., and Seymour, D.A. (1991). Thermal denaturation of whole cells and cell components of *Escherichia coli* examined by differential scanning calorimetry. *J. Gen. Microbiol*. 137, 2361-2374.

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

Mao, Y., Doyle, M.P., and Chen, J. (2001). Insertion mutagenesis of *wca* reduces acid and heat tolerance of enterohemorrhagic *Escherichia coli* O157:H7. *J. Bacteriol*. 183, 3811-3815.

Marcos, B., Aymerich, T., Monfort, J.M., & Garriga, M. (2008). High-pressure processing and antimicrobial biodegradable packaging to control *Listeria monocytogenes* during storage of cooked ham. *Food Microbiol. 25,* 177-182.

Marti, R., Muniesa, M., Schmid, M., Ahrens, C.H., Naskova, J., and Hummerjohann, H. (2016). *Short communication*: heat-resistant *Escherichia coli* as potential persistent reservoir of extended-spectrum β-lactamases and shiga-toxin-encoding phages in dairy. *J. Dairy Sci*. 99, 8622-8632.

Masuda, N., and Church, G.M. (2002). *Escherichia coli* gene expression responsive to levels of the response regulator EvgA. *J. Bacteriol*. 184, 6225-6234.

Mazzotta, A.S. (2001). Thermal inactivation of stationary-phase and acid-adapted *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in fruit juices. *J. Food Prot*. 3, 287-429.

Mecsas, J., Rouviere, P.E., Erickson, J.W., Donohue, T.J., and Gross, C.A. (1993). The activity of σ^E, an *Escherichia coli* heat-inducible σ-factor, is modulated by expression of outer membrane proteins. *Genes Dev*. 7, 2618-2628.

Mejía, R., Gómez-Eichelmann, M.C., and Fernández, M.S. (1995). Membrane fluidity of *Escherichia coli* during heat-shock. *Biochim. Biophys. Acta*. 1239, 195-200.

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

Missiakas, D., Betton, J.M., and Raina, S. (1996). New components of protein folding in the extra-cytoplasmic compartments of *Escherichia coli*: SurA, FkpA and Skp/OmpH. *Mol. Microbiol*., 21, 871-884.

Mizunoe, Y., Wai, S.N., Ishikawa, T., Takade, A., Yoshida, S. (2000). Resuscitation of viable but nonculturable cells of *Vibrio parahaemolyticus* induced at low temperature under starvation. *FEMS Microbiol. Lett*. 186, 115-120.

Mogk, A., Deuerling, E., Vorderwulbecke, S., Vierling, E., and Bukau, B. (2003). Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. *Mol. Microbiol*. 50, 585-595.

Mogk, A., Tomoyasu, T., Goloubinoff, P., Ru□diger, S., Röder, D., Langen, H., and Bukau, B. (1999) Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. *EMBO. J.* 18, 6934-6949.

Mohácsi-Farkas, Cs., Farkas, J., Mészáros, L., Reichart, O., and É. Andrássy. (1999). Thermal denaturation of bacterial cells examined by differential scanning calorimetry. *J. Therm. Anal. Calorim*. 57, 409-414.

Molina-Gutierrez, A., Stippl, V., Delgado, A., Gänzle, M.G., Vogel, R.F. (2002). In situ determination of the intracellular pH of *Lactococcus lactis* and *Lactobacillus plantarum* during pressure treatment. *Appl. Environ. Microbiol*. 68, 4399-4406.

Molina-Höppner, A., Doster,W., Vogel, R.F., & Gänzle, M.G. (2004). Protective effect of sucrose and sodium chloride for *Lactococcus lactis* during sublethal and lethal highpressure treatments. *Appl. Environ. Microbiol. 70*, 2013-2020.

Morishige, Y., Fujimori, K., Amano, F. (2013). Differential resuscitative effect of pyruvate and its analogues on VBNC (viable but non-culturable) *Salmonella*. *Microbes Environ.* 28, 180-186.

Murphy, R.Y., Marks, B.P., Johnson, E.R., Johnson, M.G., (1999). Inactivation of Salmonella and Listeria in ground chicken breast meat during thermal processing. *J. Food Prot*. 9, 975-1096.

Nadarajah, D., Han, J.H., & Holley, R. A. (2005). Inactivation of *Escherichia coli* O157:H7 in packaged ground beef by allyl isothiocyanate. *Int. J. Food Microbiol. 99*, 269-279.

Nair, M.S., Lau, P., Belskie, K., Fancher, S., Chen, C., Karumathil, D.P., et al. (2016). Potentiating the heat inactivation of *Escherichia coli* O157:H7 in ground beef patties by natural antimicrobials. *Front. Microbiol*. 7, 15.

Nakamura, T., Kawai, Y., Kitamoto, N., Osawa, T., & Kato, Y. (2009). Covalent modification of lysine residues by allyl isothiocyanate in physiological conditions: Plausible transformation of isothiocyanate from thiol to amine. *Chem. Res. Toxicol. 22*, 536-542.

Nakayama, H., Mitsui, T., Nishihara, M., and Kito, M. (1980) Relation between growth temperature of *Escherichia coli* and phase transition temperatures of its cytoplasmic and outer membranes. *Biochim. Biophys. Acta*. 601, 1-10.

Neetoo, H., and Chen, H. (2011). Individual and combined application of dry heat with high hydrostatic pressure to inactivate *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. *Food Microbiol*. 28, 119-127.

Ng, H., Bayne, H.G., Garibaldi, J.A. (1969). Heat resistance of Salmonella: the uniqueness of *Salmonella* Senftenberg 775W. *Appl. Microbiol*. 17, 78-82.

Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol. Mol. Biol. Rev*. 64, 548-572.

Nishino, K., Inazumi, Y., and Yamaguchi, A. (2003). Global analysis of genes regulated by EvgA of the two-component regulatory system in *Escherichia coli*. *J. Bacteriol*. 185, 2667-2672.

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

Ogawa, T., Nakatani, A., Matsuzaki, H., Isobe, S., & Isshiki, K. (2000). Combined effects of hydrostatic pressure, temperature, and the addition of allyl isothiocyanate on inactivation of *Escherichia coli*. *J. Food Prot. 63*, 884-888.

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

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

Parsell, D.A., and Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet*. 27, 437-496.

Paton, A.W., Ratcliff, R.M., Doyle, R.M., Seymour-Murray, J., Davos, D., Lanser, J.A., & Paton, J.C. (1996). Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli*. *J. Clin. Microbiol. 34*, 1622-1627.

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

Pereira, R., Martins, J., Mateus, C., Teixeira, J.A. and Vicente, A.A. (2006) Death kinetics of *Escherichia coli* in goat milk and *Bacillus licheniformis* in cloudberry jam treated by ohmic heating. *Chem. Papers.* 61, 121-126.

Pleitner, A., Zhai, Y., Winter, R., Ruan, L., McMullen, L.M., and Gänzle, M.G. (2012). Compatible solutes contribute to heat resistance and ribosome stability in *Escherichia coli* AW1.7. *Biochim. Biophys. Acta*. 1824, 1351-1357.

Podolak, R., Enache, E., Stone, W., Black, D.G., and Elliott, P.H. (2010). Sources and risk factors for contamination, survival, persistence, and heat resistance of *Salmonella* in low-moisture foods. *J. Food Prot*. 73, 1919-1936.

Privalle, C.T., and Fridovich, I. (1987). Induction of superoxide dismutase in *Escherichia coli* by heat shock. *Proc. Natl. Acad. Sci. USA*. 84, 2723-2726.

Quinn, P.J. (1981). The fluidity of cell membranes and its regulation. *Prog. Biophys. Mol. Biol*. 38, 1-104.

Rajic, A., Waddell, L.A., Sargeant, J.M., Read, S., Farber, J., Firth, M.J., et al. (2007). An overview of microbial food safety programs in beef, pork, and poultry from farm to processing in Canada. *J. Food Prot.* 70, 1286-1294.

Ramos, A., Raven, N.D.H., Sharp, R.J., Bartolucci, S., Rossi, M., Cannio, R., et al. (1997). Stabilization of enzymes against thermal stress and freeze-drying by mannosylglycerate. *Appl. Environ. Microbiol.* 63, 4020-4025.

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

Riemann, H. (1968). Effect of water activity on the heat resistance of *Salmonella* in "dry" materials. *Appl. Microbiol*. 16, 1621-1622.

Riordan, D.C.R., Duffy, G., Sheridan, J.J., Whiting, R.C., Blair, I.S. and McDowell, D.A. (2000) Effects of acid adaptation, product pH, and heating on survival of *Escherichia coli* O157:H7 in pepperoni. *Appl. Environ. Microbiol.* 66, 1726-1729.

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

Robey, M., Benito, A., Hutson, R.H., Pascual, C., Park, S.F., and Mackey, B.M. (2001). Variation in resistance to high hydrostatic pressure and *rpoS* heterogeneity in natural isolates of *Escherichia coli* O157:H7. *Appl. Environ. Microbiol*. 67, 4901-4907.

Rosen, R., Biran, D., Gur, E., Becher, D., Hecker, M., Ron, E.Z. (2002). Protein aggregation in *Escherichia coli*: role of proteases. *FEMS Microbiol. Lett*. 207, 9-12.

Ruan, L., Pleitner, A.M., Gänzle, M.G., and McMullen. L.M. (2011). Solute transport proteins and the outer membrane protein NmpC contribute to heat-resistance of *Escherichia coli* AW1.7. *Appl. Environ. Microbiol*. 77, 2961-2967.

Ryu, J., and Beuchat, L.R. (1998). Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. *Int. J. Food Microbiol*. 45, 185-193.

Ryu, J., and Beuchat, L.R. (1999). Changes in heat tolerance of *Escherichia coli* O157:H7 after exposure to acidic environments. *Food Microbiol*. 16, 317-324.

Sánchez-Maldonado, A.F., Schieber, A., Gänzle, M.G. (2011). Structure-function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. *J. Appl. Microbiol*. 111, 1176-1184.

Santillana Farakos, S.M., Schaffner, D.W., and Frank, J.F. (2014). Predicting survival of salmonella in low-water activity foods: an analysis of literature data. *J. Food Prot*. 77, 1448-1461.

Sharma, M., Adler, B.B., Harrison, M.D., and Beuchat, L.R. (2005). Thermal tolerance of acid-adapted and unadapted *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* in cantaloupe juice and watermelon juice. *Lett. Appl. Microbiol*. 41, 448-453.

Sikkema, J., Debont, J.A.M., & Poolman, B. (1994). Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem. 269*, 8022-8028.

Sinensky, M. (1974). Homeoviscous adaptation: a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 71, 522-525.

Smiddy, M., Sleator, R.D., Patterson, M.F., Hill, C. & Kelly, A.L. (2004). Role for compatible solutes glycine betaine and L-carnitine in listerial barotolerance. *Appl. Environ. Microbiol. 70,* 7555-7557.

Smith, S.E., Maurer, J.L., Orta-Ramirez, A., Ryser, E.T., and Smith, D.M. (2001). Thermal inactivation of *Salmonella* spp., *Salmonella Typhimurium* DT104 and *Escherichia coli* O157:H7 in ground beef. *J. Food Sci*. 66, 1164-1168.

Sofos, J. (2008). Challenges to meat safety in the 21st century. *Meat Sci. 78*, 3-13.

Strandberg, L., and Enfors, S. (1991). Factors influencing inclusion body formation in the production of a fused protein in *Escherichia coli*. *Appl. Environ. Microbiol*. 57, 1669-1674.

Studer, P, Heller, W.E, Hummerjohann, J., and Drissner, D. (2013). Evaluation of aerated steam treatment of alfalfa and mung bean seeds to eliminate high levels of *Escherichia coli* O157:H7 and O178:H12, *Salmonella enterica*, and *Listeria monocytogenes*. *Appl. Environ. Microbiol*. 79, 4613-19.

Suutari, M., and Laakso, S. (1994). Microbial fatty acids and thermal adaptation. *Crit. Rev. Microbiol*. 20, 285-328.

Swartz, R.S., Luchansky, J.B., Kulas, M., Shoyer, B.A., Shane, L.E., Strasser, H., et al. (2015). Thermal inactivation of shiga toxin-producing *Escherichia coli* cells within cubed beef steaks following cooking on a griddle. *J. Food Prot.* 78, 1013-1017.

Syamaladevi, R.M., Tang, J., Villa-Rojas, R., Sablani, S., Carter, B., and Campbell, G. (2016). Influence of water activity on thermal resistance of microorganisms in low-moisture foods: a review. *Compr.Rev. Food Sci. Food Saf*. 15, 353-370.

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

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

Teixeira, J.S., Maier, M.B., Miller, P., Gänzle, M.G., McMullen, L.M. (2016). The effect of growth temperature, process temperature, and sodium chloride on the high pressure inactivation of *Listeria monocytogenes* on ham. *Eur. Food Res. Tech.* 242, 2021-2029.

Thomas, J.G., and Baneyx, F. (1998). Roles of the *Escherichia coli* small heat shock proteins IbpA and IbpB in thermal stress management: comparison with ClpA, ClpB, and HtpG in vivo. *J. Bacteriol*. 180, 5165-5172.

Tilden, J., Young, W., McNamara, A.M., Custer, C., Boesel, B., Lambert-Fair, M.A., Majkowski, J., Vugia, D., Werner, S.B., Hollingsworth, J., & Morris, J.G. (1996).

A new route of transmission for *Escherichia coli*: infection from dry fermented salami. *Am. J. Public Health*. *86*, 1142-1145.

Torok, Z., Horvath, I., Goloubinoff, P., Kovacs, E., Glatz, A., Balogh, G., and Vigh, L. (1997) Evidence for a lipochaperonin: association of active protein-folding GroESL oligomers with lipids can stabilize membranes under heat shock conditions. *Proc. Natl. Acad. Sci. USA*. 94, 2192-2197.

Tsuchido, T., Katsui, N., Takeuchi, A., Takano, M., and Shibasaki, I. (1985). Destruction of the outer membrane permeability barrier of *Escherichia coli* by heat treatment. *Appl. Environ. Microbiol*. 50, 298-303.

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

Vaara, M. (1992). Agents that increase the permeability of the outer-membrane. *Microbiol. Rev.* 56, 395-411.

Van Opstal, I., Vanmuysen, S.C., & Michiels, C.W. (2003). High sucrose concentration protects *E. coli* against high pressure inactivation but not against high pressure sensitization to the lactoperoxidase system. *Int. J. Food Microbiol. 88*, 1-9.

Vasan, A., Geier, R., Ingham, S.C., and Ingham, B.H. (2014). Thermal tolerance of O157 and non-O157 shiga toxigenic strains of *Escherichia coli*, *Salmonella*, and potential pathogen surrogates, in frankfurter batter and ground beef of varying fat levels. *J. Food Prot*. 77, 1501-1511.

Veinger, L., Diamant, S., Buchner, J., and Goloubinoff, P. (1998). The small heat-shock proteins IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *J. Biol. Chem*. 273, 11032-11037.

Verbeke, W., Pérez-Cueto, F., de Barcellos, M., Krystallis, A., & Grunert, K. (2010). European citizen and consumer attitudes and preferences regarding beef and pork. *Meat Sci. 84*, 284-292.

Vlahova-Vangelova, D., & Dragoev, S. (2014). Marination: effect on meat safety and human health. A review. *Bulg. J. Agric. Sci. 20*, 503-509.

Wang, H., Yao, J., Erin, K., & Gänzle, M. (2015). Effect of pressure on quality and shelf life of marinated beef semitendinosus steaks. *Meat Sci. 99*, 148.

Weibel, H., & Hansen, J. (1989). Interaction of cinnamaldehyde (a sensitizer in fragrance) with protein. *Contact Derm. 20*, 161-166.

Weiss, A., and Hammes, W.P. (2005) Efficacy of heat treatment in the reduction of Salmonella and *Escherichia coli* O157: H^- on alfalfa, mung bean, and radish seeds used for sprout production. *Eur. Food Res. Technol*. 221, 187-191.

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

Whitfield, C., and Valvano, M.A. (1993). Biosynthesis and expression of cell surface polysaccharides in gram-negative bacteria. *Adv. Microb. Physiol*. 35, 135-246.

Winkler, J., Seybert, A., Konig, L., Pruggnaller, S., Haselmann, U., Sourjik, V., et al. (2010). Quantitative and spatio-temporal features of protein aggregation in *Escherichia coli* and consequences on protein quality control and cellular ageing. *EMBO J*. 29, 910-923.

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

Woodward, D.L., Clark, C.G., Caldeira, R.A., Ahmed, R. and Rodgers, F.G. (2002). Verotoxigenic *Escherichia coli* (VTEC): a major public health threat in Canada. *Can. J. Infect Dis.* 13, 321-330.

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

Yang, J., Bingol, G., Pan, Z., Brandl, M.T., McHugh, T.H., and Wang, H. (2010). Infrared heating for dry-roasting and pasteurization of almonds. *J. Food Eng.* 101, 273-280.

Yeni, F., Yavas□, S., Alpas, H., and Soyer, Y. (2015). Most common foodborne pathogens and mycotoxins on fresh produce: a review of recent outbreaks. *Crit. Rev. Food Sci. Nutr*. 56, 1532-1544.

Yoon, Y., Lee, H., Lee, S., Kim, S., and Choi, K.H. (2015). Membrane fluidity-related adaptive response mechanisms of foodborne bacterial pathogens under environmental stresses. *Food Res. Int*. 72, 25-36.

Yuk, H., and Marshall, D.L. (2003). Heat adaptation alters *Escherichia coli* O157:H7 membrane lipid composition and verotoxin production. *Appl. Environ. Microbiol*. 69, 5115-5119.

Zhang, Y., and Griffiths, M.W. (2003). Induced expression of the heat shock protein genes uspA and grpE during starvation at low temperatures and their influence on thermal resistance of *Escherichia coli* O157:H7. *J. Food Prot*. 66, 2045-2050.

Zhang, Y., and Rock, C.O. (2008). Membrane lipid homeostasis in bacteria. *Nat. Rev. Microbiol*. 6, 222-233.

Zhao, G., Ceci, P., Ilari, A., Giangiacomo, L., Laue, T. M., Chiancone, E., et al. (2002). Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of *Escherichia coli*. *J. Biol. Chem*. 277, 27689-22796.

Zhi, S., Banting, G., Li, Q., Edge, T.A., Topp, E., Sokurenko, M., Scott, C., Braithwaite, S., Ruecker, N.J., Yasui, Y., McAllisterm T., Chui, L., and Neumann, N.F. (2016). Evidence of naturalized stress-tolerant strains of *Escherichia coli* in municipal wastewater treatment plants. *Appl. Environ. Microbiol*. 82, 5505-5518.

Zolkiewski, M. (1999). ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. *J. Biol. Chem.* 274, 28083-28086.