Heat and Pressure Resistance of *Escherichia coli* and Its Inactivation In the Presence of Antimicrobial Compounds

by

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

Doctor of Philosophy

in

Food Science and Technology

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Abstract

Verotoxigenic *Escherichia coli* (VTEC) are pathogens causing severe foodborne disease. *E. coli* AW1.7 is a heat resistant beef carcass isolate that may be used as a surrogate organism to study the survival of VTEC on food. This dissertation examines the heat and pressure resistance of *E. coli* and the application of antimicrobial compounds to achieve its inactivation in food.

The pressure resistance of *E. coli* AW1.7 was compared to the resistance of other foodborne pathogens and spoilage organisms relevant in meat. *E. coli* AW1.7 was the most pressure resistant organism tested. Moreover, the ability of *E. coli* AW1.7 to resist pressure was comparable to the pressure-resistant mutant *E. coli* LMM1030.

To further study the heat- and pressure resistance of *E. coli, E. coli* AW1.7 was compared to the heat- and pressure resistance of VTEC strains from different serotypes and phylotypes. *E. coli* AW1.7 exhibited a higher heat resistance than VTEC strains; however, some VTEC strains also survived in hamburgers grilled to a core temperature of 71°C. Several strains of VTEC exhibited a higher resistance to pressure than *E. coli* AW1.7. Over one third of tested strains showed 3 log CFU/g or less cell count reduction under high pressure treatment of 600 MPa for 3 min. Therefore, additional treatment processes are required for elimination of VTEC in pressure treated food.

Additional treatments were evaluated to achieve the elimination of *E. coli* in buffers and food matrices. Chitosan was effective in injury *E. coli* in imidazole and

potassium phosphate buffer; chitosan, nisin and lactate combination were effective in yogurt serum; chitosan, nisin and heat were effective in apple juice; and chitosan, Micocin® X and heat had small bactericidal effects in ground beef. A combination of chitosan and high pressure was not effective against *E. coli* AW1.7 in potassium phosphate buffer, but addition of Micocin® X to increased killing of *E. coli* AW1.7.

In conclusion, this thesis demonstrated that high pressure alone is insufficient to kill *E. coli* in meat. Bacteriocins, lactate, and chitosan can be applied as additional antimicrobial treatments to kill *E. coli* in food. And the bactericidal effect is strongly dependent on the food matrix and the preservation method. Some of the VTEC strains tested survived heat treatment and high hydrostatic pressure (HHP) permitted recommendations of *E. coli* by Canadian regulatory agencies (Health Canada and Canadian Food Inspection Agency). More studies need to be conducted to validate the results.

Preface

This thesis is an original work by Yang Liu. Some of the research conducted for this thesis forms part of a national research collaboration, led by Dr. M. Gänzle at the University of Alberta, with Dr. A. Gill being the lead collaborator from Health Canada / Sante Canada, Microbiology Research Division, Bureau of Microbial Hazards. The screening for heat- and pressure resistant strains in phosphate buffered saline were designed and conducted by M. Meymandy and Dr. A. Gill from Health Canada, and data analysis of the experiment was illustrated in Figure 1 and 2 of chapter 4. Chapter 5 includes experimental data from the Ph.D. thesis of ZiYi Hu (Figure 5-3, 5-4 and 5-6). Data analysis in chapter 2, 3, 4, 5 and concluding discussion are my original work, as well as the literature review in chapter 1.

Chapter 2 of this thesis has been published as Y. Liu, M. Betti, and M. G. Gänzle, "High pressure inactivation of *Escherichia coli, Campylobacter jejuni*, and spoilage microbiota on poultry meat" Journal of Food Protection, vol. 75, No. 3, 497-503. I was responsible for the data collection and analysis as well as the manuscript composition. Drs. M. G. Gänzle and M. Betti were involved with concept formation and manuscript composition.

Chapter 3 of this thesis has been published as Y. Liu, A. Gill, M. Betti, L. McMullen, and M. Gänzle, "Determination of heat resistance of 101 strains of verotoxigenic *Escherichia coli*, " Proceedings of the 58th International Congress of Meat Science and Technology, *SAFMICROP-35*, 1-4. I was responsible for the data collection and analysis as well as the manuscript composition. Dr. A. Gill

collaborated for the work and contributed to manuscript edits. Drs. M. Betti, L. McMullen, and M. Gänzle were involved with concept formation and manuscript composition.

Chapter 4 of this thesis has been accepted for publication as Y. Liu, A. Gill, L. McMullen, and M. Gänzle, "Variation in heat and pressure resistance of verotoxigenic and non-toxigenic *Escherichia coli*" Journal of Food Protection, Manuscript #: JFP-14-267. I was responsible for the data collection and analysis as well as the manuscript composition. Dr. A. Gill collaborated for the work and contributed to manuscript edits. Drs. L. McMullen, and M. Gänzle were involved with concept formation and manuscript composition.

To my wife and my parents,

Thank you for your support and love

Acknowledgements

I would like to express my sincere gratitude for the guidance and support of my supervisors Dr. Michael Gänzle and Dr. Mirko Betti, who took me in with open arms and taught me about how to become a researcher. Their honesty, integrity and dedication served me as a role model both personally and professionally. My supervisory committee member and candidacy examination chair Dr. Lynn McMullen, thank you for enlighten me on the area of food science and being a knowledgeable mentor to me. My collaborator Dr. Alexander Gill from Health Canada, who gave me the opportunity to work in his lab in Ottawa, and introduced me to various members of the research community, thank you for your kindness and hospitality. I have always enjoyed our discussion on life and I have taken your advice whole heartily.

I would also like to express my appreciation to Ken Fahner, Ross Lowe, Mahdid Meymandy, Kamila Moquin, and Heather Vandertol-Vanier for technical assistance throughout my research. As well, I thank Jody Forslund for being patient and always answer my concerns in time. I am grateful to all colleagues who supported my research. It has been a great pleasure to work with you. I would like to express my gratitude to Alberta Livestock and Meat Agency for funding my research.

Although learning and research formulated an important part of my life in the past five years, more importantly, I see it as a journey of personal growth with confidence, integrity and dedication. For that, I want to express my gratitude towards

those who played a part in it. Particularly, I would like to thank my beautiful and brilliant wife Xiawei Liu, who motivates me, supports me and listens to both my successes and failures on a daily basis. Thank you for taking my hand, standing by me, and encouraging me to follow my dreams to become a better person. Thank you to Xiaoxi Lin, for being a wonderful and trustworthy friend to me, who has always giving me straight answers, thoughtful advise and helped me make difficult decisions. Thank you to Petr Miller, Linda Ho, and Rigoberto Garcia-Hernandez for making life more enjoyable in and out of the lab. I am thankful to Xiaoyan Chen, Yuanyao Chen, Lihui Du, Mengmeng Feng, Ziyi Hu, Tingting Ju, Hui Li, Xiaoji Liu, Yalu Yan, Yan Yang, Jing Zhao and Xin Zhao, who I have the pleasure to meet across the ocean and become good friends with. You have been a family to me, thank you for all of the lovely memories here at the university. Finally I would like to thank my parents for their unconditional love, support and selflessness throughout my life. They left everything behind to move far away and start a new life so I can have a brighter future. Mom and Dad, thank you for raising me and leading by example that passion and hard work would ultimately lead to success.

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Introduction and Literature Review

1.1 High Hydrostatic Pressure Technology and Applications

High hydrostatic pressure (HHP) technology was first proposed as a viable food preservation application in the late 19th century [47]. A typical HHP unit is composed of one or more motors, compression medium and a sealed pressure vessel. In common practices of HHP processing, hydrostatic pressure of up to 800 MPa is uniformly and nearly instantaneously applied to sealed packages of liquid or solid food products suspended in transmission pressure medium in a sealed pressure vessel, the compression power is driven by one or more motor unit/units [55,87]. Common pressure media used for food manufacturing and research are water and ethylene glycol.

The physical process of compression during pressure treatment results in adiabatic heating of the food. The adiabatic effect depends on food, pressure medium and heat transfer between food, pressure medium and pressure vessel. In general 3 to 10 °C increases of temperature per 100 MPa of pressure can be observed after initial compression depending on the water and fat content of the food. If the food product contains a high amount of fat or oil, the temperature will rise higher compared to the one which is mainly water based. When the pressure is released from the pressure vessel, the temperature of the food returns to/or close to the temperature before pressurization, by the process of decompression cooling.

When pressure is applied to the food package, the food decreases in volume as a function of the pressure applied and an equal expansion occurs on

decompression; therefore the package must withstand a change in volume corresponding to the compressibility of the food product [50,82]. For this reason, the packaging material used must accommodate up to 15% of reduction and return to original volume without breaching the seal or barrier of the package [82].

High hydrostatic pressure can be used as an alternative to thermal preservation. Thermal treatment may kill vegetative bacteria and bacterial spores, thus extending shelf-life and improving the safety of food products; however it also causes enzyme inactivation, decreases in nutrient level, and alters the flavor of the food. HHP uses a minimal amount of heat and can modify the functionality of the protein which can yield products with desired texture compared to thermal preservations while they retain high amounts of vitamins, minerals. In addition, HHP is also effective against vegetative bacteria. The HHP technology had been comprehensively reviewed by Norton and Sun, San Martín et al. and Yaldagard et al. [82,98,118].

1.1.1 High hydrostatic pressure and industrial applications

High hydrostatic pressure has the ability to inactivate a wide range of vegetative foodborne pathogens, parasites, molds, fungi and viruses [10,14,36,56,63,93,101]. One important application of HHP is as a post packaging treatment of ready-to-eat (RTE) meats. HHP is effective against *Listeria monocytogenes* in RTE meats [120]. A letter of no objection for the use of HHP to control *L. monocytogenes* in RTE Meats and Poultry had been issued by Health Canada, and similar letter had been issued by USDA-Food Safety and Inspection Service for the use of HHP as an effective post-packaged intervention method for

the US companies in controlling *L. monocytogenes* in RTE Meats and Poultry [44,109]. There is no evidence that the application of HHP decreases food safety of the food. After the 2002 multistate outbreak of listeriosis linked to turkey deli meat in the United States [38], and the 2008 Canada listeriosis outbreak [37], new regulations were issued in both countries to control these pathogens, which accelerated the application of HHP technologies in the food industry. Currently, HHP is used commercially to extend the storage-life of a variety of products in North America, Europe and Japan, including RTE meats and vegetables, ground beef products, fruit juices and smoothies. HHP has also been applied for shucking of oysters, increasing product yield while providing antimicrobial effect against *Vibrio parahaemolyticus* [53]. The use of HHP to control foodborne pathogens in raw meat products is currently undetermined.

1.1.2 Endospores and pressure-assisted thermal sterilization

Endospores are extremely heat resistant and can survive more than 1 GPa pressure treatment [101,107]. Inactivation or killing of endospores can be achieved by combinations of HHP with additional treatments. The HHP treatment initiates germination of endospores and allows secondary treatments such as nisin, change of pH and addition of heat to inactivate these spores [7,48,74,105]. In recent years, pressure-assisted thermal sterilization (PATS) had been proven to be effective against thermally resistant *Bacillus* and *Clostridium* endospores [70]. Although combination of high pressure with modest or high temperature negates the 'non-thermal' aspect of HHP, shortening the exposure time and lowering the exposure temperature can improve overall food product quality comparing to conventional

heating [12,71]. One drawback of PATS is the consistent tailing behavior after treatment [6,70]. The tailing effect occurs when the spore population is under treatment processes, the resistant subpopulation is not inactivated by pressure application while majority of the population is inactivated, which leaves a number of cells to be counted persistently throughout the sampling period and makes the cell count reduction versus time curve resembling a tail. Interpretation of the tailing phenomenon includes: tailing is a normal feature, bound to the mechanism of inactivation and resistance; tailing is independent from the mechanism of inactivation and likely arising from genetic heterogeneity, treatment process, spore clumping and enumeration technique; and lastly, tailing phenomenon could be neutral observers [18]. Spore population heterogeneity and/or pressure stabilization during HHP treatments can be contributors to the tailing phenomenon [12].

1.1.3 Multiple-cycle high hydrostatic pressure treatments

A maximum of three HHP cycles are allowed to process the food during processing when failure occurs in the first attempt. Some authors have reported multiple-cycle HHP treatments to be more effective compared to single-cycled treatments for inactivating various bacteria species [76,77], for example multiple-cycle HHP achieved a higher inactivation of *Escherichia coli* O157:H7 CECT 4972 than did single-cycle treatments for the same total length of treatment [76]. Multiple-cycle HHP treatments are not likely to be adapted by the industry, as the process is not only time-consuming, but also causes intensive wear on the machine.

In addition, HHP resistant strains can arise following multiple-cycle HHP in *E. coli* and *L. monocytogenes* [52,101]. Subjecting *E. coli* to repeated cycles of

lethal pressure treatment followed with outgrowth could generate pressure resistant variants [41,107]. The variants are likely to rise from the accumulation of multiple mutations over many generations instead of spontaneous mutation of the wild type strain [41,68]. Upon comparison of the intrinsic potentials for HHP resistance development among strains of *E. coli*, *Shigella flexneri*, *Salmonella* Typhimurium and *Salmonella* Enteritidis, *Yersinia enterocolitica*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Listeria innocua* using a selective enrichment approach, of all strains examined, the acquisition of extreme HHP resistance could be detected in only some of the *E. coli* strains [110]. Specific genetic predisposition of the bacteria might be required for resistance development [110].

1.2 HHP and *E. coli*

The HHP inactivation of vegetative bacteria has very variable effects that relate to the use of different food matrices, different strains of bacteria and the potential for generation of pressure resistant mutants. Greater insight of the mechanisms of HHP is crucial for the understanding and effective application of the technology. *E. coli* is an ideal model organism to discuss mechanisms of HHP inactivation. The presence of generic *E. coli* is an indicator for potential enteric pathogens. Also *E. coli* physiology is extremely well studied, and inactivation of *E. coli* by HHP is well documented.

Verotoxigenic *Escherichia coli* (VTEC), also known as Shiga toxigenic *E. coli* (STEC) are an important group of foodborne pathogens. The potential outcomes of VTEC infection include uncomplicated diarrhea, bloody diarrhea, kidney failure and death [60]. Survivors may also face long-term health problems

[60]. Some VTEC strains are heat resistant [103,116], which makes HHP an apparently attractive alternative non-thermal process to inactivate these pathogens. The physiological characteristics (outer membrane, cytoplasmic membrane, the cytoplasm, regulation of gene expression) of *E. coli* that contribute to HHP resistance and the mechanisms of HHP inactivation of *E. coli* will be outlined in the following paragraphs.

1.3 Mechanisms of *E. coli* Inactivation by HHP and *E. coli* Stress Responses to HHP

1.3.1 HHP induced damage to the outer membrane

The primary target site of HHP is believed to be the bacterial membranes and membrane bound proteins [108]. HHP induces physical damage to the membranes, disappearance of both outer and inner membrane proteins, as well as change to the cell wall structure [Figure 1-1;95,102]. Scanning electron microscopy imaging of *E. coli* cell exposed to 200 MPa pressure, revealed that the normally rough surface became smoother and the surface area appeared to be larger than the untreated cells; in addition, the bacterial membrane was distorted after 250 MPa treatments [89].

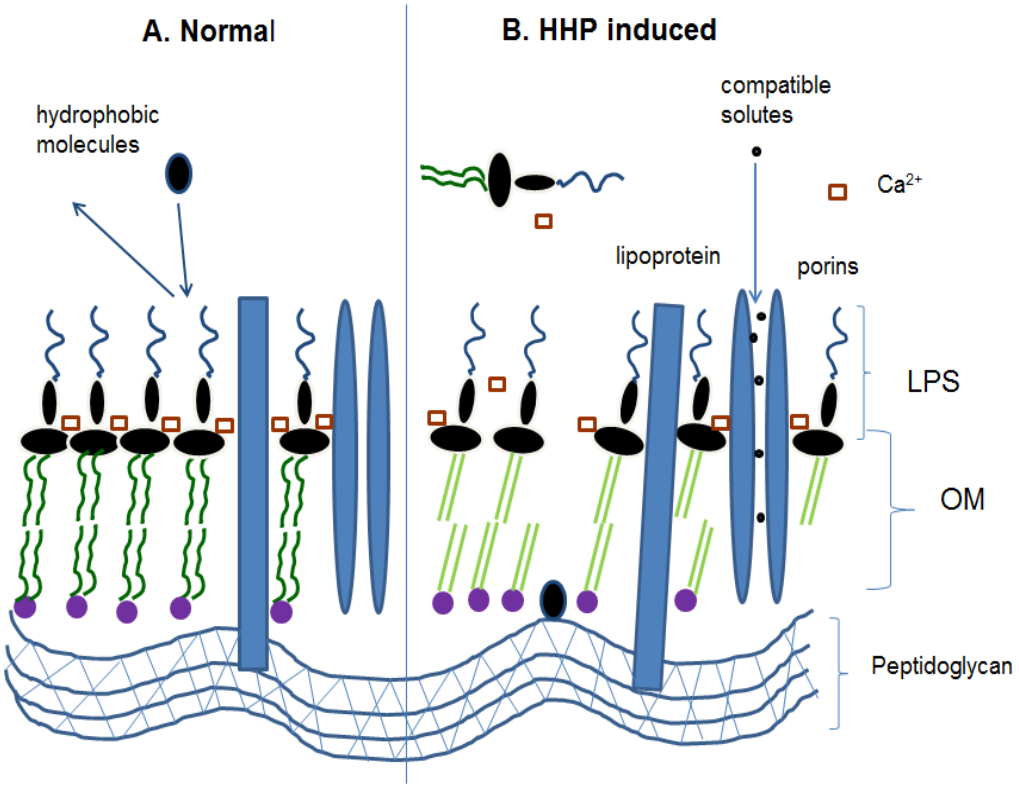

Figure 1-1: HHP and the outer membrane. Panel A represents the intact *E. coli* outer membrane (OM). The lipopolysaccharide (LPS) layer on the surface prevents penetration of hydrophobic molecules. Panel B represents an HHP treated OM. HHP breaks electrostatic interaction between positively charged bivalent cations, such as Ca^{2+}, and negatively charged LPS molecules. The process weakens membrane integrity and facilitates entry of otherwise impenetrable hydrophobic molecules. HHP also causes tight packing and phase transition of the phospholipid layer from a liquid crystal phase to a gel phase. Accumulation of compatible solutes from the environment by an uncommon OM porin NmpC contributes to HHP resistance in *E. coli* AW1.7 [97], and OmpX OM porins are expressed when *E. coli* is grown under pressurized conditions [78]. OM lipoprotein contributes to structural integrity of *E. coli.* OM lipoprotein mutants [one under RpoS control (OsmB) and the other inducible by pressure (NlpI)], are more pressure sensitive than the wild type strains [19].

The outer membrane (OM) of *E. coli* contains two leaflets, the outer leaflet is composed of a lipopolysaccharide (LPS) layer which is stabilized by divalent cations, while the inner leaflet is composed of lipoproteins and phospholipids [61,92]. HHP damages the electrostatic linkage between divalent cations and LPS molecules, resulting in the release of LPS and increaing membrane permeability as

determined by diffusion of a fluorescent dye, lysozyme, and antimicrobial compounds [33,40,67,85,89]. Increased membrane operability makes cells vulnerable to antimicrobial peptides that normally do not penetrate the outer membranes [72,102,119]. At ambient pressure, lysozyme cannot penetrate the intact OM, however addition of lysozyme enhanced killing of *E. coli* by HHP [40]. Another example is Nisin, a positively charged Class I lantibiotc. Nisin is not effective against Gram-negative bacteria with an intact OM [29], but is effective when used in conjunction with HHP [31, 34].

Porins are responsible for cellular homeostasis, and compatible solute uptake [88], and are important contributors to pressure resistance in *E. coli*. Quantification of gene expression of the heat and pressure resistant *E. coli* (*E. coli* AW1.7), revealed the increased expression of an uncommon OM porin NmpC and several transport proteins which contributed to heat resistance in *E. coli* [25,66,97]. NmpC is not expressed in most strains of *E. coli* [46]; it is responsible for accumulation of compatible solutes and contributes to heat resistance of bacteria [97]. When *E. coli* cells were grown under pressurized conditions of 0.1 MPa, the expression of OM porins ompF and ompC were significant reduced and ompX was expressed upon changes of osmolarity [78].

The accumulation of compatible solutes is important for heat resistance in *E. coli* [90,97], and likely also contributes to its pressure resistance. The presence of compatible solutes such as sugar and salt in the system increased bacteria survival under pressurized conditions [21,75,111]. *E. coli* obtain available compatible solute from the environment and can produce compatible solutes endogenously. In high

osmotic environments, the accumulation of compatible solutes leads to the restoration and maintenance of cellular turgor pressure [100]. The preferential hydration models indicate that the preferential exclusion of compatible solutes forms a hydration shell around the immediate surface of protein, and protects the protein from unfolding due to HHP treatment [5,23]. The presence of sugar in milk is important in resistance of *E. coli* to HHP induced osmotic stress [34]. Increased resistance to high pressure of *E. coli* O157:H7 was observed in milk compared to poultry meat and phosphate buffer [86]. Compatible solute Uptake by solute transporter proteins at cytoplasmic membrane and accumulation of compatible solutes by passing through the cytoplasmic membrane had also contributed to HHP resistance [111].

Studies have also demonstrated that the OM lipoprotein and anchor proteins also contributed to HHP resistance. Under HHP conditions, the wild type strain was compared to *osmB* gene and *nlpI* gene mutants. Results from the studies revealed that the OM lipoprotein is important in resistance of *E. coli* to HHP [19,68]. The *osmB* gene is induced in the stationary phase or by hyperosmotic stress conditions [57] and *nlpI* encodes for an OM anchor protein which may be involved with cell division [83].

1.3.2 HHP induced damage to the cytoplasmic membrane

The cytoplasmic membrane is composed of phospholipid bilayer and protein molecules. Pressure resistance is influenced by membrane fluidity and fatty

acid composition; cells with more fluid membranes are more pressure resistant [Figure 1-2;16].

The HHP-induced membrane damage facilitates disruption of electron transport components, results in leakage of ATP and impairs the acid efflux mechanism [64,101]. Impaired activity of membrane bound ATPase had been previously shown to contributes to cell injury [102,117]. HHP treatment of *Lactobacillus plantarum* facilitated the reduction of F_0F_1-ATP synthase, caused injury to the bacterial cell.

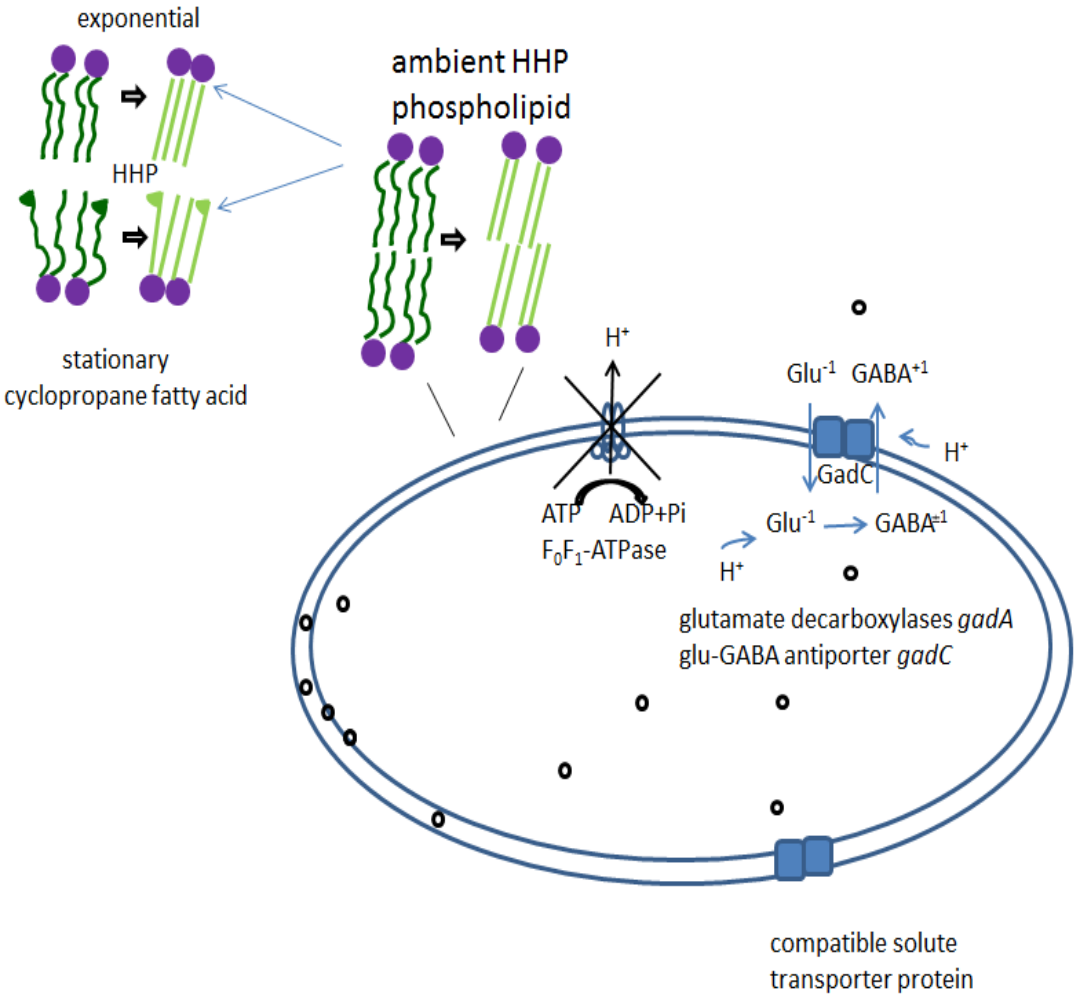

Figure 1-2: The impact of HHP on the cytoplasmic membrane. High pressure decreases lateral motion and induces phase transition in the phospholipid bilayers of *E. coli*, and promotes gelation of the membrane lipids [69,85]. Pressure resistance is influenced by membrane fluidity and fatty acid composition [16]. HHP inactivates F_0F_1-ATPase, which causes disruption of the acid efflux system [117]. Presence of glutamate in the system and activation of glutamic acid survival pathways improved the survival of *E. coli* during post-pressure acid challenge [62]. Exponential phase cell are more sensitive to HHP compared to stationary phase cells [15]. Stationary phase cell express the *cfa* gene that encodes for cyclopropane fatty acyl phospholipid synthase that converts unsaturated fatty acids to their cyclopropane form, which contribute to acid resistance in *E. coli* [13,39]. Cyclopropane fatty acids and accumulation of compatible solutes improves the post-pressure acid survival of *E. coli.*

In an acidic environment, the killing effects of high pressure against vegetative cells were enhanced [5]. The HHP treatments of strains of *E. coli* O157:H7 at 25 ^{o}C, 345 MPa for 5 min in citric acid and lactic acid (pH 4.5, 5.5 and 6.5) decreased cell viability by between 1.8 and 4.9 CFU/mL with increasing lethality corresponding to decrease in pH [5]. At low pH, bacteria are not able to immediately repair themselves, and thus sensitized to further injury.

Availability of glutamate in the environment and the presence of a glutamate survival pathway under acidic conditions and or at the stationary phase strongly improves the post-pressure acid survival of *E. coli* [62,94]. Survival of an acid resistant strain under HHP treatment was manifested as a long shoulder followed by a more rapid drop in cell number [9]. The author of the study suggested that the breach of the acid resistance barrier of the cell resulted in cell death.

The survival of *E. coli* under HHP can be different dependent on the growth stage of the cell. Important contributors for survival of exponential phase cells under pressure are growth conditions and membrane fluidity [16,17]. Exponential phase cells stained with a lipophilic dye that mainly binds to the cytoplasmic membrane showed physical disruption of the membrane after HHP and resulted in formation of vesicles, areas of engrossment, and invagination toward the cytoplasm [69]. However, stationary phase cells behaved differently under HHP. Under stationary phase, the cytoplasmic membrane of HHP sensitive strains underwent similar irreversible disruption as an exponential phase cell, but for HHP resistant strains, membranes are able to re-seal after HHP [85]. Stationary phase *E. coli* O157:H7 C940 was resistant to HHP, acid, oxidative and osmotic stress [9]. In

the stationary phase, the role of the cytoplasmic membrane is more complicated due to differences in the action of σ^S (RpoS) status [96]. When cells enter into the stationary phase, a morphological and physiological change occurs due to the action of RpoS. Stationary phase cells have a higher degree of crosslinking among membrane proteins and are less prone to lateral phase transition [73,104]. RpoS controls the transcription of >50 genes [51], including those that control cyclopropane fatty acid conversion and the glutamate survival pathway [20,94]. Variation in the RpoS activity correlates with the pressure resistance of isolates of *E. coli* O157:H7 [17,20,26,27,30,45]. A study had shown variation in the RpoS activity correlated with the pressure resistance of natural isolates of *E. coli* O157:H7 [96], and RpoS null mutants are significantly more pressure sensitive compared to a wild type strain [19].

1.3.3 HHP induced changes to the cytoplasmic contents

High hydrostatic pressure can inflict considerable cytoplasmic damage to *E. coli* (Figure 1-3). Intracellular, pressure induces oxidative stress as it releases iron from the Iron-sulfur cluster (Fe-S cluster), which reacts with hydroxyl free radicals and facilitates the generation of reactive oxygen species though a Fenton reaction [68]. Up-regulation of thiol-disulfide redox system increases antioxidant defense and balances cellular homeostasis after HHP [51,68,69]. When pressurized cells were incubated anaerobically, their survival increased significantly compared to when they were aerobically incubated [3].

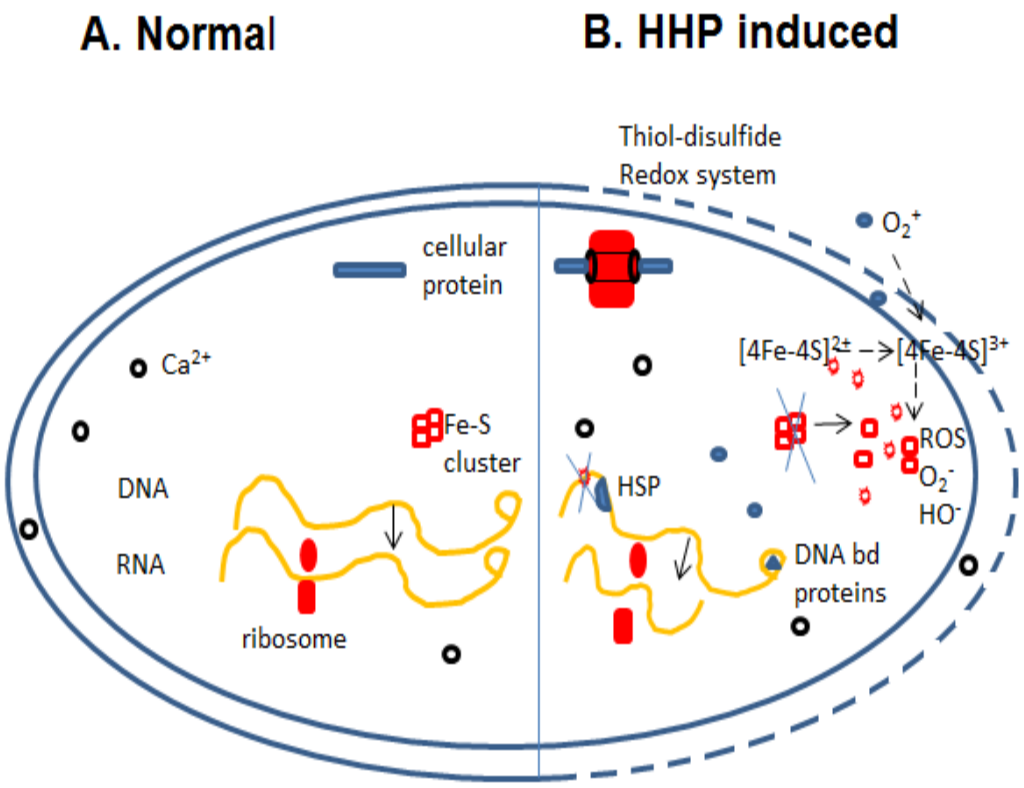

Figure 1-3: HHP and the impact on the cytoplasm. HHP sensitizes the cytoplasmic and membrane enzymes, ribosomes and proteins, results in dissociation of ribosomes [80], condensation of the nucleoid and aggregation of intracellular proteins [69] and leakage of RNA to the extracytoplasmic medium in exponential phase [69]. Influx of oxygen molecules leads to imbalance of cellular metabolism, dissociation of the Fe-S cluster and generation of reactive oxygen species (ROS) [68]. ROS in the form of ions, free radicals or peroxides can damage different parts of the cell, including DNA, lipids and proteins, which ultimately leads to cell death. Damage to cytoplasmic proteins caused by HHP and/or oxidized by ROS induces a heat shock response, production of heat shock proteins (HSP) aiding in refolding of the damaged proteins. Expression of DNA binding proteins (DNA bd proteins) and up-regulation of Thiol-disulfide redox system increased antioxidant defense, contributes to refolding of DNA and proteins balances cellular homeostasis after HHP [68]. Ca^{2+} binding affinity may also contribute to HHP resistance [42].Filled circles represents O_2^+; Open circles represents Ca^{2+}.

High hydrostatic pressure treatment of *E. coli* resulted in dissociation of ribosomes at 50 MPa [80], condensation of the nucleoid and aggregation of intracellular proteins at 200 MPa [69]. The ribosome is essential for protein

synthesis. The RNA from exponential phase cells is lost to the extracytoplasmic medium from HHP, and condensation of the nucleoid occurs; however, the stationary phase cells also has condensed RNA but to a lesser extent and very little reduction of RNA has been observed [69]. Condensation of the nucleoid is not observed in untreated cells, indicating that pressure induces a conformational change of the ribosome [69].

High hydrostatic pressure treatment induces cellular stress mechanisms. High pressure could turn on both cold and heat shock proteins by acting on the ribosome either due to the stress response or the state of the ribosomes [113]. Pressure treatment associated denaturation of proteins can induce a heat shock response which aids the refolding of proteins. The drastic change of the nucleoid from exponential phase cells compared to stationary phase cells observed Mañas et al. [69] is likely due to the absence of DNA-binding proteins synthesized in response to oxidative stress and during stationary phase growth [51,69].

Comparison of the wild type *E. coli* MG1655 and pressure resistant mutants *E. coli* LMM1010, LMM1020 and LMM1030 revealed that the presence of divalent cations such as Ca^{2+} in the testing solutions increased HHP resistance of *E. coli* and the effects were cation dose dependent [42]. Ca^{2+} ions are involved in regulation of DNA replication and cell division [81], therefore are important for bacterial survival under HHP.

1.3.4 HPP induced changes of gene expression

The resistance of *E. coli* to HHP can be multifactorial (Table 1-1); with more than 100 *E. coli* genes responding to sub-lethal HHP treatment [68,113]. Exposure of *E. coli* to high pressure also induces an SOS response [1,96]. Malone et al. [68] demonstrated that HHP induces up-regulation of the universal stress protein (UspA) family (*uvrA, recA*, and *sulA*) and down-regulation of *dps.* The universal stress protein family is involved in stopping cell replication, fixing DNA damage and inducing mutagenesis. Reduced transcription of *dps* in response to HHP may allow the renaturation of chromosomal DNA to its protective state [68]. However, from the same study, *E. coli* mutants lacking *dps* (DNA-binding protein) were sensitive to HHP [68], which suggests the importance of the contribution of DNA binding proteins to HHP resistance.

Aertsen et al. [2] showed up-regulation of *lon*, *clpPX*, and *dnaK* genes after HHP which suggested activity of σ^H. Heat shock proteins and cold shock proteins can act as chaperones that help correct folding of cellular proteins and ribosomes damaged by HHP.

Spontaneous mutations can also involve in HHP resistance. Spontaneous mutations are rare changes of bacterial genetic material and allow cells to develop different types of resistance to survive under stressful environments. Spontaneous mutation genes *yafO* and *yafN* are up-regulated in response to HHP [68].

As mentioned above, the Fe-S cluster can be detrimental to the cell under high pressure. Genes that contribute to oxidative stress survival are also important for HHP resistance. HHP causes up regulation of an oxygen sensor (*fnr*) and down regulation of the Fe-S cluster related operons (*isc* operon) and up-regulation of the operon repressor *iscR* [68]. Pressure resistant mutants, such as *E. coli* LMM1010 are more resistance to the oxidative stress than wild type strain [41], and mutants with impaired peroxide (*katE, katF*, *oxyR*) and superoxide (*sodA, sodB*, *soxS*) stress genes are sensitive to HHP [3]. Up-regulation of the thiol-disulfide redox system has also been observed [68], and its importance for survival in response to HHP has been mentioned in section 1.3.3.

The production of compatible solutes and presence of the transcription dual-regulator proteins also contributes to HHP resistance. Trehalose synthesis by trehalose synthase (*otsA, otsB*) increased pressure resistance of *E. coli* O157:H7 compared to an $otsA^{-}$, $otsB^{-}$ mutant [68]. Transcription dual-regulator protein genes *hns* and *stpA* double mutants were significantly more pressure sensitive compared to the wild type [68].

Table 1-1: Genes that contribute to HHP resistance in *E. coli*

Gene	Functional category	Regulation
trxA, trxC, grxA, nrdHIEF operon, *katE, katF, oxyR, sodA, sodB, soxS, fnr*	Oxidative stress genes	up-regulated
UvrA, recA, sultA, Ion, clpP, clpX, dnaK dps	σ^S and σ^H factor	up-regulated down- regulated
cspA	cold shock protein	up-regulated
yafO, yafN	spontaneous mutation	up-regulated
otsA, otsB	trehalose synthase	up-regulated
hns, stpA	Transcription dual-regulator and H-NS-like protein	up-regulated
iscR *suf* operon	Fe-S cluster	up-regulated down regulated
UspA family	Universal stress protein	down regulated

1.4 Inactivation of Different Strains of *E. coli* by HHP

A comparison of the inactivation of *E. coli* in different food matrices including meats, dairy and fruit products is show in Table 1-2. The variability of the lethal effects of HHP indicates that HHP resistance is strain and food matrix dependent. In meat products, a 1 to 5 log reduction in cell count was observed, and in fermented dairy and fruit products 1 to 8 logs reduction in cell count was observed. Meat matrix is an extremely complicated complex where many factors could interfere with treatment processes. Fermented dairy products and fruit products represent much simpler matrices.

The lethal or sublethal outcome of HHP induced injury is dependent on the strain and on the matrix. For example, treatment at 550 MPa for 2 min in apple juice of six strains of VTEC resulted in a range of cell reductions from 1.25 to 4.39 log CFU/mL [114]. In another study, a cocktail of VTEC was treated at 615 MPa for 2 min in grapefruit juice and apple juice, and a 8.34 log CFU/mL cell reduction was observed in grapefruit juice; however, only a 0.41 log CFU/mL cell reduction was observed in apple juice [106]. Pressure treatment of *E. coli* O157 C9490 in apple juice and tomato juice resulted in 5 log CFU/mL cell reduction, but only 1 to 2 log reductions was observed in orange juice [58]. The large variability of HHP results suggests that additional hurdles should be applied to improve the efficacy.

Table 1-2: Overview on inactivation of different strains of *E. coli* by HHP in food

E. coli Serotype	Strain #[1]	P/T (GPa/°C)	Time (min)	Lethality[2]	Products (reference)
		Meat and meat products			
O103:H5	O103:H25rifR	0.6/24-30	3.3	3.3	Sausage (84)
O157:H7	FSIS OB070361, JBL2139, JBL2347, and JBL1411	0.6/28-37	1-5	>4.7	RTE meats (89)
O157:H7	00-3581, 02-0304, 02-0627, and 02-0628	0.6/34	3	4	RTE meats (55)
O157:NM	02-1840				
O157:H7	CECT 4972	0.4/12	20 5x5x5	4.39 4.96	Ground beef (76)
O157:H7	250, 251, H1730, 52 and Cider	0.4/20 0.4/-5	10	3 1	Ground beef (11)
		Milk and dairy products			
O59:H21 **O157:H7**	CECT 405 CECT 5947	0.4/20	10	4.28 4.05	Cheese (24)
	ATCC 11229	0.59/5	1x1x1	4	Milk (28)
O157:H7	ATCC 43888	0.35/25	15	~1 ~2.7	Skim milk Banana juice (79)
O157:H7	933 931	0.35/50	5	>8 >8	Milk orange juice (4)
		Fruit juices (ju.) and fruit products			
O157:H7	H1730, Cider, 250, 251, J58	0.45/21	2	6	Strawberry puree (49)
O157:H7	C9490	0.5/20	5	1-2 5 5	Orange ju. Tomato ju. Apple ju. (58)
O157:H7	SEA13B88, ATCC 43895, and 932	0.62/15	2	8.34 0.41	Grapefruit ju. Apple ju. (106)
O157:H7	E009	0.55/6	2	1.92	Apple ju. (115)
O157:H7	E009 E0019 E994 Cider F4546 H1730	0.55/6	2	1.25 1.61 1.81 4.39 1.9 2.77	Apple (App) ju. (114)
	ATCC 25922	0.4/25	0.5 3	2.19 4.82	Cashew apple ju. (65)
	ATCC 11775	0.3/20	5 5x 1	4 1 4.5 2.1	Kiwi, pineapple ju. Kiwi, pineapple ju. (15)
	MG1655 LMM1010	0.3/20 0.4/20 0.3/20 0.4/20	15	>4.4, 3.5 >4.4, >4.4 1.1, 0.8 >4.7, 1.5	App, orange ju. App, orange ju. App, orange ju. App, orange ju. (51)
	LMM1010	0.3/40 0.5/40	10	>5.8	App pieces App in syrup(112)

[1.] Underlined are VTEC; [2.] Lethality: Reduction log CFU/g or CFU/mL

1.5 Use of HHP with additional antimicrobial hurdles to inactivate *E. coli*

The pressure resistance of *E. coli* is highly variable and is strain dependent (Table 1-1); thus combinations of HHP with additional antimicrobial hurdles should be considered. Combination of CO_2 and HHP has been studied in orange juice, but the results indicated the combination was ineffective in reducing cell counts of *E. coli* [22]. HHP followed by low temperature storage can dramatically increase the killing [11,58]. The presence of lactic acid in dairy products can have additional antimicrobial effects [33]. Increased acidity in combination with mild temperature (25 ^{o}C) reduced cell counts of *E. coli* [9,58]. Membrane damage induced by HHP enables the inactivation of *E. coli* by bacteriocins in buffer, milk and meat model systems which are otherwise ineffective [34,35,58]. Chelators such as EDTA also increased killing of *E. coli* by HHP [42]. Thus HHP in combination with low temperature storage, acid and antimicrobial compounds could, in theory, inactivate strains of *E. coli* which are otherwise HHP resistant.

1.6 Hypothesis and Objectives

To investigate the application of HHP processing in combination with other treatments to inactivate *E. coli* in food (especially meat) products, the following studies were conducted:

1) To compare the pressure resistance of a heat resistant VTEC surrogate *E. coli* AW1.7 to that of the other foodborne pathogens and spoilage organisms relevant in meat;

2) To compare the heat resistance of *E. coli* AW1.7 with VTECs in media;

3) To compare the heat- and pressure resistance of *E. coli* AW1.7 with the heat- and pressure resistance of VTECs in media and meat; and

4) To evaluate bacteriocins, lactate, and chitosan with pressure or heat treatment to reduce the cell counts of *E. coli* in different media and food matrices.

Cross-resistance of heat and pressure has been observed in *E. coli*. The thesis research would not only generate a large amount of data on the heat and pressure resistance of VTEC, but it also would test the effects of hurdles on killing these pathogens based on the selection of different food matrices. Results of the thesis may help in the design of treatment processes for food preservation to improve the microbiological safety of the food products. I hypothesis that many strains of *E. coli* are heat- and pressure resistant and *E. coli* cell survival after treatments dependent on the food matrices. Additional treatment such as bacteriocins, lactate and low temperature storage would increase the killing of *E. coli* in foods.

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2. High Pressure Inactivation of *Escherichia coli*, *Campylobacter jejuni*, and Spoilage Microbiota on Poultry Meat

2.1 Introduction

High pressure processing is an alternative to thermal processing of food. The application of high pressure at ambient temperature inactivates microorganisms. In addition to the preservative effect, pressure application may improve the texture of whole cuts of meats. Increased tenderness, juiciness, springiness and chewiness of meat products were achieved by pressure treatment of red meats and poultry meat [26,40,42]. Pressure and temperature treatments in the range of 200 – 800 MPa and 20 - 50 °C had a synergistic effect on increasing the hardness in chicken breast muscle [47]. Temperature-assisted high pressure processing of chicken breast meat achieved a texture similar to cooked poultry products after a treatment at 400 – 600 MPa and 40 °C [30]; however, poultry meat has a relatively short storage-life due to a high prevalence of pathogenic and spoilage organisms [23,31]. This chapter studies the microbial safety of pressure-treated poultry products.

Campylobacter species are an important cause of foodborne gastroenteritis in developed nations [8,36]. *Campylobacter jejuni* accounts for the majority of cases of campylobacteriosis. Foodborne outbreaks are predominantly linked to handling and consumption of raw or undercooked poultry products [16,36]. Although bacterial resistance to pressure is highly variable even among strains of the same species [3,6,20], data on the pressure resistance of *C. jejuni* are available only for few strains [25,41]. Other relevant pathogens on fresh poultry

meat include *Staphylococcus aureus* and pathogenic *Escherichia coli.* Both species exhibit high resistance to pressure compared to other vegetative bacterial cells [6,56]. *E. coli* AW1.7 is a beef carcass isolate that is highly resistant to heat [11] and was used as a model organism for this study.

Extension of the refrigerated storage life of poultry meat by pressure processing requires the control of psychrotrophic spoilage microbiota such as *Carnobacterium* spp. and other psychrotrophic lactic acid bacteria, *Pseudomonas* spp. and allied Gram-negative organisms, and *Brochothrix thermospacta* [22,28]. Data on the pressure resistance of *Carnobacterium* spp. and *B. thermospacta* is unavailable.

2.2 Objective and Hypothesis

The aim of this study is to determine the pressure resistance of *C. jejuni*, *E. coli*, and spoilage organisms of the genera *Brochothrix, Carnobacterium,* and *Pseudomonas*. Pressure processing conditions were selected to match treatment parameters that provide products with similar texture compared to cooked meat products [30]. The strain selection included meat isolates for each target organism, and all pressure treatments were performed on aseptically prepared poultry meat. It was hypothesized that the proposed HHP parameters can be used to inactivate all tested organisms including *C. jejuni*.

2.3 Material and Methods

2.3.1 Aseptically comminuted chicken breast meat preparation

Skinless chicken breasts were retrieved from whole chicken carcasses obtained at a local retail store and stored at -20 °C. Breast meat was thawed, washed with tap water, air-dried for 1 min, soaked in 3% hydrogen peroxide for 2 min, air dried again, soaked in 98% ethanol for 1 min, and flamed. The outer layer of the meat was removed with a sterile surgical blade to remove meat that was denatured by decontamination treatments and the remaining meat was divided into approximately 5 g portions and stored in sterile plastic bags at -20 °C until use. Representative samples from each batch were plated on BHI-YE (brain-heart infusion yeast extract) agar (BHI, *BD*, *Bacto*, Spark, MD and 5 g/L Yeast extract (*BD*) to ensure the absence of contaminating microbiota from the meat. Before each experiment, 5-g portions of aseptic poultry meat were thawed, stomachered for 1 min in sterile bags, minced with a sterile surgical blade to achieve a particle size of approximately 1 mm^3 or less, and manually homogenized for 1 min.

2.3.2 Bacterial strains and growth conditions

Bacterial strains and culture conditions are listed in Table 2-1. Strains FUA 2053-2057 and FUA 1232 were isolated from chicken breast and identified by 16S rRNA gene sequencing. Cultures were maintained at -80 °C in 65% glycerol. Cultures were initially streaked on agar plates as listed in Table 3-1. *C. jejuni* was incubated for 48 h in anaerobic jars supplemented with GasPak EZ Campy Container System (*BD Diagnostics*, Sparks, MD) to generate microaerophilic

conditions (5% O_2, 10% CO_2, and 85% N_2). Other organisms were incubated aerobically for 24 h. One colony from each culture was suspended in either Luria-Bertani- Miller media (*BD*, *Difco*) or BHI-YE (*Bacto*) and incubated for 24 h or 48 h at 30 °C or 37 °C (Table 2-1).

Table 2-1: Bacterial strains and culture conditions

Organism	Origin(reference)	Culture media / agar for selective enumeration
Brochothrix thermosphacta FUA2054	Poultry meat (this study)	BHI-YE broth / STAA[b] agar aerobic, 30 °C
Carnobacterium divergens FUA2053	Poultry meat (this study)	BHI-YE broth / APT[c] agar aerobic, 30 °C
Campylobacter jejuni ATCC700819	33	BHI-YE broth, mCCDA[d] agar, microaerophilic, 42 °C
C. jejuni FUA1220	Poultry meat (this study)	as above
C. jejuni HCJ2002, HCJ2082, HCJ2241, HCJ2316, HCJ3400, HCJ3599, HCJ4132, HCJ4763	Human clinical isolate	as above
C. jejuni PCJ420, PCJ426, PCJ470, PCJ472, PCJ481, PCJ490, PCJ494, PCJ497, PCJ498	Poultry isolate	as above
Escherichia coli AW1.7	Beef carcass (11)	BHI-YE broth / Endo[e] agar or LB[f] agar, aerobic, 37 °C
E. coli FUA1041 (STEC)	Cow rectum (25)	BHI-YE broth / LB agar, aerobic, 37 °C
E. coli FUA1233	Poultry meat (this study)	as above
E. coli FUA1234	Poultry processing facility (this study)	as above
E. coli MG1655	43	as above
E. coli LMM1030	43	as above
Pseudomonas fluorescens FUA1232	Poultry meat (this study)	BHI-YE broth / BHI-YE-AB[g] agar, aerobic, 30 °C
Salmonella enterica ATCC13311		BHI-YE broth / Endo agar, aerobic, 30 °C
Staphylococcus condimenti FUA2057	Poultry meat (this study)	BHI-YE broth / BP[h] agar, aerobic, 30 °C
Staphylococcus saprophyticus FUA2056	Poultry meat (this study)	as above
Staphylococcus sciuri FUA2055	Poultry meat (this study)	as above

[a] Brain Heart Infusion with Yeast extract, (*BD Diagnostics*, Sparks, MD)

[b] Streptomycin-thallous acetate-actidione with STAA supplement, (*Oxoid*, Lenexa, KS, U.S.A.)

[c] All-Purpose Tween, (*Difco Laboratories*, Detroit, MI, U.S.A.)

[d] modified Charcoal Cefoperazone Deoxycholate Agar with mCCDA supplement (*BD*)

[e] Endo agar, (*Difco*)

[f] Luria- Bertani- broth, (*Difco*)

[g] Brain Heart Infusion with Yeast extract, Bacto, supplement with Amphotericin B solubilized (*Sigma Co.,* St. Louis, MO) and Vancomycin hydrochloride (Sigma)

[h] Baird- Parker agar Difco, supplemented with egg yolk-tellurine emulsion, (*Oxoid*)

2.3.3 Pressure resistance of *C. jejuni*

Stationary-phase (48 h) cultures of *C. jejuni* were centrifuged at 6000 x g for 10 min at room temperature, and the cell pellets were resuspended in an equal volume of buffered peptone water (BP, 0.1% peptone, pH adjusted to 7.0 with 1M NaOH). Cell suspensions (0.5 mL) were mixed with 3 g of aseptically prepared breast meat to achieve a cell count of about 10^6 CFU/g. Approximately 0.3 g of the mixture was packed into 3 cm Tygon R3603 tubing (Akron, USA) and heat sealed at both ends. The sample was placed in a 2-mL Wheaton™ Cryovial (*Wheaton*, Millville, NJ, USA) filled with 5% bleach and treated at 300 MPa and 30 °C for 3 min in a Multivessel Apparatus U111™ (*UNIPRESS equipment division*, Warsaw, Poland). Maximum sample envelope dimensions are 12.4 x 60 mm. Polyethylene glycol (100%) was used as pressure transmission fluid. Compression and decompression rates were 300 MPa/min. The temperature of the unit was maintained by a thermostat jacket coupled to an external water bath. The temperature in the pressure vessels was monitored by internal thermocouples to ensure that temperature changes during compression and decompression did not exceed 2 °C. After decompression, serial dilutions of untreated and pressure-treated samples with BP were plated on BHI-YE and modified charcoal cefoperazone deoxycholate (mCCDA) plates, and incubated for 48 h.

2.3.4 Recovery of sublethally injured *C. jejuni* after pressure treatment

C. jejuni HCJ2316 was treated in minced poultry meat at 300 MPa at 30 °C for 3 min as described above. Untreated and pressure-treated samples were

diluted and plated on mCCDA agar and BHI-YE-agar, or on BHI-YE agar with the following additives: 0.30 g/L $FeSO_4$ (Fe); 3.0 g/L tryptone (T); 1.0 g/L of sodium desoxycholate x H_2O (SD); BHI-YE (Fe + T); BHI-YE (Fe + SD); BHI-YE (SD + T); or BHI-YE (Fe + SD + T). Plates were incubated for 48h.

2.3.5 Survival of *C. jejuni* and growth of meat microbiota during refrigerated storage of meat

Aseptically prepared minced poultry meat was inoculated with *C. jejuni* HCJ2316 alone, or with *C. jejuni* HCJ2316 together and with a cocktail of the meat spoilage organisms *B. thermosphacta* FUA2054, *Carnobacterium divergens* FUA2053, and *Pseudomonas fluorescens* FUA1232, and the heat-resistant beef isolate *E. coli* AW1.7. *C. jejuni* was inoculated to a level of ~6.5 log CFU/g, spoilage organisms were added to a cell count of 4.0 log CFU/g (*B. thermosphacta*) or ~6.5 log CFU/g each (all other organisms). Inoculated minced meat was divided into portions of 0.3 g, which were individually packaged with an oxygen-permeable film OPE 1950 R® (*Winpak*, Winnipeg, Canada), or vacuum-packaged with a Multivac T200 Tray Sealer (Wolfertschwenden, DE) under modified atmosphere (30 % CO_2, 0.39 % CO, balance N_2) in Mapfresh ™ packaging trays (Winpak, Winnipeg, Canada) covered with a ESXE 1250R® film (*Winpak*, Winnipeg, Canada). Samples were stored at 4 °C for 8 days. Organisms on the meat samples were enumerated by plating on selective agar (Table 2-1) after packaging and after 2, 4, or 8 days of storage. The survival of *C. jejuni* on meat during refrigerated storage was assessed in three independent experiments and results are reported as means ± standard deviation.

2.3.6 Survival of *C. jejuni* and growth of meat microbiota after pressure treatment and refrigerated storage

Aseptically prepared minced poultry meat was inoculated with C. *jejuni* HCJ2316 and the strain cocktail and treated at 400 MPa and 40 °C for 30 min with sample preparation same as described above. Samples were vacuum packaged in ESXE 1250R® film immediately after treatment and stored at 4 °C for 21 days. Bacterial cell counts were determined by surface plating on selective media (Table 2-1) after packaging and after 2, 4, 8, 14, or 21 days of storage at 4°C.

2.3.7 Pressure resistance of *E. coli* AW1.7 in comparison to other *E. coli* strains, *Salmonella* Typhimurium and *Staphylococcus* spp

Aseptically prepared minced poultry meat was inoculated with one of the following strains: *E. coli* strains AW1.7, FUA1041, or FUA1234, *Salmonella enterica* serovar Typhimurium ATCC13311, *Staphylococcus sciuri* FUA2055, *Staphylococcus saprophyticus* FUA2056, or *Staphylococcus condimenti* FUA2057. Cell counts of inoculated but untreated meat samples ranged from 10^7 - 10^8 CFU/g. Inoculated meat samples were treated with 600 MPa, 40 ºC for 30 min as outlined above and cell counts were enumerated by surface plating on LB agar. To compare the pressure resistance of *E. coli* AW1.7 to the pressure-resistant mutant *E. coli* LMM1030 and its parent strain *E. coli* MG1650, the experiment was performed under otherwise identical conditions with treatment parameters of 400 MPa, 40 ºC for 30 min. Surviving cells were enumerated by surface-plating on LB agar and BHI-

YE agar. All experiments were carried out in three independent replicates and data were reported as means ± standard deviations.

2.3.8 Statistical analysis

Experiments were carried out at least in duplicate. Results represent the average values of two independent treatments, or the mean ± standard deviation of three independent treatments as indicated. Welch's two sample t-test was performed to determine whether differences of bacterial survival were significantly different between treatments or storage conditions.

2.4 Results

2.4.1 Pressure resistance and pressure-induced sublethal injury of *C. jejuni*

All pressure treatments and storage experiments in this study were performed using aseptically prepared minced poultry meat. Plating of representative samples from each batch of aseptically prepared poultry meat confirmed that bacterial contaminants were absent. To investigate the variation of pressure resistance of different strains of *C. jejuni,* 19 strains were treated with 300 MPa and 30 °C for 3 min. Surviving cells were determined on non-selective BHI-YE agar and selective mCCDA agar (Figure 2-1). Remarkably, the bactericidal effect of pressure as assessed by plating on the non-selective BHI-YE was greater when compared to enumeration on the selective agar mCCDA (Figure 2-1). *C. jejuni* strains varied in their response to pressure. Pressure treatment reduced the cell counts of sensitive strains by about 3 log CFU/g (mCCDA) and 5 log CFU/g (BHI-YE). *C. jejuni*

HCJ2316 was the most resistant strain with a reduction of 0.5 log CFU/g (mCCDA) and 2.8 log CFU/g (BHI-YE). This strain was selected for subsequent experiments.

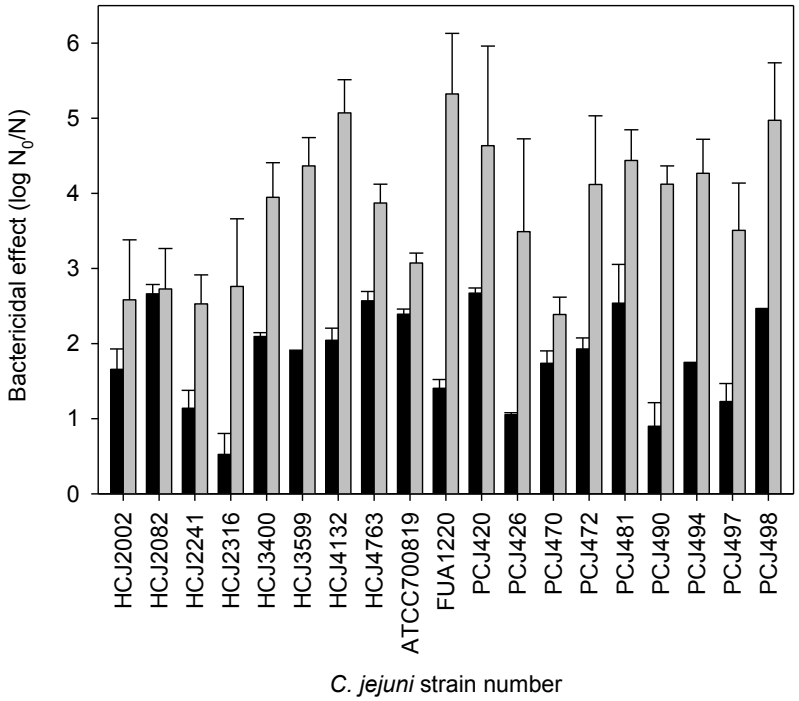

Figure 2-1: Pressure resistance of 19 strains of *C. jejuni*. Cell counts were determined on mCCDA agar or on BHI-YE agar. Data for mCCDA agar (black bars) represent means of two independent experiments, data for BHI-YE agars (grey bars) represent means ± standard deviation of four independent experiments.

To investigate the unexpected differences in recovery on mCCDA agar and BHI-YE agar, the composition of the two media was compared and BHI-YE was supplemented with media components that are present in mCCDA but not in BHI-YE. Poultry meat was inoculated with *C. jejuni* HCJ2316 and treated at 300 MPa, 30 °C for 3 min. Surviving cells were enumerated on mCCDA, BHI-YE, and BHI-YE supplemented with Fe^{2+}, tryptone, sodium deoxycholate, or combinations of the three components (Figure 2-2). Supplementation of BHI-YE with sodium deoxycholate or

tryptone did not improve the recovery of pressure-treated *C. jejuni* (data not shown). However, the recovery of *C. jejuni* on BHI-YE media supplemented with Fe^{2+} was comparable to the recovery on mCCDA (Figure 2-2). These results demonstrated that iron is required for recovery of *C. jejuni* after pressure-induced sublethal injury.

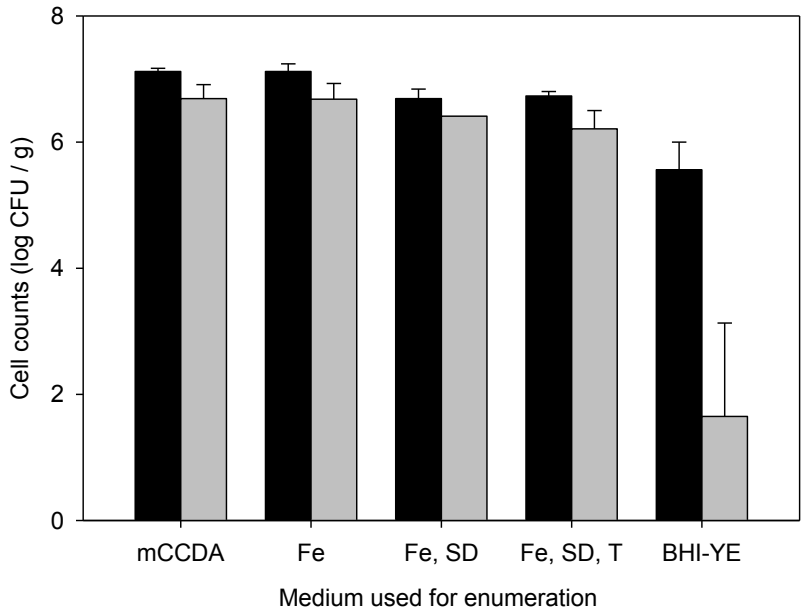

Figure 2-2: Recovery of *C. jejuni* HCJ2316 after treatment at 300 MPa and 30 ºC for 3 min. Grey bars represent the treated samples; dark bars represent the untreated controls. mCCDA: *C. jejuni* selective agar; Fe: BHI-YE supplemented with $FeSO_4$; Fe, SD: BHI-YE supplemented with $FeSO_4$ and sodium deoxycholate; Fe, SD, T: BHI-YE supplemented with $FeSO_4$, sodium deoxycholate and tryptone; BHI-YE: non-selective BHI-YE agar. Data are shown as the average of two independent experiments.

2.4.2 Survival or growth of *C. jejuni* and meat microbiota during refrigerated storage of meat

Survival of *C. jejuni* HCJ2316 on poultry meat was evaluated under two storage conditions (aerobic conditions or vacuum packaging), and in the presence or absence of competing microbiota. In vacuum-packaged meat, *Cb. divergens* grew to

cell counts exceeding 10^7 CFU/g during 8 days of storage (data not shown). In meat packaged with an oxygen-permeable film, *P. fluorescens. B. thermosphacta*, and *Cb. divergens* grew to cell counts exceeding 10^7 CFU/g (data not shown). Cell counts of *C. jejuni* decreased by 0.2 – 0.5 log (CFU/g) over the storage period (data not shown). This decrease was independent of the presence of competing microbiota ($p > 0.05$) or the presence of oxygen ($p > 0.05$).

The survival of *C. jejuni* HCJ2316 and the strain cocktail during refrigerated storage following pressure treatment at 400 MPa and 40 ºC for 30 min is shown in Figure 3-3. These treatment parameters yield ready-to-eat poultry meat products with a texture comparable to heat treated products [30]. Pressure treatment reduced cell counts of all organisms except *E. coli* AW1.7 to levels below the detection limit of 1.48 log CFU/g. Pressure treatment of meat inoculated with *B. thermosphacta* at 400 MPa and 40 °C for 30 min also reduced the cell counts by more than 6 log CFU/g (data not shown). Cell counts of *E. coli* AW1.7 were reduced by 3 log CFU/g and essentially remained unchanged during subsequent refrigerated storage (Figure 2-3).

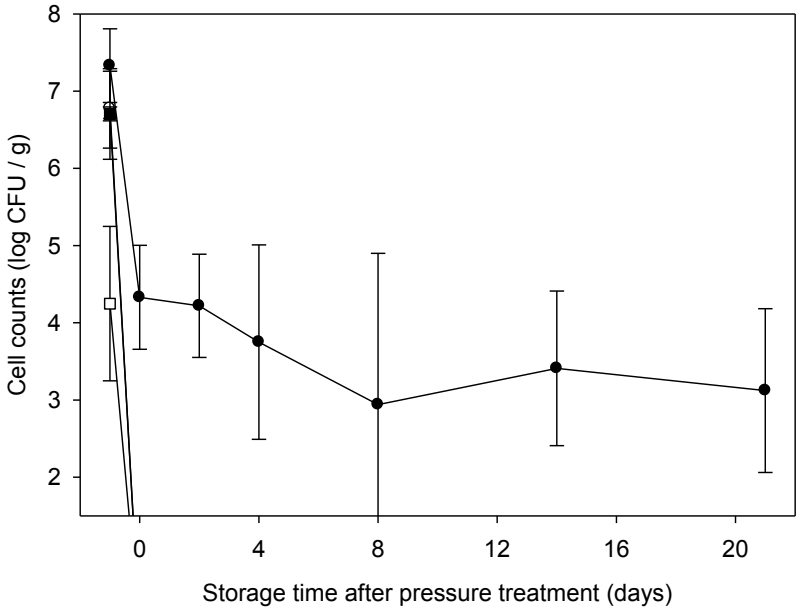

Figure 2-3: Survival of *B. thermosphacta*, *C. divergens*, *E. coli*, *C. jejuni* and *P. fluorescens*. Strain cocktail was mixed in aseptically prepared chicken meat after treatment at 400 MPa and 40 ºC for 30 min, followed by storage at 4°C. (●) *E. coli*, (□) *Cb. divergens*, (▲) *C. jejuni*, (■) *P. fluorescens*; and (□) *B. thermosphacta*. Lines dropping below the x-axis indicate cell counts that were reduced to levels below the detection limit of 1.48 log CFU/g after high pressure treatment, and remained below the detection limit throughout storage. Data represent means ± standard deviations of three independent experiments.

2.4.3 Pressure resistance of *E. coli* AW1.7 in comparison to other *E. coli* strains and poultry isolates

To determine whether the pressure resistance of *E .coli* AW1.7 exceeds the pressure resistance of other *E. coli* strains, foodborne pathogens, or poultry isolates, the strain was treated at 600 MPa and 40 °C for 40 min. Survival of *E. coli* AW1.7 was compared to three other *E. coli* strains, poultry meat isolates *E. coli* FUA1233 and FUA1041, and the cattle isolate *E. coli* FUA1041 (STEC); *S.* Typhimurium ATCC13311, and three poultry isolates of *Staphylococcus* spp, *Staphylococcus sciuri*

FUA2055, *Staphylococcus saprophyticus* FUA2056 and *Staphylococcus condimenti* FUA2057. Pressure treatment reduced cell counts of all strains by more than 6 logs (CFU/g) to levels below the detection limit; however, *E. coli* AW1.7 was reduced by only 4.5 ± 0.5 log CFU/g.

The pressure resistance of *E. coli* AW1.7 was also compared to the pressure resistant mutant *E. coli* LMM1030 and its pressure-sensitive parent strain *E. coli* MG1655. Treatment at 400MPa and 40 ºC for 30 min reduced cell counts of *E. coli* AW1.7 by 3.2 ± 0.8 log (CFU/g). *E. coli* LMM1030 was more sensitive to pressure treatments in poultry meat ($p < 0.05$) and cell counts were reduced by 4.6 ± 0.6 log (CFU/g). Cell counts of *E. coli* MG1655 were reduced by more than 6.5 log (CFU/g) to levels below the detection limit. These results confirm that the meat isolate *E. coli* AW1.7 exhibits exceptional resistance to pressure.

2.5 Discussion

This study evaluated the pressure resistance of *C. jejuni*, *E. coli*, and other pathogens or spoilage organisms in poultry meat. To the best of our knowledge, this is the first study that used aseptically prepared poultry meat without treatment such as radiation or thermal sterilization. The use of aseptically prepared meat is essential to study bacterial pressure-resistance in a meat matrix without interference from contaminating microbiota. To ensure the absence of contaminants, each batch of meat was tested for the presence of indigenous microbiota after the preparation.

Because the resistance to pressure may be highly variable among strains of the same species [3,6,20], challenge studies to ensure food safety require the use of strain cocktails or pressure resistant representatives of the target organisms. This study demonstrated that strains of *C. jejuni* exhibit an intra-species variation of pressure-resistance that is comparable to other foodborne bacteria. The resistance of *C. jejuni* HCJ2316, identified as the most pressure resistant among nineteen strains, matches or exceeds the pressure resistance of the few other strains of *C. jejuni* for which data are available [7,25,41].

From this study, three components of mCCDA agar ($FeSO_4$, tryptone, and sodium deoxycholate) were tested for its role upon the recovery of sublethally injured *C. jejuni*. Tryptone provide organisms with source of amino acids. Sodium deoxycholate inhibit the growth of Gram-positive bacteria. Both tryptone and sodium deoxycholate was not effective in increase recovery *of C. jejuni* after HHP. Remarkably, iron was an essential factor for the enumeration of *C. jejuni* after pressure-induced sublethal injury. This result contrasts previous studies on the role of

iron for survival of pressure-treated *E. coli*. Pressure-induced membrane damage caused intracellular oxidative stress in *E. coli* [1,68]. Different from *E. coli*, however, iron uptake and oxidative stress defensive mechanisms are regulated separately in *C. jejuni* [45]. Outer membrane proteins contributing to the defense against oxidative stress, including CfrA and ChuA, were repressed in presence of an abundance of iron [44]. From this study, the iron content of meat clearly aids in survival and recovery of *C. jejuni* after pressure treatment. Another beneficial effect of FeSO4 supplementation to the media could be its neutralizing effect of oxygen radicals. *C. jejuni* are microaerophilic organism which does not grow in the presence of air and grow optimally in atmospheres containing 5% of oxygen [32]. Thermal sterilization of media stimulate autoxidation of compounds such as phosphate, causes formation of reactive oxygen species (ROS) leading to the substrate- accelerated bacterial inactivation and reduced recovery of sublethally injured cells [5,13.21.35.46]. Supplementation of ferrous sulfate (FeSO4) in Trypticase soy agar (TSA) significantly improved the recovery of heat- injured *Salmonella* from egg albumen, whereas another iron source ferric ammonium citrate which does not have the ROS-reducing power of FeSO4 supported the recovery of fewer cells than with TSA+ 1.0 g/L FeSO4 [13]. The reducing effect may also improve the survival and increase the recovery of sublethally injured *C. jejuni* during HHP and subsequent enumeration.

Past studies on the survival of *C. jejuni* during refrigerated storage of meat used *C. jejuni* alone, in combination with undefined meat microbiota, or in combination with a single species of competing bacteria [8,15,19,31]. The strain cocktail employed in this study consisted of the meat isolates *B. thermosphacta*

FUA2054, *Cb. divergens* FUA2053, *P. fluorescens* FUA1232, and *E. coli* AW1.7, representing major spoilage microbiota of aerobically and vacuum-packaged meat and potential foodborne pathogen. Growth of organisms in the strain cocktail during aerobic storage or during storage of vacuum-packaged meat is well in agreement with previous studies. *Cb. divergens* grows during both aerobic and vacuum-packaged storage [24,28]. *P. fluorescens* and *B. thermosphacta* grow at aerobic conditions but are out-competed by *Cb. divergens* in vacuum-packaged meat [23,39]. The observation that survival of *C. jejuni* on poultry meat was not affected by the presence of oxygen or other bacteria contrasts with results from previous studies using surface inoculation on beef or pork [4, 12] or *in vitro* model systems [14]. In this study, however, *C. jejuni* was mixed with aseptically prepared minced poultry meat rather than inoculated on the surface of a muscle. The surface topology of poultry skin or meat affected survival of *Campylobacter* [10] and the limited diffusion of oxygen to the interior of the meat samples likely improved survival of *C. jejuni* even in the absence of other bacteria.

Treatment of chicken breast meat with 400 MPa and 40 ºC for 30 min resulted in a product with a texture that is comparable to current ready-to-eat poultry meat products [30]. From this work, we demonstrated that high pressure treatments to obtain chicken meat products with suitable texture eliminated *C. jejuni* as well as the meat spoilage microbiota by more than 6 log CFU/g. The study is the first to document the response of *B. thermosphacta* and *Cb. divergens* to pressure treatment in meat products. Treatment at 600 MPa and 40 °C also reduced cell counts of *S.* Typhimurium, three *Staphylococcus* spp., and three *E. coli* strains, including the

shiga-toxin producing *E. coli* FUA 1041, by more than 6 log CFU/g. *S. aureus* are known to exhibit a relatively high resistance to pressure [3,56]. However, pressure treatments at 400 MPa or 600 MPa and 40 °C failed to reduce cell counts of *E. coli* AW1.7 by more than 4.5 log CFU/g. Comparison of the pressure resistance of *E. coli* AW1.7 and *E. coli* LMM1030 an extremely pressure resistant mutant indicates that *E. coli* AW1.7 is among the most pressure resistant vegetative bacterial cells described to date [56]; however, *E. coli* AW1.7 is a meat isolate representing organisms present in fresh meat whereas *E. coli* LMM1030 is a pressure resistant mutant selected through repeated cycles of treatment and re-growth. Based on the results from this study, the current use of pressure processing in the food industry (600 MPa, 25 °C for 3 min) is unlikely to support the selection of pressure resistant mutants.

In conclusion, the evaluation of the pressure resistance of 19 strains of *C. jejuni* confirmed that this species is relatively sensitive to pressure; however, the elimination of more pressure resistant meat microbiota, particularly *E. coli* and *Staphylococcus* spp. may require a combination of high hydrostatic pressure and elevated temperature. These processing conditions result in ready-to-eat poultry products with a texture that is comparable to heat processed products [30]. However, other fresh meat products may require the use of additional treatments such as reduced pH or antimicrobial agents to reduce the treatment intensity. The identification of *E. coli* AW1.7 as a heat- and pressure resistant meat isolate is consistent with resistance development that is based on accumulation of compatible solutes [97]. *E. coli* AW1.7 expresses unusual nmpC porins which increases the

accumulation of compatible solutes that helps the cell survives under stress conditions [90,97]. Compatible solutes are small organic solutes which cells can accumulate under stress without the change of intracellular pH. The presence of heat- and pressure resistant *E. coli* on meat may pose additional challenges for a safe food supply.

2.6 References

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3. Determination of Heat Resistance of 101 Strains of Verotoxigenic *Escherichia coli*

3.1 Introduction

Several *E. coli* pathotypes cause gastrointestinal infections owing to the presence of specific virulence factors [8]. Pathotypes causing foodborne illness include Verotoxigenic *E. coli* (VTEC), also known as Shiga toxigenic *E. coli* (STEC). VTEC are distinguished by the production of one or more verotoxins (shiga toxins) and may possess additional virulence factors, including the locus of enterocyte effacement [8]. VTEC infection results in diarrhea, followed by hemorrhagic colitis (HC), which in a minority of cases develops into hemolytic uremic syndrome (HUS). HUS often results in long term health impacts, commonly as a consequence of kidney failure, and has a significant risk of death [9]. The development of HC and HUS result from the production of verotoxin in the victim's intestine and uptake of the toxin by a specific receptor on human kidney cells [9]. HUS is particularly life threatening in young children and elderly [9].

E. coli strains are serotyped on the basis of the antibodies for the O-antigen (lipopolysaccharide) and H-antigen (flagellin). VTEC of the serotype O157:H7 and nonmotile variant (O157:H- or NM) account for two thirds of reported VTEC illness in the USA [1]. A wide diversity of serotypes have been isolated that are responsible for the remaining cases. However, certain serotypes predominate in cases of human illness; of 940 non O157 VTEC isolates submitted to the CDC between 1983 and 2002, seventy % of isolates belonged to 6 O-types (O26, O45, O103, O111, O121 and O145) [4]. These serotypes were recently classified as adulterants by the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS). It was

announced in Sept, 2011 that these serotypes will be included together with O157:H7 in the routine sampling of beef products; and the presence of these microorganisms in raw ground beef or its precursors would lead to the prohibition of these products to enter commerce [13].

VTEC of unusual serotypes should not be ignored as major outbreaks are not limited to O157 and the top 6 US serotypes. In May of 2011 an outbreak of HC and HUS caused by *E. coli* O104:H4 initiated in Germany, resulted in 4075 cases of illness including 908 cases of HUS and 50 deaths across 16 countries [7,14]. There was only one previous report of VTEC O104:H4 case of hemolytic uremic syndrome from Korea [2].

Heat treatment is a common intervention to reduce the numbers of vegetative cells on animal carcasses and as part of food preparation. In *E. coli,* and specifically in *E. coli* O157:H7's, resistance to heat is highly variable between strains [6]. Moreover, heat resistant strains of *E. coli* are cross-resistant to high hydrostatic pressure [10].

3.2 Objective and Hypothesis

The aim of the study was to compare the heat resistance of a wide variety of VTEC strains. It was hypothesized that VTEC are not significantly more heat resistant than non-pathogenic *E. coli.* In addition, the suitability of *E. coli* AW1.7 as a surrogate for VTEC will be evaluated.

3.3 Material and Methods

3.3.1 Bacterial strains and growth conditions

E. coli AW1.7 and 87 VTEC strains and 14 VT negative *E. coli*, of 15 different serotypes, were used in this study. All strains were maintained frozen at -80 °C and resuscitated before use by streaking onto Luria- Bertani agar, Miller (LB, *Difco*, Detroit, MI, USA) and incubating at 37 °C for 24 h.

For experimental use a single colony of each strain to be tested were inoculated in 10 mL of LB media or LB with 2% NaCl. The broth was incubated in a shaking incubator at 200 rpm for 24 h at 37 °C.

3.3.2 Screening for heat resistant strains

Screening experiments were conducted in duplicate for cells grown in LB or LB with 2% NaCl. From the overnight stationary phase *E. coli* culture 1 mL was withdrawn and diluted in 9 mL of 0.1% buffered peptone water. For each strain to be tested 100 µL of cell suspension was transferred to the wells of a Twin. tec 96 well PCR microtiter plate (*Eppendorf AG*, Hamburg, DE). Four plates were prepared for each experiment; an untreated control, and for exposure to 60 °C for 5, 15 or 30 min in an Eppendorf PCR thermal cycler (*Eppendorf AG*, Hamburg, DE). Following heat treatment, the microtiter plates, including the control, were incubated at 37 °C for 48 h. After incubation, the wells of the microtitre plate were examined for turbidity. Growth, indicating survival, was recorded if a plaque of cells formed at the bottom of the plate well.

3.3.3 Enumeration of survivors following heat treatment

Strains of *E. coli* for which increased turbidity was observed following exposure to 60 °C for 5 min. were selected for enumeration of survivors following heat treatment. Cells in heated and control samples were enumerated by plating with a Whitley Automatic Spiral Plater (*Don Whitely Scientific*, Shipely, UK). Cells grown in LB broth were diluted in 0.1% buffered peptone water and plated onto LB agar. Cells grown in LB with 2% NaCl were diluted in 0.1% buffered peptone water with 0.85% NaCl and plated onto LB agar with 1% NaCl. Plates were incubated at 37 °C for 48 h. Colonies were counted and the reduction of cell number by heat treatment was calculated in log CFU/m. Enumeration experiments were conducted in triplicate.

3.3.4 Statistical analysis

Mean and standard deviation were calculated for colony count data. Welch's two-sample *t*-test was performed to determine whether the addition of NaCl in growth media affected the number of *E. coli* recovered following heat treatment.

3.4 Results and Discussion

3.4.1 Screening for heat resistant strains

No visible growth was observed following incubation for any of the *E. coli* strains that had been exposed to 60 °C for 15 or 30 min, whether grown in LB or LB with 2% NaCl, with the exception of *E. coli* AW1.7. Of the VTEC tested, 25 strains showed visible turbidity following the incubation at 60 °C for 5 min (Table 3-1).

The results indicate that some of the *E. coli* strains tested are substantially more heat resistant than the majority of *E. coli* strains [5,6]. All of VTEC tested, however, were less heat resistant than *E. coli* AW 1.7. Moreover, heat resistance is not dependent upon serotype.

Table 3-1: VTEC and other *E. coli* demonstrating resistance to 60 °C for 5 min

Strain ID	Serotype	Isolation	stx1	stx2	eae
1935	O157:H7	human	+	+	+
EC99	O157:H7	unknown	+	+	+
7236	O157:H7	human	+	+	+
7283	O157:H7	hamburger	+	+	+
C0283	O157:H7	cattle feces	+	+	+
E0122	O157:H7	cattle	-	+	+
E0139	O157:H7	deer jerky	-	+	+
CA 334	O145:H34	unknown	-	-	+
CA 728	O145:H34	unknown	-	+	-
03-6430	O145:NM	human	+	-	+
05-6544	O26:H11	human	+	-	+
99-4610	O26:H11	stool	+	-	+
00-4748	O111:NM	human	-	+	+
P 447	O111:NM	unknown	-	-	+
06-0434	O103:H2	human	+	-	+
P 444	O103:H2	unknown	-	-	-
06-0434	O45:H2	human	+	-	+
09-0525	O113:H4	unknown	+	+	-
92-0275	O117:H4	unknown	+	+	-
09-0523	O76:H19	unknown	+	+	-
03-2642	O121:H19	stool	-	+	+
03-4064	O121:NM	human	-	+	+
03-2832	O121:H19	human	-	+	+
96-0120	O121:H10	unknown	-	+	-
09-414	O104:H7	unknown	-	-	-

'+' represents positive, and '-' represents negative of target gene

3.4.2 Enumeration of survivors following heat treatment

To determine the magnitude of heat resistance of the screened *E. coli*, cells were challenged at 60 °C for 5 min after grown with or without NaCl. Of the 25 strains tested, only six strains had a lower than 5 log reduction (E0122, 03-6430, 05-6544, 03-2832, 09-0525) (Figure 3-1). *E. coli* AW 1.7 was significantly more heat

resistant than the *E. coli* strains tested ($P < 0.01$), with reductions of 1.08 and 0.34 log for cells grown in LB or LB with NaCl, respectively. Reduction of 3 log or greater were observed for all other *E. coli* strains. The observed sensitivity of the *E. coli* strains to 60 °C was within the range previously reported in studies of heat resistance of *E. coli* [3,6].

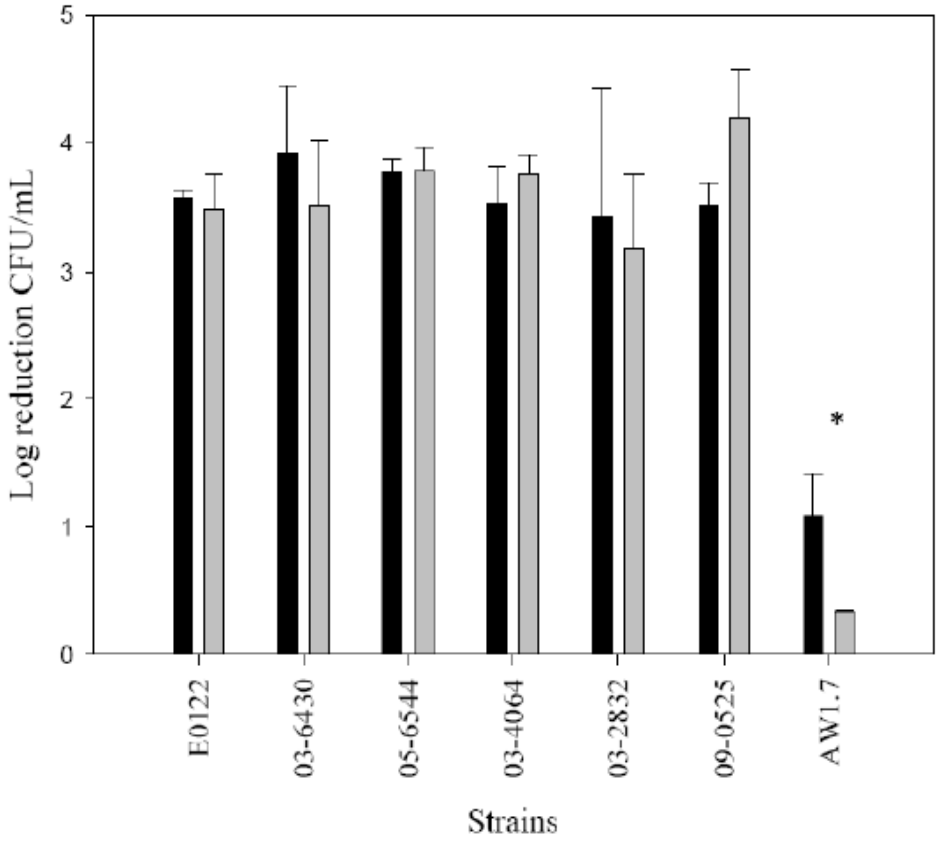

Figure 3-1: Survival of *E. coli* after heating for 5 min at 60 °C. Black bars indicate cells grown and enumerated on LB agar; grey bars indicate cells grown and enumerated on 1% NaCl LB. Error bars represent one standard deviation for triplicate experiments. * indicates statistically significant differences between cultures enumerated on LB or LB 1% NaCl ($P < 0.01$).

Comparison of *E. coli* recovered following heat treatment in LB with those in LB with NaCl did not indicate a significantly greater recovery ($P > 0.05$). The

addition of NaCl to the growth medium did not improve the survival of *E. coli* strains in the screening test. However, the recovery of *E. coli* AW1.7 was significantly greater ($P < 0.01$) with the addition of NaCl to growth media. The protective effect of NaCl on *E. coli* AW1.7 to heat stress likely is a consequence of accumulation of compatible solutes inside the cell by outer membrane transport protein NmpC and transport proteins in the cytoplasmic membrane [11,12]. These results confirm the exceptional heat resistance of *E. coli* AW1.7 [5]. Pleitner et al. [11] observed heat resistance of *E. coli* AW1.7 was maximized with addition of 2 to 4% NaCl, our data are in agreement with those findings.

3.5 Conclusion

With 87 strains of VTEC and 14 additional *E. coli* tested, this is the largest study on the heat resistance of VTEC. Though six VTEC strains were able to withstand 60 °C for 5 min with less than a 5 log reduction there is no indication that VTEC as group are significantly more heat resistant than other *E. coli*. None of the 101 strains tested demonstrated greater heat resistance than *E. coli* AW1.7. This indicates the suitability of *E. coli* AW1.7 as a surrogate of VTEC in thermal challenge studies. Addition of 2% NaCl improved the recovery of *E. coli* AW1.7, but not of the other strains.

3.6 References

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4. Variation in Heat- and Pressure Resistance of Verotoxigenic *Escherichia coli* and Non-toxigenic Reference Strains

4.1 Introduction

Escherichia coli pathotypes causing food-borne disease include Verotoxigenic *E. coli* (VTEC). *E. coli* O157 is the most commonly reported serotype of VTEC in North America. However, a large proportion of *E. coli* O157 do not harbor genes coding for Stx [24], and VTEC of other serotypes (non-O157 VTEC) are recognized to cause 40 % or more of VTEC illness in the US and Canada [6,16,39,49]. The estimated incidence of VTEC infections in the U.S. in 2012 was 1.12 cases per 100,000 populations for O157 and 1.16 per 100,000 population for non O157 VTEC [6]. Among non O157 VTEC isolates submitted to the U.S. Centers for Disease Control and Prevention between 1983 and 2002, the serotypes O26, O45, O103, O111, O121 and O145 represented 70% of isolates [13]. However, the relative incidence of clinical VTEC isolates varies between regions [9] and new VTEC pathogens of previously obscure serotypes can periodically emerge, due to the mobility of the verotoxin genes, which are encoded by the stx-prophage. For example, the largest outbreak of VTEC illness reported to date was caused by *E. coli* O104:H4 in 2011 [22].

The resistance of strains of *E. coli* O157:H7 to intervention treatments in food processing has been well studied; however, strains of the serotype O157:H7 represent a distinct and narrow phylogenetic group in the species *E. coli* and thus are unlikely to represent the physiological diversity of VTEC or of the species *E. coli* [27,46]. Though all *E. coli* strains are rapidly inactivated at temperatures greater than

70 °C, as temperature decreases the resistance of individual strains to heat becomes highly variable and the D_{60} values of individual strains may range from less than 0.1 to more than 30 min [19,21]. Likewise, the resistance of *E. coli* to HHP, an alternative to thermal decontamination is variable between strains [3,14].

Previous studies of the pressure resistance of VTEC have focussed on strains of *E. coli* O157 [3,8]. Wild type strains of *E. coli* with significant pressure resistance at 600 MPa have been described [chapter 2, 35], but it remains unknown whether VTEC strains exhibit comparable resistance.

4.2 Objective and Hypothesis

To be able to assess the potential for VTEC to survive HHP treatment of foods, it requires data on the range and maximum HHP resistance that may be found in VTEC strains. The study aims to collect data on the resistance of *E. coli* strains (belonging to 23 O groups, including 94 VTEC) in which commercial HHP treatment condition (600 MPa, 3 min) is applied. *E. coli* strains were simultaneously assessed for resistance to heat treatment (60 °C for 5 min) to investigate if there was any relationship between pressure and heat resistance, O serogroup or phylogenetic group.

In the second phase of this study, the response to HHP and heat treatment in broth media and ground beef was studied for a smaller group of 24 strains to determine whether sodium chloride concentration and fat content affected survival. These experiments included two strains of *E. coli*, AW1.7 [19] and LMM1030 [25] with high heat and pressure resistance respectively, to assess their suitability as model organism for challenge studies.

It was hypothesized that VTEC are not significantly more heat- and pressure resistant than non-pathogenic *E. coli.* Some VTEC strains can be both heat- and pressure resistant in broth and meat challenge studies independent of serotype or phylotype. Addition of NaCl can improve the resistance of *E. coli*.

4.3 Material and Methods

4.3.1 Bacterial strain and growth conditions

The *E. coli* strains used in this study are listed in Tables 4-1 and 4-2. All strains used were stored as glycerol stocks at -80 °C. Strains used in screening experiments in phosphate buffered saline were cultured by streaking onto Brain Heart Infusion agar (BHI, *BD Diagnostics*, Sparks, MD, USA) and incubated for 24 h at 35 °C. Strains used in experiments with broth media or ground beef were streaked onto LB agar, Miller (LB, *Difco*, Detroit, MI, USA) plates and incubated for 24 h at 37 °C.

Table 4-1: 100 Strains of *E. coli* for initial heat and HHP screening test

Serotype	Strain ID	Ph.	Isolation	Serotype	Strain ID	Ph.	Isolation
O26:NM	11-6009	B1	human	O121:NM	03-4064	B1	human
O26:H11	CFS2, F5-28-2	B1	cattle	O121:H1	11-5594, 11-5597	B1	human
O26:H11	05-7321,02-6737, 05-6544,99-4610	B1	human	O121:H19	00-5288, 11-3925, 11-4440, 03-2642, 03-2832	B1	human
O26:H21	11-5130, 11-5805	B1	human	O121:H10	96-0120	B1	unknown
O26:H21	11-5593	A	human	O121:H19	O157-3	B1	cattle
O45:H2	05-6545,04-2445, 3267-95, 3285-96, 89-39	B1	human	O145:NM	03-6430, 04-7099, 04-1449, 03-4699	D	human
O45:H2	85-X-40c R3	B1	cattle	O145:NM	PARC449 *	D	unknown
O91:NM	40-4, 770-3	B1	sheep	O145:H2	A9619.C2	A	human
O91:H14	09-1768, 09-1769	B1	unknown	O145:H2	75-83	D	human
O91:H21	EC-CFIA-CA 574, 94-0297	B1	unknown	O145:H34	EC-CFIA-CA 334	B2	unknown
O91:H21	85-489	B1	human	O145:H25	2769	A	human
O91:H21	B2F1	B1	human	O145:H34	EC-CFIA-CA 728	A	unknown
O91:H21	ECI-1285	B1	cattle	O157:H7	87-1215, 1935, LCDC	D	human

					7236		
O91:H28	03-5074*	B1	unknown	O157:H7	1934	D	beef
O103:H2	PARC 445*	B1	unknown	O157:H7	1933	D	pork
O103:H2	01-6102, 2109-01, 06-0434	B1	human	O157:H7	EC99	D	unknown
O103:H2	112.1	B1	sheep	O157:H7	C0283, E0122	D	cattle
O103:H2	PARC 444*	B1	unknown	O157:H7	LCDC 7283	D	hamburger
O103:H11	04-3973	B1	human	O157:H7	E0139	D	deer jerky
O103:H21	11-4211, 11-5595	B1	human	O5:NM	03-2682	A	human
O103:H25	03-2444	B1	human	O6:H34	03-5166	B1	human
O104:H4	11-3088	B1	human	O46:H38	97-0757	B1	human
O104:H7	09-417, 09-414*	B1	unknown	O55:H7	05-0376	D	human
O104:H21	3024-94	B1	unknown	O69:H11	11-5596	B1	human
O111:NM	00-4748, 11-6320, 05-4161, 03-3991	B1	human	O76:H19	09-0523	B1	unknown
O111:NM	PARC 447*	B1	unknown	O98:H29	09-5073	B1	human
O111:NM	LC#2, CFS4	B1	cattle	O108:H11	11-3580	B1	human
O111:H8	79-C-43hiiR3, 8448-100.8	B1	cattle	O117:H4	92-0275	B1	unknown
O111:H11	OLC 455	B1	unknown	O123:H2	11-4968	B1	human
O113:H4	F17-A BEADS,	B1	cattle	O128:NM	H2954/96	B1	human

	FM4-5-B						
O113:H21	93-0016, 04-1450, 89-972, 91-0415	B1	human	O165:H25	00-4540	A	human
O113:H4	09-0525	B1	unknown	O177:NM	03-3974	A	human

Ph: phylotype; ***** Negative for VT1 and VT2. The presence of STEC virulence genes *stx1*, *stx2*, *eae*, and EHEC *hlyA* was determined by the PCR protocol of Paton and Paton *(40)*.

Grey and white color alternation for serotype clarity.

Table 4-2: *E. coli* strains involved with resistance studies from 4.3.3 to 4.35

Serotype	Strain ID	Isolation	Phylotype
O26:H11	PARC 448*	unknown	B1
O26:H11	05-6544	human	B1
O44:H18	PARC 450*	unknown	D
O45:H2	05-6545	human	B1
O76:H19	09-0523	unknown	B1
O103:H2	PARC 444*, PARC 445*	unknown	B1
O103:H25	338	unknown	B1
O104:H4	11-3088	human	B1
O111:NM	PARC 447*,583	unknown	B1
O113:H4	09-0525	unknown	B1
O121:NM	03-4064	human	B1
O121:H19	03-2832	human	B1
O145:NM	03-6430	human	D
O145:NM	PARC 449*	unknown	D
O157:H7	E0122	cattle	D
O157:H7	C0283	cattle feces	D
O157:H7	1935	human	D
O157:H7	C06CE1943,C06CE900 C06CE2940,C09CE1353	unknown	D
UNK	AW1.7*,AW1.7ΔpHR1*	Beef, 43	B1
UNK	MG1655*,LMM1030*	25	A

***** Negative for VT1 and VT2; UNK: unknown

4.3.2 Screening for heat- and pressure resistant strains in phosphate buffered saline

Single colonies of *E. coli* strains to be tested (Table 4-1) were inoculated to 10 mL of BHI broth (test tube dimension 13 x 100 mm) and incubated for 16-24 h at 35 °C. The cultures were harvested by centrifugation (4800 g for 10 min), the supernatant was removed from the cell pellet which was resuspended in phosphate buffered saline (PBS, 0.2 M sodium phosphate, 0.85% NaCl pH 7). The cell washing process was repeated, and following a second resuspension the OD_{600nm} of the cell suspensions was adjusted to 0.100 ± 0.005, corresponding to a concentration of approximately 7.5 log CFU/mL. Aliquots of the suspension were withdrawn immediately for enumeration, and for pressure and thermal treatment. The initial concentration of the cell suspension was determined by spiral plating (WASP, *Don Whitley Scientific Ltd.*, Shipley UK) onto Trypticase Soy agar (TSA, Difco, Detroit, MI, USA), which was incubated for 48 hr at 35 °C.

For pressure treatment, 100 µL of 0.1 OD_{600nm} cell suspension was diluted in 9.9 mL of PBS. Five mL of the diluted cell suspension (*ca.* 5.5 log CFU/mL) was transferred into a sterile Whirl-Pak bag (*Nasco*, Fort Atkinson, WI, USA) which was heat sealed, without head space. The individual bags of cell suspension were labeled and then placed into a larger plastic bag filled with 1% bleach, as a precaution against release of pathogens during pressure treatment.

HHP treatment of 600 MPa (6000 bar) was applied to samples using a high pressure pilot unit manufactured by *Dustec Hochdrucktechnik* GmbH (Wismar, DE).

The pressurizing medium was water, and the temperature of the pressure medium was measured throughout the experiment at three locations by thermocouples on the internal wall of the pressure vessel. Prior to pressure treatment the pressure vessel and the fluid inside the pressure vessel were equilibrated to 25 °C by heating with an external heating element. Samples were treated with 600 MPa for a 3 min hold period. The compression rate was 600 MPa/min, and the decompression rate was 600 MPa/30s. Adiabatic heating during compression resulted in an average temperature increase in the pressure vessel of 10.8 °C (standard deviation 2.4 °C, n = 16) as measured across the three thermocouples.

Bags containing pressure treated samples were removed from the outer bag, dried with paper towel and then opened with a flame sterilized blade. The surviving cells were immediately enumerated by spread plating 250 µL manually or spiral plating 50 µL onto TSA agar, which was incubated at 35 °C. Colonies were counted after 48 h incubation.

Survival at 60 °C was assessed by diluting 100 µL of the OD_{600nm} 0.1 cell suspension in 9.9 mL of PBS in a test tube that was submerged and equilibrated in a 60 °C water bath. After 5 min at 60 °C the test tube was removed from the water bath. The contents were mixed by vortexing and a 1 mL aliquot was transferred to a chilled test tube, on ice. The concentration of surviving cells in cell suspension was determined by plating onto TSA as described above. Experiments were replicated twice with each strain.

4.3.3 Heat- and pressure treatment in LB broth

A group of 24 strains of *E. coli*, representing a diversity of heat- and pressure sensitivity, were selected to compare the results obtained by treatment in PBS. Single colonies of the strains were inoculated into 10 mL of LB (Miller) broth and incubated at 37 °C on an orbital shaker (200 rpm) for 24 h. For thermal treatment, 100 µL aliquots of the culture, were transferred to 200 µL PCR tubes and heated to 60 °C in an Eppendorf thermocycler (*Eppendorf*, Hamburg, DE) for 5 min. For pressure treatments, 300 µL of culture was transferred to Tygon R3603 tubing (inner diameter 1.6 mm, outer diameter 3.2 mm, Akron, USA) and heat sealed. The tubing containing the culture was then placed in a secondary container, a 2 mL Cryovial (*Wheaton*, Millville, NJ, USA) filled with 5% bleach. Samples were treated at 600 MPa and 25 °C for 3 min in a Multivessel Apparatus U111™ *(Unipress Equipment*, Warsaw, Poland). Polyethylene glycol was used as pressure transmission fluid. The temperature was maintained with a thermostat jacket coupled to an external water bath and monitored by internal thermocouples. Compression and decompression rates were 600 MPa/min; the temperature changes due to adiabatic compression were 3 °C or less. Cell counts in heat- and pressure treated samples were determined by surface plating on LB agar and incubation at 37 °C for 48 h. Experiments were replicated at least twice with each strain.

4.3.4 Heat- and pressure treatment in ground beef

Ground beef with 15% fat or 35% fat was obtained from a local Federally inspected processing plant. Ground beef was stored at -20 °C until used in experiments. The microbiota of each batch of meat was determined by surface plating onto LB agar and Violet Red Bile agar (VRBA, *Difco*, Sparks, MD, US).

The following strains were inoculated into ground beef for thermal or pressure treatment *E. coli* AW1.7, *E. coli* AW1.7ΔpHR1, *E. coli* O26:H11 05-6544, *E. coli* O104:H4 11-3088, *E. coli* O111:NM PARC447, *E. coli* O121:H19 03-2832 and *E. coli* O157:H7 1935. The selected strains were grown to the stationary phase in LB (Miller) broth as previously described. Ten mL of culture was mixed with 200 g of ground beef with 15% fat content (*ca.* log 10^7 CFU/g). The inoculated meat was massaged by hand for 1 min and molded into a hamburger patty with a hamburger mold (1.2 to 1.5 cm thick). Approximately 20 g of the patty was removed prior to heating and the cell count was determined by surface plating onto LB agar. The remaining beef patty was grilled to an internal temperature of 63 °C ± 0.2 °C or 71 °C ± 0.2 °C with an electrical clamshell grill Cuisinart® 5-in-1 Griddler™ (Woodbridge, Canada). The temperature was monitored with a digital thermometer inserted into the geometric centre of the patty. A core temperature of 63 °C required approximately 2 min of cooking and 71 °C required 3.5 min of cooking. After treatment, patties were sliced in half (90 g). The two 90 g patty halves were each suspended in 100 ml of chilled PBS and stomached for 2 min using a Steward Stomacher. One of the patty halves was diluted and used for enumeration of surviving *E. coli* by spread plating onto LB agar to recover the entire population. One hundred mL of TSB was added to the second half of the patty and the mixture

was incubated for 48 h at 37 ºC and the enrichment media was plated onto coliform selective media (VRBA) and incubated at 37 °C for 24 h. No colonies observed on VRBA plates after incubation represented inactivation of *E. coli* by the treatment. Experiments were replicated twice with each strain.

For pressure treatment of inoculated ground beef, the selected strains were prepared as previously described. A 0.5 mL aliquot of culture was mixed with 100 g of ground beef (approx. log 10^6 CFU/g). Approximately 0.3 g of the mixture was packed into Tygon R3603 tubing, heat sealed, placed in a secondary container, and treated at 600 MPa and 25 °C for 3 min in a Multivessel Apparatus U111™ as described at section 4.3.3. The cell concentration in the treated and untreated samples of ground beef was determined by surface plating on LB agar that was incubated at 37 °C for 48 h prior to enumeration. Experiments were replicated three times with each strain.

4.3.5 Heat treatment of ground beef with different NaCl and fat content

E. coli AW1.7, *E. coli* AW1.7ΔpHR1, *E. coli* MG1655 and *E. coli* LMM1030 were grown in either LB broth without NaCl (LB 0% NaCl, 10 g/L tryptone and 5 g/L yeast extract) or with an additional 20 g/L NaCl (LB broth 2% NaCl, 20 g/L NaCl, 10 g/L tryptone and 5 g/L yeast extract). A 10 mL aliquot of the culture was added to 200 g 15% or 35% fat ground beef, with or without the addition of 4 g of NaCl (2% w/w) at *ca*. log 10^7 CFU/g. The inoculated ground beef was moulded into beef patties as described above and patties were grilled to an internal temperature of 63 °C ± 0.3 °C with a commercial gas grill (Garland™ H286 Starfire, Mississauga, Canada). The temperature was monitored with a digital thermometer

inserted into the geometric centre of the patty, and patties were flipped every 30 sec. The desired internal temperature was reached after approximately 4.5 min; patties were immediately chilled in 100 ml of ice cold PBS prior to enumeration, stomached, and the cell counts were determined by surface plating onto LB agar, which were incubated at 37 °C for 48 h. Experiments were replicated three times with each strain.

4.3.6 Determination of the phylogenetic group of *E. coli* strains

DNA from bacterial cultures was extracted using the QIAamp DNA Stool Mini Kit (*Qiagen*, Mississauga, Canada). Phylogenetic group scheme was established by Clermont et al. [17], reagents and PCR cycle conditions for TSPE4.C2 marker was established by Clermont et al. [17]. The chuA and yjaA primer pair and PCR conditions were established by Doumith et al. [20]. Primers used for the assays are listed in Table 4-3.

Table 4-3: Primer sequences for the phylogrouping assay

Marker	Primer	Primer sequence (5'–3')	An.	Pro. (bp)	Ref.
chuA	Forward Reverse	ATGATCATCGCGGCGTGCTG AAACGCGCTCGCGCCTAAT	62° C	281	20
yjaA	Forward Reverse	TGTTCGCGATCTTGAAAGCAAAC GT ACCTGTGACAAACCGCCCTCA	62° C	216	20
TSPE4.C 2	Forward Reverse	GAGTAATGTCGGGGCATTCA CGCGCCAACAAAGTATTACG	59° C	152	17

An.: annealing temperature, Pro.: product, Ref. :reference

4.3.7 Statistical analysis

Average of cell counts was determined from at least two independent experiments. Student's T-test was applied ($P<0.001$) for cell counts involved triplicate experiments.

4.4 Results

4.4.1 Heat- and pressure resistance in phosphate buffered saline

Considerable variation was observed in the resistance of individual *E. coli* strains suspended in PBS to treatment with pressure (600 MPa for 3 min at 25 °C) or heat (60 °C for 5 min) (Figure 4-1 and Figure 4-2). The strain most resistant to pressure treatment was *E. coli* O111:NM CFS4 which was reduced by 1.1 log CFU/mL. The median reduction was 3.9 log CFU/mL and 9 strains were reduced by 5.5 log CFU/mL. The strain most resistant to heat treatment was *E. coli* O113:H21 04-1450 which was reduced by 2 log CFU/mL. The median reduction for *E. coli* tested was 4.0 log CFU/mL and cell counts of 18 strains were reduced by 5.5 log CFU/mL.

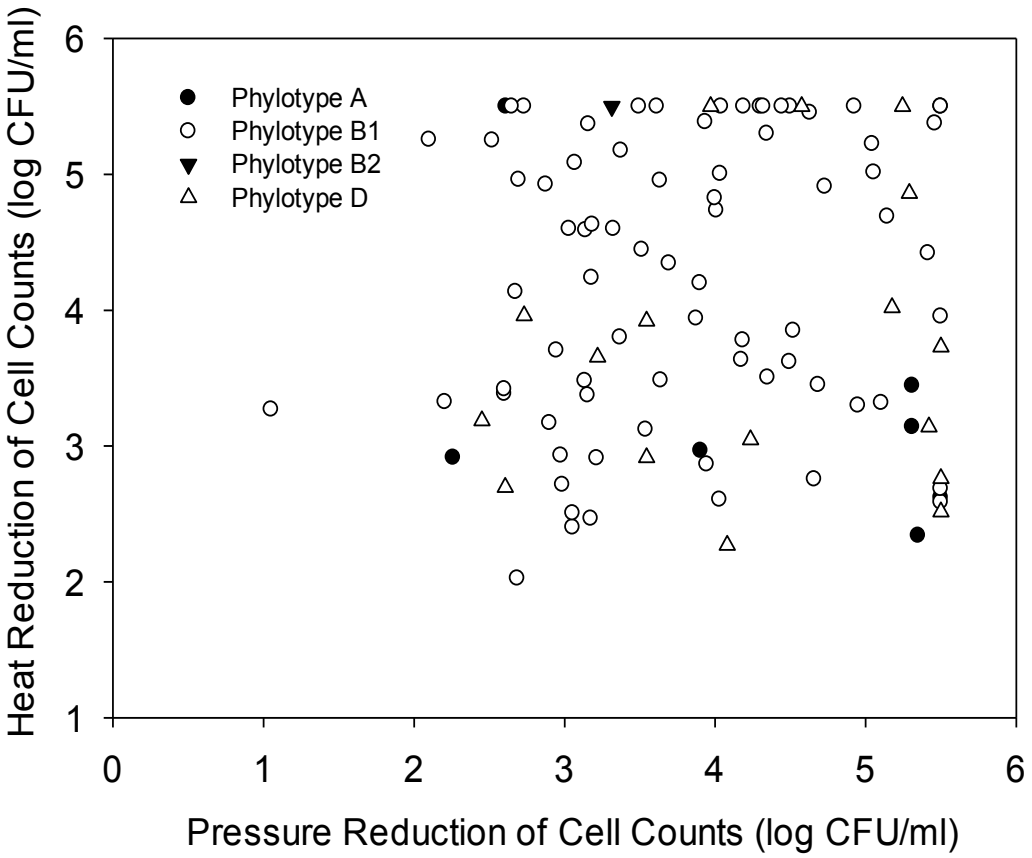

Figure 4-1: Reduction of cell counts of 100 strains of *E. coli* by heat- and pressure treatment. Plotted on the y-axis is the reduction of cell counts (log) by treatment at 600 MPa and 25 °C for 3 min; plotted on the x-axis is the reduction of cell counts of the same strains by treatment at 60 °C for 5 min. Values for cell counts reductions of 5.5 log (CFU/mL) indicate cell counts below the detection limit.

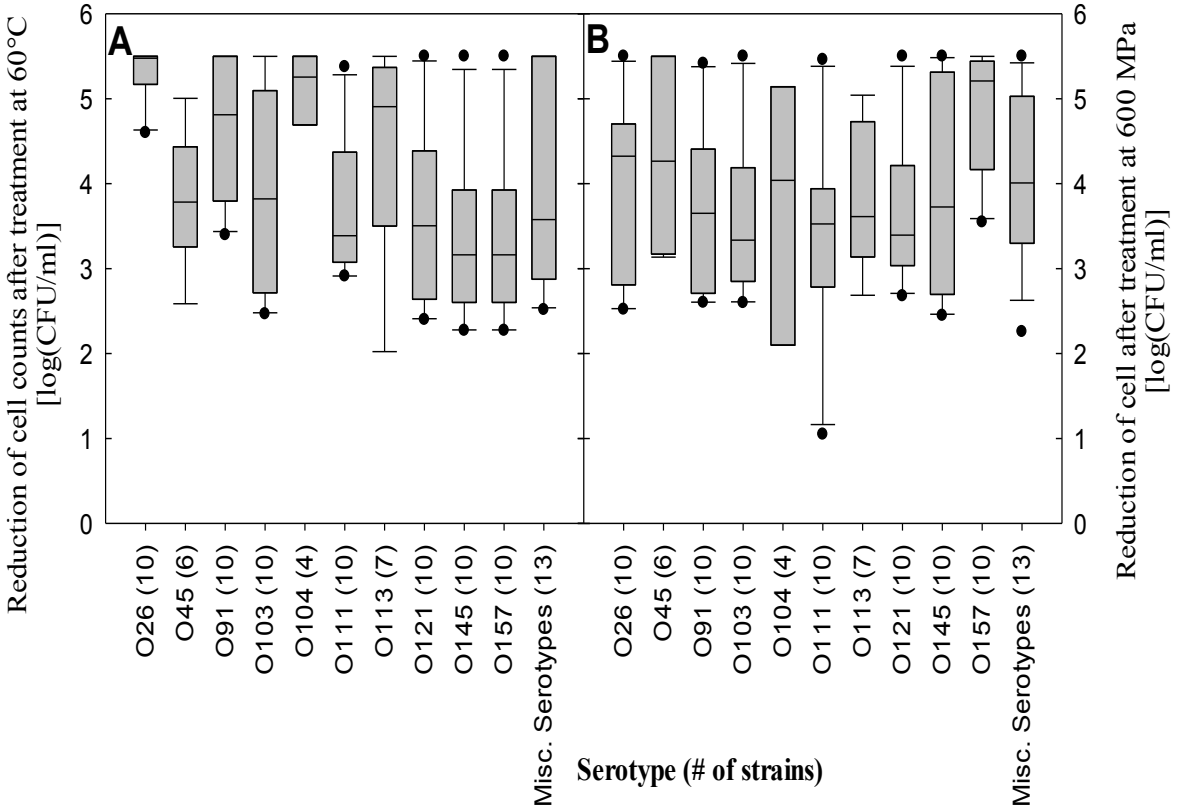

Figure 4-2: Heat- and pressure resistance of strains representing different serotypes of *E. coli.* Shown is the inactivation by treatment with 60 °C for 5 min (Panel A), and the inactivation by treatment with 600 MPa at 25 °C for 3 min (Panel B). Data shown in Figure 4-1 were re-plotted to allow the comparison of VTEC of different serotypes. The serotypes (number of strains) are indicated on the x-axis. Miscellaneous serotypes are represented by 13 strains of 13 different serotypes. Boxes indicate reduction range, line indicates median, whiskers indicate standard deviations and dot symbols indicate outliers.

The log reduction due to pressure and temperature was plotted, with different symbols assigned to the four phylogenetic groups represented, B1 (75 strains), D (17 strains), A (7 strains) B2 (1 strain) (Figure 4-1). The phylogenetic group D as determined by the Clermont scheme includes all strains of *E. coli* O157 which are separated into the closely related phylogenetic group E by multi-locus sequencing or whole genome comparison [27,46]. There was no evidence of a

relationship between resistance to pressure treatment and resistance to heat treatment and there was no evidence of an association of the phylogenetic group with resistance to either treatment.

Since the O-antigen is used in the risk assessment of VTEC strains, the treatment resistance data was also grouped on the basis of the O-antigen (Figure 4-2). Thirteen strains with unique O-antigens were grouped together.

4.4.2 Heat- and pressure treatment in LB broth

To investigate whether the cell suspension medium alters the heat- and pressure resistance of *E. coli*, in total 24 strains including 15 strains with a diversity of heat- and pressure sensitivity in PBS were compared to *E. coli* AW1.7 and 8 additional strains in LB broth (Table 4-4). Generally, the screening for heat resistance of strains in LB broth and PBS provided similar results. *E. coli* O104:H4 11-3088, a clinical isolate from the *E. coli* O104 outbreak in Germany in 2011 [22], is a noticeable exception. Treatment at 60 °C in LB broth reduced the cell counts of this strain by 3.3 log (CFU/mL); the reduction of cell counts after an equivalent treatment in PBS was more than 5.0 log (CFU/mL). Cell counts of *E. coli* AW 1.7 were reduced by 0.3 log (CFU/mL) only; this strain was significantly more heat resistant ($P < 0.01$) than other strains of *E. coli* (Table 4-4). Six of the heat sensitive strains were also pressure sensitive (Table 4-4). Remarkably, 10 of the 23 strains of VTEC were highly resistant to pressure in LB and their cell counts were reduced by less than 2 log (CFU/mL) (Table 4-4).

Table 4-4: Response of selected stains to pressure or heat treatment in LB broth

Strain ID	Bactericidal effect (logN0/N)	
	Pressure (600 MPa 3 Min)	**Heat (60 °C 5 min)**
05-6544	1.38	**3.78**±0.18
05-6545	>5.00	**>5.00**
09-0523	1.62	**>5.00**
PARC 444*, PARC 445* , PARC 447*	**>5.00**	**>5.00**
11-3088	1.96	**3.32**±0.05
09-0525	1.57	**4.19**±0.38
03-4064	>5.00	**3.76**±0.14
03-2832	1.25	**2.82**±0.40
03-6430	1.35	**3.50** ±0.52
PARC 449*	**>5.00**	**>5.00**
E0122	2.66	**2.28**
C0283	>5.00	**>5.00**
1935	2.13	**>5.00**
AW1.7*	**1.46**±0.38	**0.34** ±0.01
PARC 448*	**>5.00**	**>5.00**
PARC 450*	**>5.00**	**>5.00**
338	1.23	**2.24** ±0.62
583	3.25	**3.60**±0.67
C06CE1943	1.35	**4.52**±0.12
C06CE900	1.81	**3.18**± 0.97
C06CE2940	2.15	**4.36**±0.48
C09CE1353	3.91	**4.33**±0.19

***** Negative for VT1 and VT2. Results are shown as means of duplicate independent experiments or in bold as means ± standard deviation of triplicate independent experiments. >5.00 represent cell counts below the limit of quantification after treatments.

4.4.3 Enumeration of survivors following pressure treatment in ground beef

Because the suspension medium influences the resistance of *E. coli* to pressure, the response of selected strains to treatment at 600 MPa and 25 ºC for 3

min in ground beef as a model food matrix was studied. The strains tested in ground beef included four that exhibited pressure resistance in LB (AW1.7, 05-6544, 03-2832, 03-6430) and two pressure sensitive strains (C0283 and AW1.7ΔpHR1). The strains that were resistant to pressure treatment in LB broth (Table 4-4) were also resistant to pressure treatment in ground beef (Figure 4-3). Cell counts of the most resistant VTEC strain, 05-6544, were reduced by 2.0 log CFU/g only. Cell counts of *E. coli* O157:H7 C0-2832 was reduced to below the detection limit (more than 5 log cell count reduction) after treatment in ground beef.

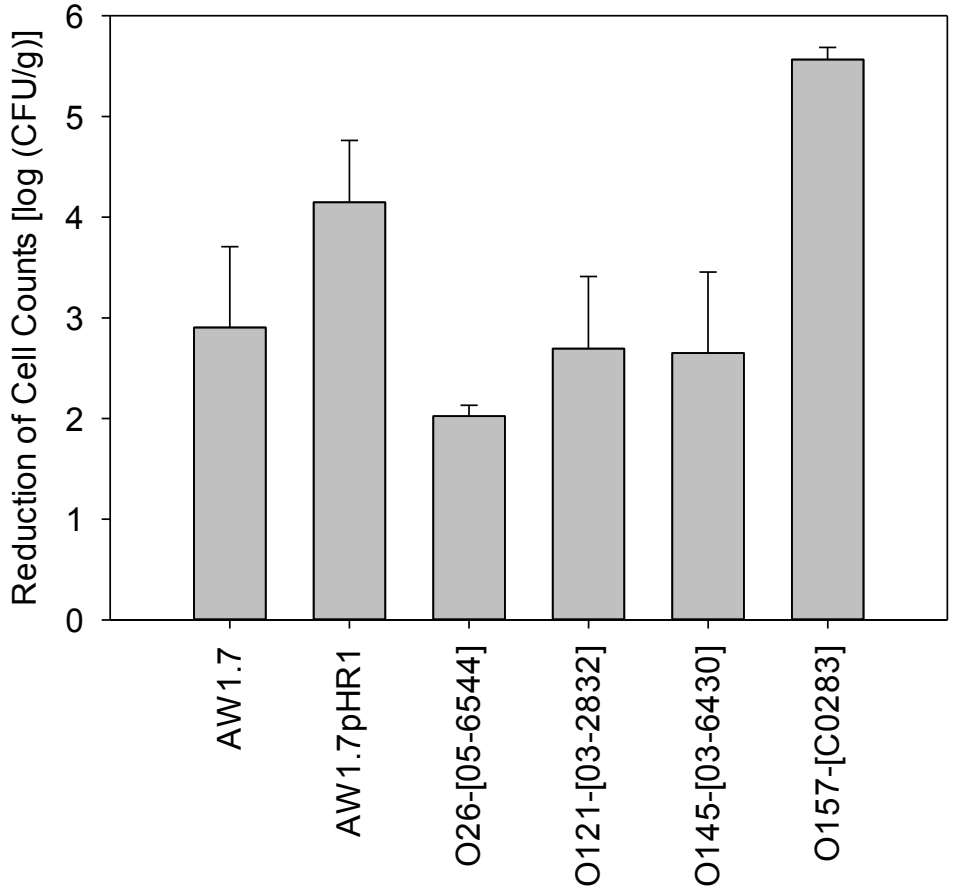

Figure 4-3: Survival of 6 strains of *E. coli* after treatment at 600 MPa and 25 °C for 3 min in ground beef samples. Cell counts were determined on LB agar. Data represent means ± standard deviation of three independent experiments.

4.4.4 Grilling of beef patties to an internal temperature of 63 °C and 71 °C

Grilling or frying of beef imposes a different temperature profile. Therefore, the survival of three pressure and heat resistant strains 03-2832, 05-6544, 11-3088, and one heat sensitive and pressure resistant strain 1935 and one heat- and pressure sensitive strain PARC 447 (Table 4-4) was determined in beef patties that were grilled to an internal temperature of 63 or 71 °C. *E. coli* AW1.7 and *E. coli*

AW1.7ΔpHR1 were used as heat-resistant and heat-sensitive reference strains, respectively.

Cell counts of *E. coli* AW1.7 were reduced by 1.4 log (CFU/g), confirming previous data on the survival of this strain during grilling of beef patties [19]. Cell counts of the most heat resistant VTEC, 03-2832, were reduced by 2.3 log (CFU/g) (Figure 4-4). Grilling to an internal temperature of 71 ºC ± 0.2 °C, equivalent to well-done burgers, reduced cell counts of *E. coli* AW1.7 by only 3.4 log (CFU/g). Cell counts of 05-6544 were reduced by 5.8 log (CFU/g) (Figure 4-4). Surviving cells were recovered by enrichment from patties inoculated with 11-3088, as observed by bacterial growth on VRBA plates. The enrichment cultures from patties inoculated with 03-2832 did not result in the detection of colonies on VRBA plates, which indicated that in the 90 g sample of cooked beef, no cells survived the treatment (Figure 4-4).

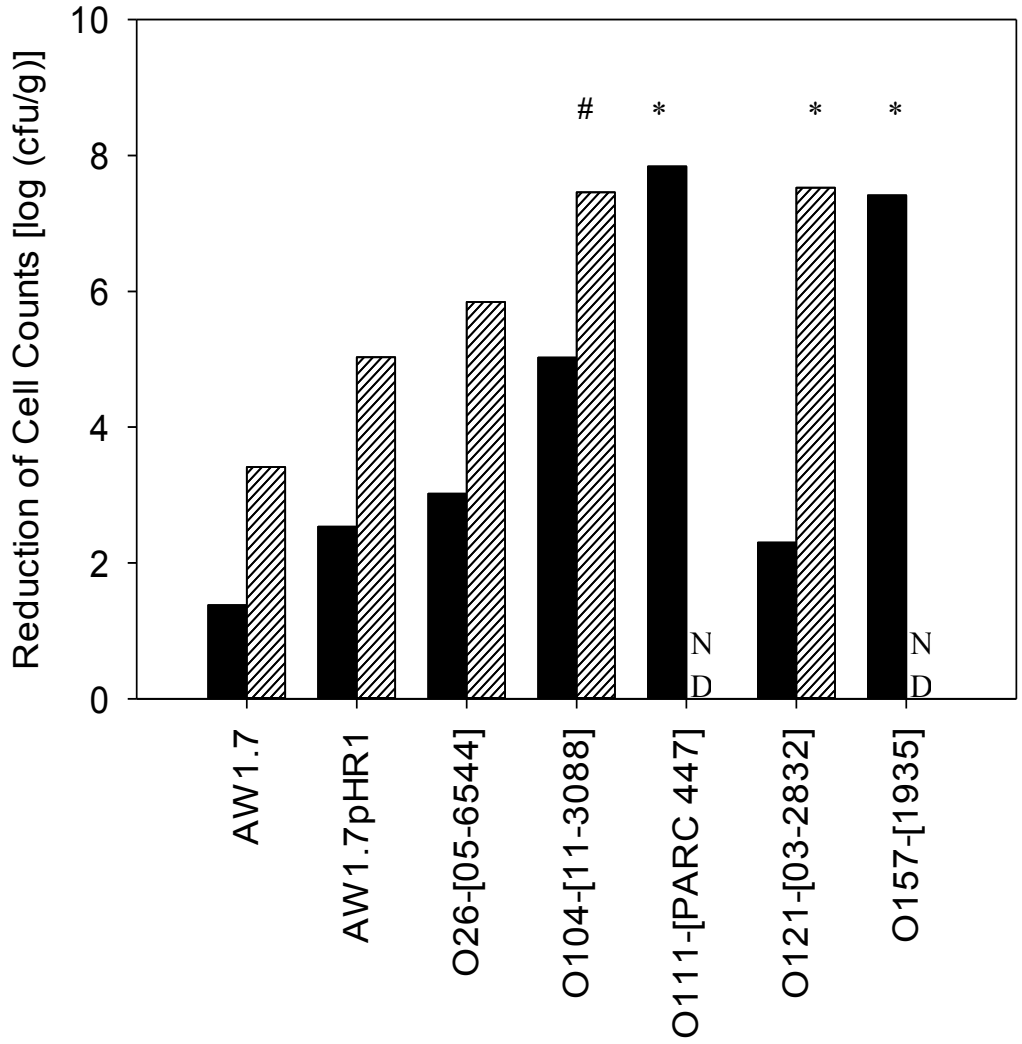

Figure 4-4: Survival of 7 strains of *E. coli* after grilling of beef patties. Ground beef patties were inoculated with *E. coli*, cooked to an internal temperature of 63 °C (black bars) or 71 °C (grey bars), and surviving cells of *E. coli* were enumerated on LB agar. ND: not determined; *: no surviving cells detected after enrichment; #: surviving cells were detected after enrichment in TSB broth overnight at 37 °C. The serotype-[strain ID] is indicated on the x-axis. Data represent the average of two independent experiments.

4.4.5 Effect of NaCl and fat content on the heat resistance of *E. coli*

The NaCl content of the growth- and treatment medium was previously shown to influence the heat resistance of *E. coli* AW1.7 [41]. To determine whether NaCl has a similar influence on the heat resistance of other *E. coli* strains, beef patties were prepared to achieve a fat content of 15% or 35% and NaCl content by adding 0 or 2% (w/w). Patties were inoculated with AW1.7, AW1.7ΔpHR1, MG1655 and LMM1030, and grilled to an internal temperature of 63 °C ± 0.3 °C corresponding to medium rare stage. The results (Figure 4-5) confirm prior data on the heat resistance of the strains in PBS or laboratory media [19,25,41]. Moreover, the NaCl content as well as the fat content strongly influenced heat resistance in ground beef in a strain-dependent manner. *E. coli* strains were generally most resistant in patties containing 15% fat and 2% NaCl. The reduction of cell counts of *E. coli* AW1.7 in patties with 15% fat and 2% NaCl was 1.3 ± 0.2 log (CFU/g) (Figure 4-5); cell counts of *E. coli* MG1655 were reduced to below the detection limit irrespective of the composition of the ground beef patties (Figure 4-5).

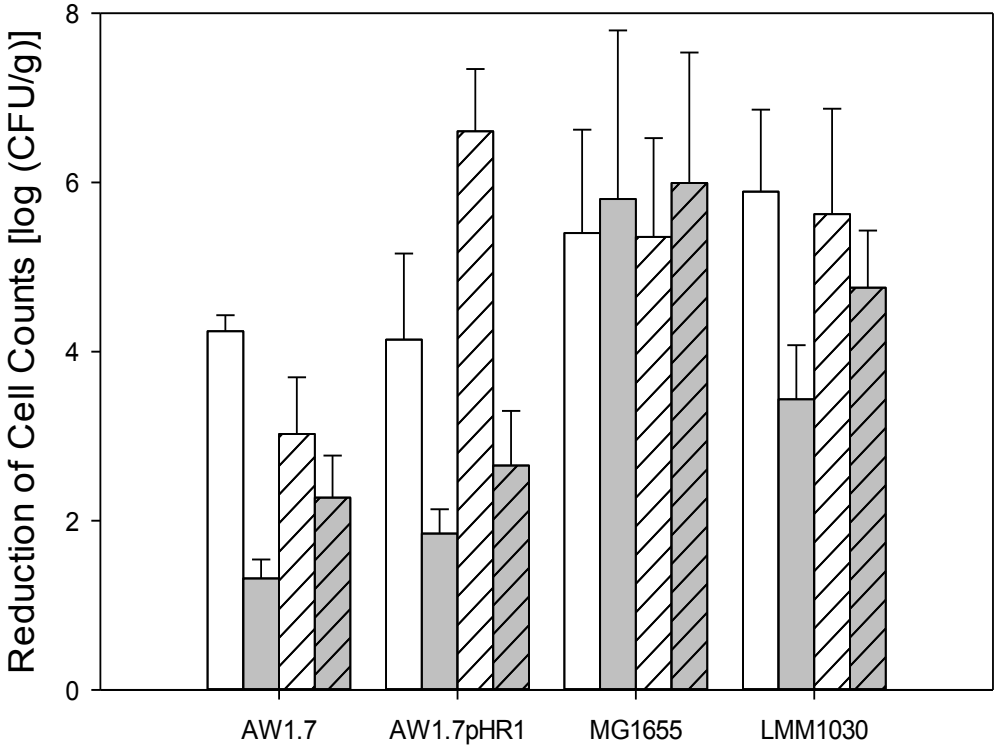

Figure 4-5: Survival of 4 strains of *E. coli* after grilling of beef patties to an internal temperature of 63 °C. Strains of *E. coli* were inoculated into ground beef with a fat content of 15% or 35%, with or without 2% NaCl added. White solid bars indicate *E. coli* heated in ground beef with 15% fat and 0% NaCl; grey solid bars indicate *E. coli* heated in ground beef with 15% fat and 2% NaCl. White hatched bars indicate *E. coli* heated in ground beef with 35% fat and 0% NaCl; grey hatched bars indicate *E. coli* heated in ground beef with 35% fat and 2% NaCl. Data represent means ± standard deviation of three independent experiments.

4.5 Discussion

The response of strains of the cells of any bacterium to physical treatment can be expected to be variable between strains of the species and to be dependent upon the physiological state of the cells. Variability between strains will be greater at relatively mild treatments and converge as the intensity of the treatment is increased. This phenomenon is clearly demonstrated by the observation that in response to

increasing temperature the D-values of strains of a bacterium will decrease and converge at the temperature at which lethality is near instantaneous.

Resistance to pressure has been previously reported to be highly variable among strains of the same bacterial species [3,8,31] and pressure resistance in some strains of *E. coli* has been observed to coincide with heat resistance [3,8,38]. Due to the variability of heat- and pressure resistance in *E. coli* strains, assessment of the risk of survival in food processing of VTEC, including *E. coli* O157, necessitates identifying the greatest potential resistance of the pathogen. This requires the collection of data from a wide variety of *E. coli* strains to identify the range of resistance occurring in the population as there is no evidence that any subgroup of *E. coli*, such as *E. coli* O157:H7 strains, are uniformly more resistant than others [31,36,48].

This study evaluated the heat- and pressure resistance of a total of 112 strains of *E. coli* representing 23 different serotypes and including 102 strains of VTEC. Our data confirm and extend prior data on the variability of heat- and pressure resistance of VTEC, and demonstrate that the resistance of strains of *E. coli* is not associated with specific serotypes and or phylogenetic groups. Previous studies linking the genotype of VTEC to heat resistance [33] of selected strains from a narrow phylogenetic lineage only; differences in the resistance to heat were less than 2 log (CFU/mL).

The expression of outer membrane porins and the accumulation of compatible solutes were demonstrated to contribute to the heat resistance of *E. coli*

AW1.7 [41,43]. Exposure to 5% of NaCl also increased the thermal resistance of *E. coli* O157:H7 [7]. Current study demonstrated that NaCl also increases resistance of this strain in ground beef; moreover, the resistance of other strains of *E. coli* also increased upon incorporation of NaCl. The addition of NaCl in meat products may thus facilitate survival of *E. coli* after cooking.

The current knowledge of the mechanisms of stress resistance in *E. coli* suggests that multiple solutions to resistance exist within this species. For example, *E. coli* AW1.7 and *E. coli* LMM1030 are equally resistant to pressure [35]; however, the overexpression of heat shock proteins contributes to resistance in *E. coli* LMM1030 but not in *E. coli* AW1.7 [25,41,43]. These "multiple solutions" to stress resistance likely account for the observation that heat resistance was generally not correlated to pressure resistance, although strains of VTEC with exceptional resistance to both heat- and pressure could be identified. The pressure resistance of strains treated in PBS medium differed from the resistance in LB broth or ground beef. The osmotic pressure of the treatment medium or the presence of compounds that prevent membrane damage or oxidative stress may account for this effect. LB broth treatments were conducted in a HHP unit with 3 ºC compression heating, the PBS treatments were conducted in a unit with 10 ºC compression heating, the temperature profile during the treatments may have contributed to the discrepancy of the results obtained in PBS and broth.

Past studies on the pressure resistance of VTEC in meat products have primarily focused on *E. coli* O157:H7. Treatment of a cocktail of strains of *E. coli* O157:H7 at 400 MPa for 10 min reduced cell counts in ground beef by 3 log

(CFU/g) [10]. The present study demonstrated that a substantial proportion which is more than one third of strains of VTEC are resistant to treatment equivalent to current commercial practice, which is 600 MPa for 3 min at ambient temperature. Their cell counts were reduced by less than 3 log (CFU/g) and their resistance thus matches the resistance of pressure resistant mutant strains [25]. Because of the high proportion of strains of VTEC that are resistant to pressure, present study suggests that pressure alone is not sufficient for elimination of VTEC in meat or meat products if they are present on meat at high numbers. Moreover, the pressure resistance of *E. coli* AW1.7 is equal or higher than the resistance of VTEC [34]; this strain can thus be used as surrogate organisms for future challenge studies.

In Canada, 17% of O157:H7 patients had consumed at least one serving of under cooked ground beef in a period of 10 days before illness [50]. In the U.S. and Australia, an estimated 40 – 60% of consumers prefer beef cooked medium-rare (internal temperature of 63 ºC) or rare (internal temperature of 58ºC) [18,45]. Moreover, consumer-style cooking methods of beef patties are based on the observation of color, appearance and texture of the meat and may result in consumption of undercooked patties [42]. In the present study, grilling of beef patties in a clamshell grill to an internal temperature of 63 ºC reduced cell counts of VTEC by less than 3 to more than 8 log (CFU/g). Surviving cells were isolated for two strains after grilling beef patties to an internal temperature of 71ºC. The range of cell count reductions is in general agreement with the range reported previously for VTEC in beef [1,23,29,37,42]. However, the use of a large number of strains allows the estimation of the proportion of heat resistant strains of VTEC that are likely to

survive cooking to 63 ºC as approximately 10% of all strains of VTEC. This proportion of heat resistant VTEC in combination with the consumer preference for medium-rare or rare beef products suggests that heat resistant characteristics of some VTEC can contribute to foodborne illness.

The survival of *E. coli* in meat is influenced by the cooking method [1,37]. A previous study indicated cooking of beef in clamshell grills was more lethal for *E. coli* when compared to cooking on an open grill [37]. In this study, *E. coli* AW1.7 cell counts reduction at 63 ºC was similar for both treatment methods (Figures 5-4 and 5-5).

Currently there is no mandatory reduction of VTEC in place for fresh meat. To provide safe food products, an additional hurdle or hurdles to heat- and pressure treatment is required. The combined effect of bioprotective cultures and EDTA to control *E. coli* O157:H7 in frozen ground-beef patties had been investigated previously and various combinations of nisin, lysozyme and monolaurin plus EDTA were tested against Gram-negative bacteria in broth medium and proven to be bactericidal; whereas none of the antimicrobials alone without EDTA showed inhibitory activity [12,15]. The presence of chelators such as EDTA removes divalent cations from the lipopolysaccharide layer of Gram-negative bacteria make them sensitive to antimicrobial peptides [11,26], and thus could provide food industry with other options to be utilized to kill these pathogens.

4.6 References

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5. Heat Sensitization of *Escherichia coli* by Chitosan and Bacteriocin

5.1 Introduction

Chitosan (poly-(ß1 → 4)-2-amino-2-deoxy-D-glucopyranose) is a partially and fully deacetylated chitin [51] that is currently used in water treatment [4,43], wound healing [2,24] and the pharmaceutical industry [47]. Chitosan is recognized as safe by Food and Drug Administration and may be used in the food industry as a packaging film, an antioxidant and has shown antimicrobial activities [22,35,38,39,46]. The mode of action of chitosan is explained by electrostatic interaction between positively charged chitosan NH_3^+ groups and the negatively charged cell membrane, which results in disruption of bacterial outer and cytoplasmic membranes [15,21]. The presence of NaCl and the pH of the food matrix impact the antimicrobial property of chitosan [10,21].

Many strains of VTEC are heat and/ or pressure resistant [3,25, chapter 4]. An additional hurdle is needed for the inactivation and elimination of these microorganisms from food. One of the potential candidates for such a process is bacteriocins.

Bacteriocins are heat stable antimicrobial peptides that inhibit the growth of closely related bacteria. Bacteriocins can be classified based on their structure and molecular weight into two classes [9]. Class I bacteriocins, also known as lantibiotics, contain lanthionine and its derivatives. Class II bacteriocins are heterogeneous and don't contain lanthionine [9]. Several bacteriocins produced by Gram-positive bacteria are used as antimicrobials [37]; however, they are ineffective against Gram-negative bacteria. The outer membrane of Gram-negative bacteria contains a lipopolysaccharide

(LPS) layer on its outer leaflet that is responsible for bacterial structure integrity, protects against hydrophobic antimicrobial compounds [52] including bacteriocins. A representative Class I bacteriocin is nisin, which is produced by *Lactococcus lactis* subsp. *lactis* [5,13]. Nisin has been used as natural food preservative to inhibit spore germination and prevent the growth of a broad range of Gram-positive bacteria [8,11,53]. Micocin® II and Micocin® X are bio-preservatives derived from a *Carnobacterium maltaromaticum* culture that produces three class II bacteriocins. Micocin® products have been applied to ready-to eat meats after cooking by spray application during packaging to control the growth of *Listeria monocytogenes*.

If the outer membrane of the Gram-negative bacteria is destabilized by EDTA, bacteriocin can penetrate into the cytoplasmic membrane and inhibit the growth of these bacteria [12,48]. High hydrostatic pressure (HHP) processing and chelating agents remove cations (for example Ca^{2+} and Mg^{2+}) from the LPS, thus destabilize the LPS structure and allow bacteriocins and other hydrophobic compounds to penetrate into cell membrane [7,19]. Outer membrane damage compromises the resistance of Gram-negative bacteria to bile acid, leads to extensive damage to intracellular components in the form of oxidative stress, and results in cell death [36].

5.2 Objective and Hypothesis

Currently, no study has been reported on the application of chitosan in conjunction with bacteriocins as the sole source of antimicrobial in food systems. The aim of this study was to determine antimicrobial property of commercially available bacteriocins and chitosan in different food and buffer systems. Three types of bacteriocin products (Nisin®, Micocin® II and Micocin® X) were tested against *E.*

coli AW1.7 and a five strain VTEC cocktail with or without the presence of high and low molecular weight (MW) chitosan in various buffers and food systems. It was hypothesized that the commercially available bacteriocins can improve the killing of *E. coli* in combination with chitosan and the effect depends on the type of food or broth.

5.3 Material and Methods

5.3.1 Bacterial strain and growth conditions

Bacterial strains are listed in Table 5-1. *E. coli* AW1.7 is a beef carcass isolate that is highly resistant to heat and HHP [14,30,31] and was used as a model organism for this study. VTEC strains were obtained from Dr. A. Gill (Health Canada, Ottawa, Canada). All cultures were cultivated in Luria- Bertani, Miller (LB, *Difco*, Detroit, MI, USA) and grown for 24 h at 37 °C prior to use in experiments. LB agar plates and Violet Red Bile agar (VRBA, *Difco*) plates were used throughout the study. LB agar represented non-selective nutrient rich agar, which allows the recovery of all surviving *E. coli* including sub-lethally injured *E. coli*. VRBA represented a selective agar, containing bile salt, where only the cells resistant to outer membrane damage would be recovered.

Table 5-1: *E. coli* strains for screening

Serotype	Strain ID	Origin	Virulence factor
O26:H11	05-6544	human	Stx1, eae, EHEC-hlyA
O121:H19	03-2832	human	Stx2, eae, EHEC-hlyA
O145:NM	03-6430	unknown	Stx1, eae, EHEC-hlyA
O145:NM	*PARC 449*	human	eae, EHEC-hlyA
O157:H7	C0283	cattle feces	Stx1/2, eae, EHEC-hlyA
Unknown	*AW1.7*	Beef carcass [14]	n/a

Non VTEC strains are underlined and italicized; n/a: not applicable

5.3.2 Antimicrobials

Chitosans used for the experiments were a gift of Ying Hu, Hubei University of Technology. Chitosan was dissolved in 1% acetic acid to obtain a final concentration of 1% chitosan. Chitosan with a MW of 400 kDa and chitosan with a MW of 10 kDa were designated as a high MW (HMW) and a low MW (LMW) chitosan, respectively. Micocin® was kindly provided by Griffith Laboratories, Toronto, CA. Laboratory grade Nisin® (Chrisin) was purchased from *MP Biomedicals*, LLC (Illkirch, France). A nisin stock solution (250 mg/L) was prepared by dissolving 0.1 g of 2.5% Nisin powder in 10 mL of 0.02 N HCl. To adjust the pH of the solution, 1% NaOH and 1% acetic acid were used throughout the experiments.

5.3.3 Survival of *E. coli* AW1.7 in presence of antimicrobials during refrigerated storage

The effect of chitosan on survival of *E. coli* AW1.7 at 4 ^{o}C was determined in imidazole buffer (IM) at pH 5.5 and in phosphate buffered saline (PBS) at pH 7.0. HMW chitosan, Micocin® X, or chitosan plus Micocin® were mixed with the buffers to achieve a final concentration of 0.1% chitosan and 1% Micocin®. Because chitosan was dissolved in acetic acid, samples that did not contain chitosan were supplemented with acetic acid (pH 5.4) to achieve an equivalent final concentration of 0.1%. Control samples were supplemented with water or acetic acid. After volume adjustment with the stock solutions, the buffer had a concentration of 40 mmol/L (chitosan plus Micocin® or acetic acid plus Micocin®) or 45 mmol/L (control, chitosan, Micocin®, and acetic acid). Buffers were chilled overnight at 4 ^{o}C in a water bath, inoculated with *E. coli* AW1.7 to a cell count of about 10^7 CFU/mL, and incubated at 4 °C for a total of 5 days. Samples were taken after 30 min, 1 d, 2 d, 3 d and 5 d after inoculation. Cell counts were determined by surface plating with a WASP2 spiral plater (*DW* Scientific, Shipely, WYK, UK) on LB agar and VRBA. Plates were incubated at 37 ^{o}C for 24 h prior to enumeration.

5.3.4 Survival of *E. coli* AW1.7 in presence of antimicrobials during refrigerated storage and heat treatment in buffer solutions with or without Ca^{2+} and Mg^{2+} supplementation

To determine whether addition of divalent cations such as Ca^{2+} and Mg^{2+} improves the survival of *E. coli* AW1.7 in pH 5.5 imidazole buffer (IM), IM buffer was supplemented with 0.08 mg/mL Ca^{2+} and 0.29 mg/mL Mg^{2+} and designated as IMS. HMW chitosan was used for the experiment. Testing solutions containing chitosan and Micocin® X were prepared as above. Buffers were chilled to 4 ^{o}C, inoculated with 10^{7} CFU/mL of *E. coli* AW1.7, and incubated at 4 °C for a total of 5 days. Samples were taken after 30 min, 1 d, 2 d and 5 d after inoculation. An additional set was taken after 5 days of storage and subjected to heat treatment at 60 ^{o}C for 30 min. Cell counts were determined by surface plating on LB agar and VRBA as outlined above.

5.3.5 Food matrix selection

The food matrices chosen for the experiments were apple juice, yogurt serum and ground beef, representing food matrices with a higher amount of divalent cations and low pH. Apple juice and yogurt were purchased from a local grocery store. Acidity of the apple juice was adjusted from original pH 3.9 to 3.8. Fresh ground beef was kindly provided by a local Federally inspected meat processing facility.

5.3.6 Survival of *E. coli* AW1.7 in presence of antimicrobials during refrigerated storage and heat treatment in apple juice

The effect of chitosan with or without nisin on survival of *E. coli* AW1.7 after refrigeration and heat treatment was determined in apple juice. The apple juice was filtered through 0.22 um syringe filter prior experiments. HMW chitosan, LMW chitosan, or both chitosan and nisin were mixed with apple juice to achieve a final concentration of 25 mg/L nisin and/or 0.1% chitosan. Apple juice contains a similar amount of Ca^{2+} as IMS buffer (0.08 mg/mL) and thus was used as a food matrix for comparison to the buffer. Control samples were supplemented with water. Apple juice was pre-chilled to 4 ^{o}C, inoculated with 10^5 CFU/mL of *E. coli* AW1.7, and incubated at 4 °C for a total of 5 days. Samples were taken after 30 min, 1 d and 5 d after inoculation. Cell counts were determined by surface plating on LB and VRBA agar. Plates were incubated at 37 ^{o}C for 24 h prior to enumeration. After 5 days of storage, an additional three sets of samples were taken for heat treatment at 60 ^{o}C for 1, 2 and 4 min. Cell counts were determined as above.

5.3.7 Survival of *E. coli* AW1.7 in presence of antimicrobials during refrigerated storage followed with heat treatment in yogurt serum

The effect of chitosan, with or without nisin on survival of *E. coli* AW1.7 after refrigeration and heat treatment was determined in yogurt serum. Yogurt was centrifuged for 10 min at 4 ^{o}C, the supernatant was collected and pH was measured as 4.2. Yogurt serum was filtering sterilized (0.22 μm) and pre-chilled at 4 ^{o}C before

experiments. The experimental design of the yogurt serum experiment was identical to that of the apple juice experiment outlined above.

5.3.8 Survival of *E. coli* AW1.7 in presence of antimicrobials during refrigerated storage followed with heat treatment in ground beef

The effect of chitosan and Micocin® II on survival of *E. coli* AW1.7 was determined in refrigerated ground beef. Due to the ineffectiveness of nisin in raw beef, experiments were done with the commercially available product Micocin® II. The presence of live live *C. maltaromaticum* could produce additional bacteriocins during 4 ^{o}C storage. Ground beef was received on the day of production, divided in aliquots and stored frozen (-20 ^{o}C). Beef was thawed overnight before use in experiments. HMW chitosan, Micocin®, or both chitosan and Micocin® were mixed in ground beef to achieve a final concentration of 0.1% chitosan and/or 1% Micocin®. Control samples were supplemented with water or acetic acid to final concentration of 0.1%. Ground beef was inoculated with 10^7 CFU/g of *E. coli* AW1.7 in a sterile bag, hand massaged for 1 min and incubated at 4 °C for a total of 5 days. Samples were taken after 30 min, 1 d and 5 d after inoculation. An additional set of samples was taken after 5 d of storage for heat treatment at 60 ^{o}C for 30 min. Cell counts were determined by surface plating on LB agar and VRBA as above. Due to the presence of live *C. maltaromaticum* culture in Micocin® II, samples containing Micocin® II and the control were also plated on All Purpose Tween (APT agar, *Difco)* plates and incubated at 25 ^{o}C for 48 h.

5.3.9 Survival of VTEC strain cocktail during refrigerated storage followed with 60 ^{o}C for 30 min heat treatment in ground beef

The effect of chitosan with or without the presence of bacteriocins on refrigerated survival of the VTEC cocktail was determined in ground beef. Samples were prepared the same as for the *E. coli* AW1.7 ground beef experiment with the exception that Micocin® X was used instead of Micocin® II. Ground beef was thawed at 4 ^{o}C overnight, inoculated with a cocktail of VTEC to a cell count of about 10^{7} CFU/g, and incubated at 4 °C for a total of 5 days. Samples were taken after 30 min, 1 d and 5 d after inoculation. Cell counts were determined by surface plating on LB agar and VRBA. Plates were incubated at 37 ^{o}C for 24 h prior to enumeration. At 5 d, heat treatments were conducted in a water bath at 60 ^{o}C for 5, 10 and 20 min. Samples were serially diluted, enumerated on LB and VRBA plates, and incubated as above. One additional set of samples was subjected to treatment at 60 ^{o}C for 20 min. After treatment, samples were cooled in an ice bath and put into 100 ml of Tryptic Soy broth (TSB, *Difco*, Detroit, MI, US) for 24 h enrichment at 37 ^{o}C.

5.3.10 Statistical analysis

Average cell counts were determined from at least two independent experiments. A Normality Test (Shapiro-Wilk) was applied ($P<0.05$) for significant difference, and tests involved triplicate experiments of different treatment groups.

5.4 Results

5.4.1 Survival of *E. coli* AW1.7 treated with antimicrobials during refrigerated storage

To determine whether HMW chitosan alone or in combination with Micocin® X reduces *E. coli* cell counts over a period of five days, *E. coli* samples were stored in IM and PBS buffer containing different treatment compounds at 4 ^{o}C. Neither chitosan nor Micocin® X treatment reduced *E. coli* cell counts on LB (Figure 5-1). However, chitosan treatment reduced the recovery of *E. coli* counts on VRBA plates in all samples, which indicated that chitosan induced sublethal injury of *E. coli* cells. This is presumed to be a result of a permeabilization of the outer membrane by chitosan [15,21]. Acetic acid, Micocin® X, and the combination of acetic acid plus Micocin® X treatment had no effect on counts of *E. coli*. Comparison of chitosan treatment in IM (pH 5.5) and PBS buffer (pH 7) confirmed that the acidity of the buffers played an important role for the antimicrobial activity of chitosan. Antimicrobial activity of chitosan was more pronounced in IM buffer.

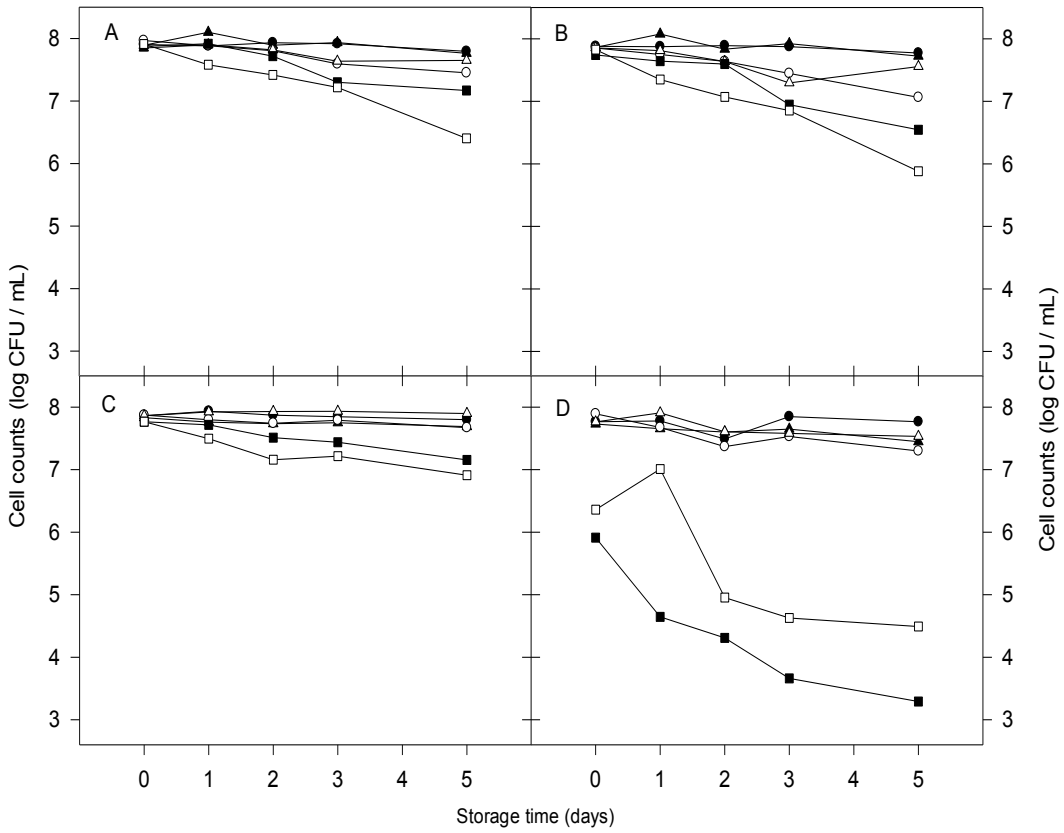

Figure 5-1: Survival of *E. coli* AW1.7 in PBS buffer (Panel A, B) and IM buffer (Panel C, D) with different compounds during storage at 4 °C. (●) control, (o) Micocin® X, (▲) acetic aicd, (Δ) acetic acid plus Micocin® X, (■) HMW chitosan, and (□) HMW chitosan plus Micocin® X. Cell counts were determined on LB agar (Panel A, C) or on VRBA (Panel B, D). Data represent means of two independent experiments.

5.4.2 Survival of *E. coli* AW1.7 in presence of antimicrobials during refrigerated storage and heat treatment in buffer solutions with or without Ca^{2+} and Mg^{2+} supplementation

To determine whether supplementation of divalent cations provides protection to *E. coli* cells in presence of antimicrobials during refrigerated storage and subsequent heat treatment, a comparison between treatments carried out in IM

and IMS (IM supplemented with Ca^{2+} and Mg^{2+}) buffer was done. There was no additive effect between Micocin® X and chitosan; supplementation of divalent cations such as Ca^{2+} and Mg^{2+} improved *E. coli* survival during storage. Chitosan was lethal to *E. coli* when combined with heat treatment in buffer systems (Figure 5-2).

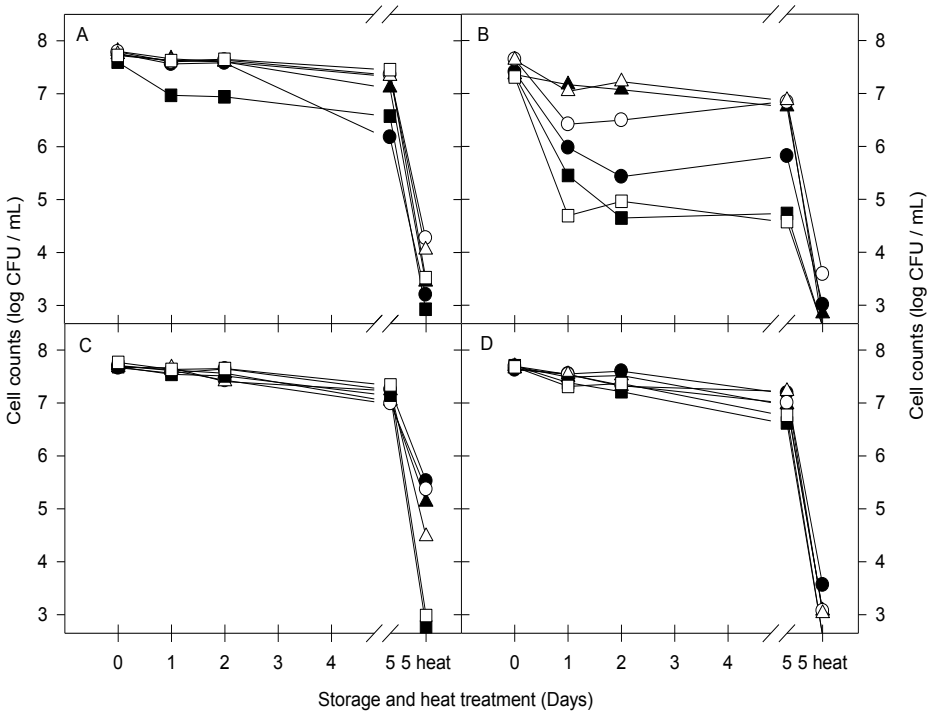

Figure 5-2: Survival of *E. coli* AW1.7 in IM buffer (Panel A, B) and IM buffer supplemented with Ca^{2+} and Mg^{2+} (Panel C, D) with different testing compounds during storage at 4°C and followed by 60 °C heat treatment of 30 min. (●) control, (o) micocin, (▲) acetic acid, (Δ) acetic acid plus micocin, (■) HMW chitosan, and (□) HMW chitosan plus micocin. Cell counts were determined on LB agar (Panel A, C) or on VRBA (Panel B, D). Lines dropping below the x-axis indicate cell counts that were reduced to levels below the detection limit of 2.6 log CFU/mL after heat treatment.

Based on acidity and composition, three food matrixes were chosen for subsequent experiments to investigate the potential application of chitosan in food systems.

5.4.3 Survival of *E. coli* AW1.7 during refrigerated storage and heat treatment in apple juice solutions

To determine whether the molecular weight of chitosan plays a role on *E. coli* reduction in a food matrix system with low pH and 0.08 mg/mL of Ca^{2+}; nisin was used in conjunction with HMW or LMW chitosan (Figure 5-3). *E. coli* AW1.7 remained viable in apple juice without heat. A Normality Test (Shapiro-Wilk) indicated cell counts of all groups during storage period are similar ($P < 0.050$). But after 1 min heat treatment, LMW chitosan plus nisin group effectively reduced cell counts of *E. coli* to below the detection limit of 2.6 log CFU/mL. After 2 min of heating, HMW chitosan plus nisin reduced the cell count of *E. coli* to below the detection limit. Nisin alone was not effective as indicated by similar *E. coli* cell count reduction as the control group. When enumerated on VRBA, LMW chitosan and both chitosan compounds plus nisin groups reduced the *E. coli* cell count below the detection limit in less than 2 min of heat treatment. *E. coli* cell counts were reduced below the detection limit after 4 min heat treatment for all tested samples. Comparison of *E. coli* count on LB and VRBA indicated chitosan injures bacteria by damage the outer membrane, and both high and low MW chitosan with nisin were effective in inactivation of *E. coli*. It was interesting to observe that after storage,

sedimentation occurred in samples containing chitosan, and the most sedimentation occurred in the LMW chitosan group.

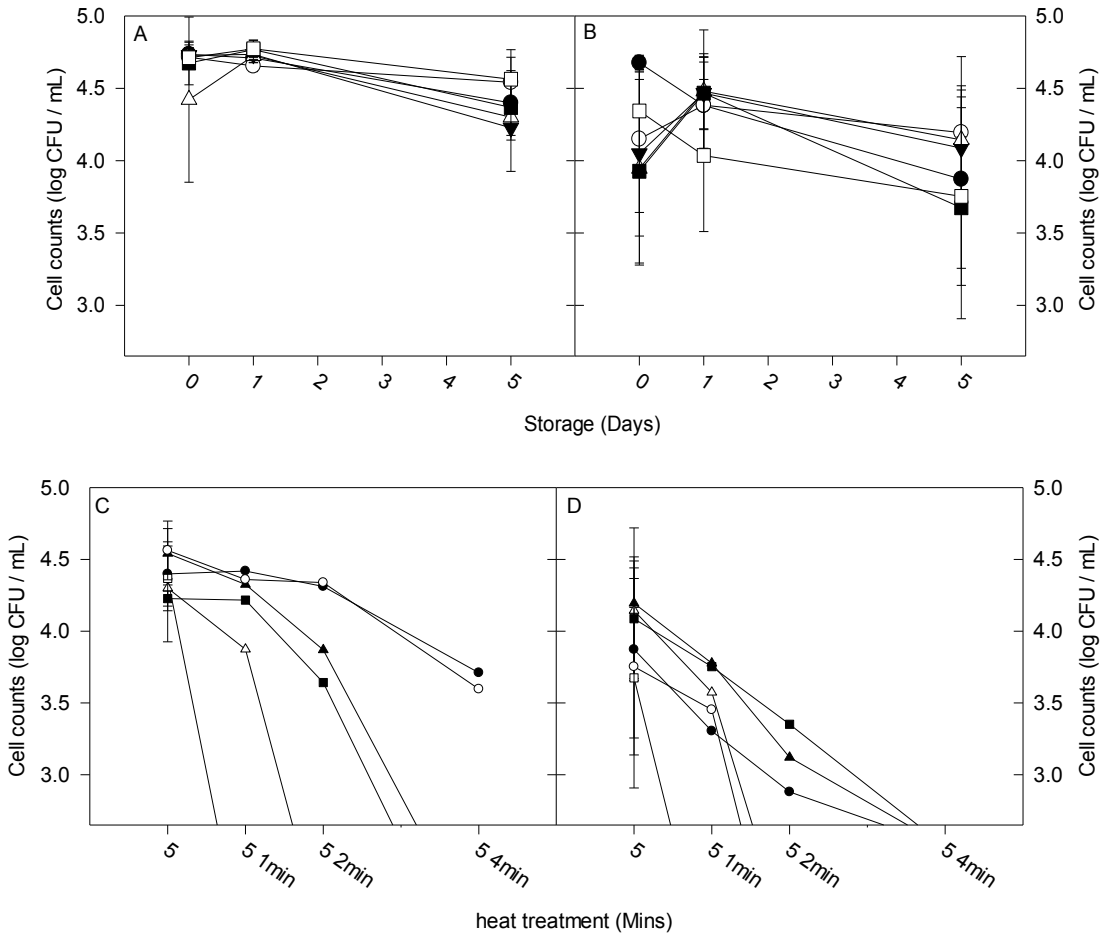

Figure 5-3: Survival of *E. coli* AW1.7 in apple juice with different compounds during storage at 4 °C (Panel A and B), and samples stored for 5 days and heated at 60 °C for 1, 2 and 4 min (Panel C and D). (●) control, (o) nisin, (▲) HMW chitosan, (Δ) HMW chitosan plus nisin, (■) LMW chitosan, and (□) LMW chitosan plus nisin. Cell counts were determined on LB agar (Panel A, C) or on VRBA (Panel B, D). Storage data represent means ± standard deviation of three independent experiments; heat treatment data represent means of two independent experiments. Lines dropping below the x-axis indicate cell counts that were reduced to levels below the detection limit of 2.6 log CFU/mL after heat treatment.

5.4.4 Survival of *E. coli* AW1.7 during refrigerated storage and heat treatment in yogurt serum

Yogurt serum was chosen as second food matrix, yogurt serum has low pH, contains lactate and Ca^{2+} (*ca.* 1 mg/mL), but yogurt serum is not a significant amount of Mg^{2+} (Figure 5-4). Experiments similar to those conducted for apple juice were done. From the first two days of storage, a Normality Test (Shapiro-Wilk) indicated no significant difference was observed from the treatment groups ($P < 0.05$). After 5 days of storage, both HMW and LMW chitosan and nisin combinations reduced *E. coli* cell counts below the detection limit when enumerated on LB plates. Heat treatment reduced *E. coli* cell counts to below the detection limit for all treatments (data not shown). Enumeration on VRBA plates after 0 and 1 day of storage showed similar cell counts for all treatments ($P < 0.05$); and no growth on VRBA was observed after 5 days of storage from all treatments. The result suggests that lactic acid and low pH increased the antimicrobial effect of a combination of chitosan with nisin. Yogurt serum alone was sufficient in inflicting outer membrane injury to *E. coli*, as demonstrated by the inability of the organism to grow on VRBA after 5 days of storage. Combinations of nisin with chitosan killed all cells, as indicated by no visible cell growth on LB agar. Size of chitosan did not affect reduction of cell counts.

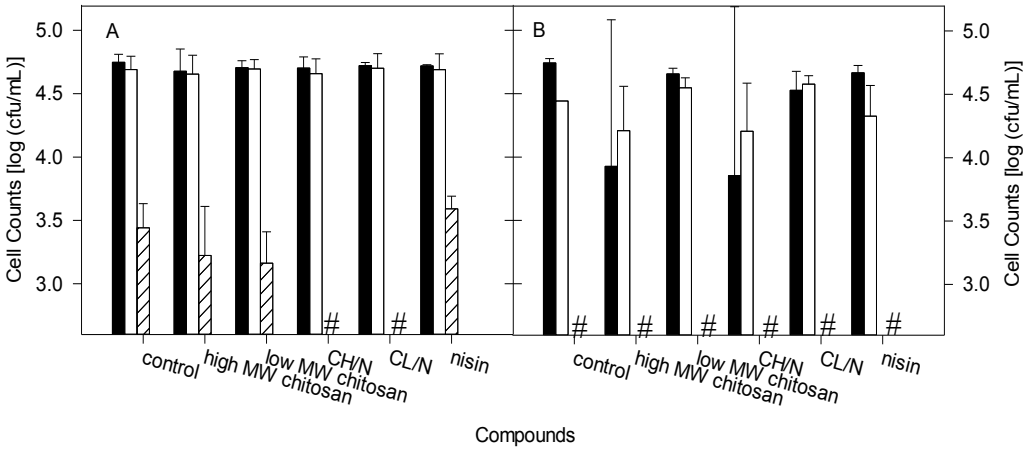

Figure 5-4: Survival of *E. coli* AW1.7 in yogurt serum with different compounds during storage at 4 °C. CH/N represents HMW chitosan and nisin combination; CL/N represents LMW chitosan and nisin combination. Black bars represent results on day 0; clear bars represent results after 1 day of storage, and hatched bars represent results after 5 days of storage. Cell counts were determinedon LB agar (Panel A) or on VRBA (Panel B). Storage data represent means ± standard deviation of three independent experiments. #: no colonies were observed on agar plates.

5.4.5 Survival of *E. coli* AW1.7 in presence of antimicrobials during refrigerated storage in ground beef followed with heat treatment

To determine whether chitosan and commercially available bacteriocin products were effective in a food matrix containing Ca^{2+}, Mg^{2+} and lactic acid, *E. coli* were inoculated into ground beef containing different antimicrobials and stored at 4 ^{o}C for 5 d and subsequently heat treated (Figure 5-5). Micocin® II was used in this experiment because the presence of live *C. maltaromaticum* in the product can potentially produce additional bacteriocins during storage and increase its antimicrobial effect. The initial cell count of *C. maltaromaticum* was approx. 3 log CFU/g in samples supplemented with Micocin® II and after 5 days of storage, counts reached approx. 6 log CFU/g (data not shown). The addition of live cells of *C.*

maltaromaticum did not enhance the antimicrobial effect as a similar reduction in counts of *E. coli* AW1.7 were obtained when meat was supplemented with Micocin® X (data not shown). A one log CFU/mL reduction of *E. coli* AW1.7 was achieved after heating at 60 ºC for 30 min in all samples. In the presence of chitosan and Micocin® X or II in the meat, no increase in *E. coli* AW1.7 reduction was observed after subsequent heat treatment when compared to the control samples.

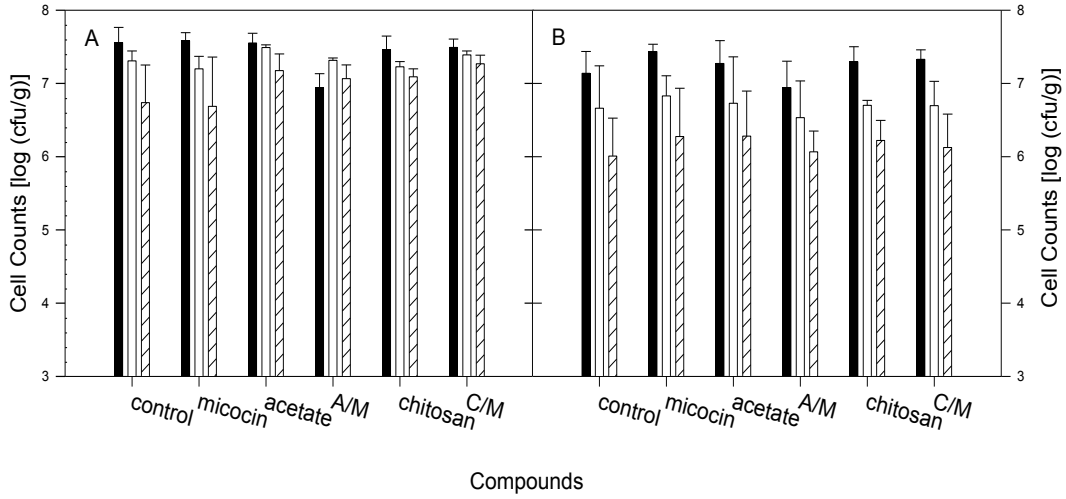

Figure 5-5: Recovery of *E. coli* AW1.7 after 5 days of storage at 4 °C in ground beef and heating at 60 °C 30 min. A/M represents acetic acid plus Micocin® II combination; and C/M represents a combination of HMW chitosan and Micocin® II. Black bars represent results at day 0; clear bars represent results after 5 d of storage, and hatched bars represent results after heating at 60 °C for 30 min. Cell counts were determined on LB agar (Panel A) or on VRBA (Panel B). Data represent means ± standard deviation of three independent experiments.

5.3.6 Survival of a VTEC strain cocktail in presence of antimicrobials during refrigerated storage in ground beef followed by heating at 60 °C

E. coli AW1.7 is the most heat resistant strain of *E. coli* (14,30), and it is not likely representative of entire *E. coli* species; therefore, a 5 strain VTEC cocktail was tested in ground beef with HMW chitosan and Micocin® X (Figure 5-5 and 5-6)

to determine their antimicrobial effect in ground beef against low dose pathogenic strains of *E. coli*. Experiments were designed in a manner similar to the previous experiment except a 5 strain cocktail of VTEC was used and a shorter heat treatment was done. All samples including the control had *ca*. 1 log CFU/g reduction after heating for 10 min at 60 ^{o}C. However, no survival of the VTEC cocktail was achieved after 20 min of heating at 60 ^{o}C in samples supplemented with HMW chitosan and Micocin® X. The addition of chitosan and Micocin® X had a small effect on the reduction of *E. coli* in ground beef after refrigerated storage and subsequent heat treatment.

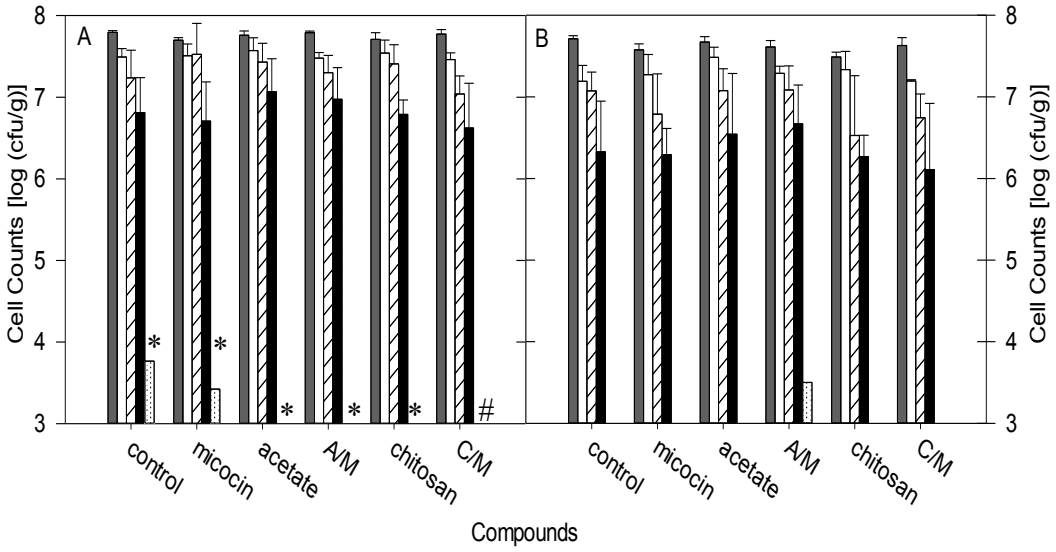

Figure 5-6: Recovery of a cocktail of VTEC in ground beef after 5 days of storage at 4 °C and following heating at 60 ^{o}C for 5, 10 and 20 min. A/M represents acetic acid plus Micocin® X combination; and C/M represents a combination of HMW chitosan and Micocin® X. Grey bars represent results on day 0; clear bars represent results after 5 days of storage, hatched bars represent results after heating at 60 ^{o}C for 5 min, black bars represent results after heating at 60 ^{o}C for 10 min, and dotted bars represent results after heating 60 ^{o}C for 20 min. *: surviving cells were detected after enrichment. #: no surviving cells detected after enrichment. Cell counts were determined on LB agar (Panel A) or on VRBA (Panel B). Data represents means ± standard deviation of three independent experiments. Enrichment was performed during two independent experiments.

5.5 Discussion

This study probed the effect of chitosan as an outer membrane permeabilizer, which can be applied to enhance the antimicrobial effects by combining with other treatments. The antimicrobial effect of chitosan is over a broad molecular weight range, from 10 kDa to 1000 kDa [29,51]. In present study both high and low MW chitosan showed additive effect with bacteriocins in apple juice, yogurt serum, and in buffer systems. This research demonstrated a bactericidal effect of chitosan when combined with heat, lactate, and bacteriocins in three different food matrices with different levels of Ca^{2+} and pH. The challenge study was originally designed for ground beef experiments through five days storage followed by heat treatment; although it does not match processing conditions for apple juice and yogurt serum. In order to make a comparison to ground beef experimental design, both apple juice and yogurt serum were treated the same as ground beef.

Reports of bactericidal activity on chitosan and its derivatives against *E. coli* are limited. Weak bactericidal activity is observed from chitosan derivatives and from chitosan at a high concentration [23,51]. The present study demonstrated that chitosan is bacteriostatic rather than bactericidal. Control of VTEC in food requires bactericidal activity, thus application of chitosan to inactivate VTEC requires additional antimicrobial treatments.

The outer membrane provides protection to cells of *E. coli*. The outer membrane contains two leaflets; the outer leaflet is composed of LPS and it serves as a barrier to prevent rapid penetration of hydrophobic molecules. The LPS is

stabilized by divalent cations, particularly Mg^{2+} and Ca^{2+} [40,52]. Chitosan remains positively charged at pH below 6. The NH_3^+ groups of the chitosan interact with the negatively charged bacterial outer membrane by competition for the Ca^{2+} specific binding site on the membrane surface [20,41, 54], This causes the release of the LPS [29] and exposes the underlying phospholipid layer to hydrophobic compounds and environmental stressors. In the current study the most successful food application for chitosan was in yogurt serum which contains high amount of lactate and Ca^{2+} compared to other tested matrices. The outer membrane permeabilization effect of lactic acid in *E. coli* had been demonstrated previously [1,18,21] and could play a major role in causing outer membrane injury of *E. coli* in yogurt serum. At low pH, chitosan interacts with the outer membrane and act additively with lactic acid produced by the lactic acid bacteria from the yogurt serum in killing the cell during the storage. Lactic acid could be a contributor to the damage during storage because outer membrane damage was inferred by the inability to grown on VRBA in yogurt serum but this was not observed from apple juice, as growth on VRBA occurred (Figures 5-3 and 5-4).

The antimicrobial effect of chitosan has previously been studied in apple juice. Kisko et al. [27] found that chitosan can provide a species specific delay of yeast spoilage in apple juice when stored at 4 ^{o}C, but can prolong the survival of *E. coli* O157:H7. In this study, the bactericidal effect of chitosan was only observed after mild heat treatment (1 to 2 min at 60 ^{o}C; Figure 5-3) in apple juice. This can provide industry a low thermal process that inactivates *E. coli* and that will potentially minimize the energy cost compared to conventional heating, but may also preserve some of the nutrients and flavor aspects of the juice. Application of chitosan

during apple juice clarification as a fining agent to separate pectin has been described previously [6,45]. Sedimentation and lighter color caused by the chitosan-pectin interaction was observed in treatments containing chitosan. Removal of pectin increases clarity and potentially increases value of the apple juice. The industrial application of chitosan during apple juice clarification can be two fold, as a fining agent and as an antimicrobial agent. However, when pectin interacts with chitosan, the outer membrane permeabilization effect of chitosan would be reduced, as the fining process would decrease chitosan concentration in the matrix.

Chitosan had small bactericidal effect in ground beef where divalent cations are abundant. Ground beef is a nutrient rich and complex food matrix. The effect of bacteriocins is influenced by the composition of the food matrix [17]. Class I lantibiotics such as nisin are not effective in meat because the presence of high amount of glutathione in meat readily inactivates it [44]; therefore, the commercially available class II bacteriocin products Micocin® X and II were chosen for ground beef experiments. Micocin® X and II are derived from *C. maltaromaticum* and contains three class II bacteriocins: piscicolin 126 and carnobacteriocin BM1 and a circular bacteriocin, carnocyclin A [33]. Class II bacteriocins produced by *C. maltaromaticum* have bactericidal activity against *E. coli* when added together with EDTA [34]. In addition, ground beef contains lactic acid, which contributes to membrane permeability [1,18] and could synergistically increase the effect of chitosan and bacteriocin. Micocin® II derives from both the live cells and the culture supernatant, while Micocin® X derived from culture supernatant. Despite that, the application of Micocin® products with chitosan in ground beef failed to reduce cell counts of *E. coli*. However, its possible application in beef where it has proven

antioxidant effects and prevents premature browning [49,50] could be more relevant as we showed its marginal bactericidal activity against several *E. coli* strains during heat treatment.

Chitosan alone is not effective in inactivation of *E. coli*; however, this research has shown a synergistic bactericidal effect with additional hurdles such as antimicrobials, heat treatment and refrigerated storage. In addition, chitosan had shown additive effect with high hydrostatic pressure [32]. These results suggested that chitosan can be applied as an additional hurdle to kill foodborne pathogens such as VTEC.

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6. General Discussion and Conclusion

6.1 Aim of the Study

Verotoxigenic *Escherichia coli* (VTEC) are food borne pathogens of particular concern, as the infectious dose of VTEC is low, and VTEC illnesses have involved a wide range of foods, including meat and dairy products. A review of the literature on *E. coli* and HHP indicated that hydrostatic pressure can sublethally injure *E. coli* by damaging its outer membrane, cytoplasmic membrane, cytoplasm and DNA/RNA components; which results in vulnerability to oxidative stress, osmotic imbalance and acid stress. Experimental work reported in this thesis demonstrated that strains of *E. coli* are among the most pressure resistant vegetative foodborne organisms tested and can survive the current commercial HHP processing parameters. In addition, there is a large variation in HHP resistance of VTEC and the variation is both strain and food matrix dependent (see Table 1-1).

The overall aim of the study was to investigate the application of HHP processing alone or in combination with other treatments to inactivate *E. coli* in food (especially meat) products. In order to achieve the objective, individual studies were conducted and discussed in the following paragraphs.

6.2 HHP Inactivation of Pathogenic and Spoilage Bacteria in Meat

To compare the HHP resistance of *E. coli* against other spoilage and pathogenic bacteria in a food matrix, a challenge study with HHP followed by storage was conducted in poultry meat. Pressure resistance of *E. coli* AW1.7 was evaluated against *C. jejuni* and other pathogens and spoilage organisms in poultry

meat. It was hypothesized that the proposed HHP parameters can be used to inactivate all tested organisms including *C. jejuni*. Results from the study indicated treatment of 400 MPa and 40 ºC for 30 min resulted in a reduction in cell counts of more than 6 log CFU/g of spoilage bacteria and *C. jejuni*. However, HHP of 400 or 600 MPa and 40 °C failed to reduce cell counts of *E. coli* AW1.7 by 5 logs CFU/g. Pressure alone was not able to produce safe to eat products from raw poultry. More study needs to be conducted on the subject and additional antimicrobial treatment processes are required to inactivate *E. coli*.

6.2 Heat and HHP Inactivation of VTEC and Non-toxigenic Reference Strains

Dlusskaya et al. [2] found *E. coli* AW1.7 to be extremely heat resistant when compared to other strains of *E. coli.* It was hypothesized that VTEC are not significantly more heat and pressure resistant than non-pathogenic *E. coli*. The screening results from chapter 3 and 4 confirmed this hypothesis, in which *E. coli* AW1.7 was the most heat resistant strain tested among VTEC and non-toxigenic strains. Challenge studies showed grilling ground beef patties to an internal temperature of 71 ºC resulted in a 3.4 log CFU/g reduction of *E. coli* AW1.7. Further study indicated the NaCl content as well as the fat content strongly influenced heat resistance of *E. coli* AW1.7 with a combination of 2 % NaCl and 15% fat resulting in the greatest heat resistance. Data on the heat resistance of VTEC in broth and ground beef demonstrated some VTEC strains are heat resistant. Further study on HHP resistance of VTEC and verotoxin negative strains indicated some VTEC strains exceeded the pressure resistance of *E. coli* AW1.7. In fact, over one third of strains

were reduced by less than 3 log CFU/g after a HHP treatment at 600 MPa for 3 min in LB broth, and with the most heat resistant strain of VTEC *E. coli* O121:H19 03-2832, cell counts were reduced by 2.7 log (CFU/g) when treated at 600 MPa for 3 min in ground beef samples. In contrast a 2.9 log (CFU/g) cell count reduction was observed with *E. coli* AW1.7). Results indicated *E. coli* strains are among the most pressure resistant vegetative bacterial cells described to date and resistance is in a range similar to that of *S. aureus* [9]. Some of the VTEC strains tested survived cooking recommendations of *E. coli* by Canadian regulatory agencies (Health Canada and the Canadian Food Inspection Agency) and in addition, some VTEC were resistant to the current industrial HHP practice parameters. More studies need to be conducted to validate the results. Additional antimicrobial treatments could be applied to kill these organisms.

6.3 Effect of Antimicrobial Treatment Processes on *E. coli*

Additional hurdles such as bacteriocin, chitosan and naturally occurring lactic acid produced from lactic acid bacteria in yogurt were tested in buffer, apple juice, yogurt serum and ground beef. It was hypothesized that the commercially available bacteriocins could improve the killing of *E. coli* in combination with the antimicrobial chitosan and the effect might be dependent on the type of food or broth tested. Results from the study indicated naturally occurring lactic acid and nisin with chitosan was the most effective combination against *E. coli* AW1.7 in yogurt serum. The outer membrane permeabilization effect of lactic acid had been demonstrated previously [4,8]. The presence of natural occurring lactic acid could provide additive effect in killing of *E. coli* and outer membrane damage is crucial for the bactericidal

effect. The bactericidal effect of chitosan was observed after heat treatment in apple juice when combined with nisin plus chitosan. Chitosan combination with heat had small bactericidal effect in ground beef against the VTEC cocktail, as ground beef is a nutrient rich and complex food matrix and can interfere with the effect of chitosan and bacteriocins. Initial studies of HHP indicated that chitosan in combination with HHP was not effective against *E. coli* AW1.7, but Micocin® X combined with HHP increased killing of *E. coli* AW1.7 (Figure 6-1). Both chitosan and HHP causes outer membrane injuries. And if the outer membrane is damaged by HHP, a bacteriocin can attach to the cell membrane, and initiate bactericidal activity. Further study of HHP and Micocin® X need to be conducted in order to confirm the additive bactericidal effect observed from this study. Addition of lysozyme and nisin prior to HHP increased killing of *E. coli* [5]. Although the bactericidal effect was observed in broth medium, the same effect may not work in a complex food matrix such as meat. Thus the killing of *E coli* AW1.7 by application of additional treatment process is strongly dependent on the food matrices and preservation methods.

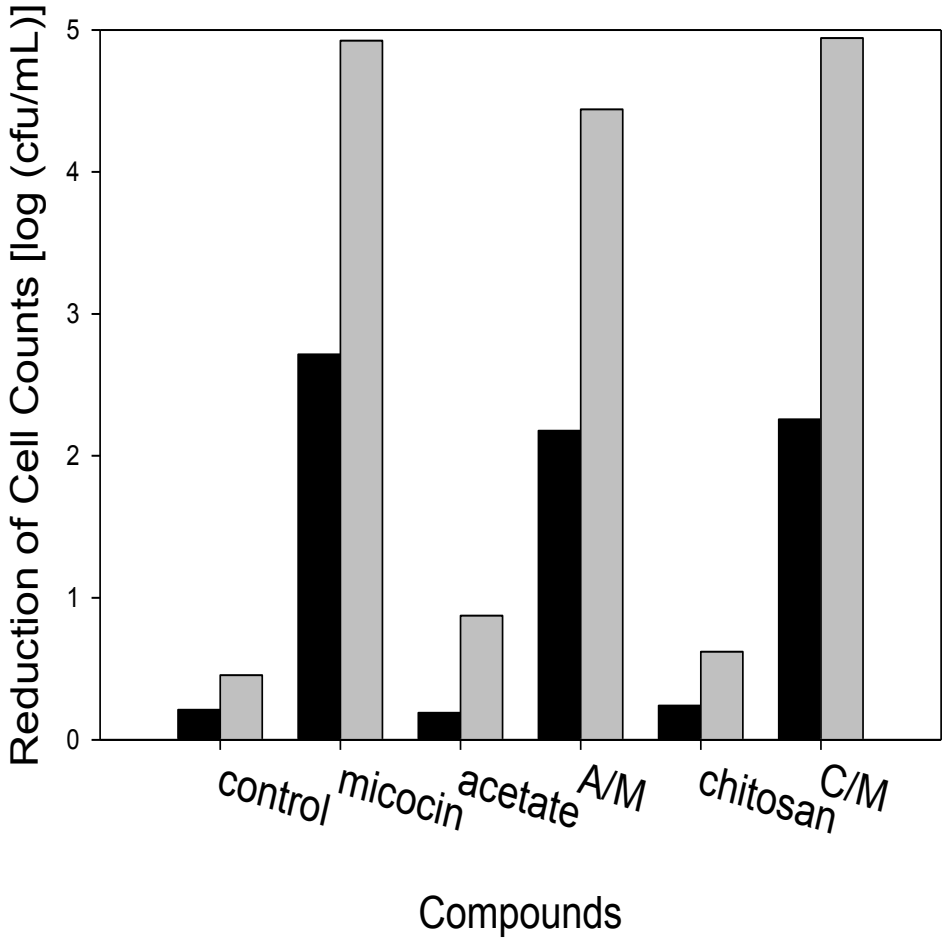

Figure 6-1: Recovery of *E. coli* AW1.7 after treatment at 600 MPa at 25 °C for 3 min in potassium phosphate buffer broth (pH 7) immediately after HHP treatment and 1 day of 4 °C storage. Bacterial cultures were prepared followed procedure of chapter 5.2.1, and antimicrobial preparation followed procedure of chapter 5.2.3. HHP sample preparation and treatment followed the procedure of chapter 4.2.3. A/M represents 0.01% acetic acid and Micocin® X combination; and C/M represents a combination of chitosan and Micocin® X. Black bars represent results immediately after treatment; grey bars represent results after 1 day of storage. Cell counts were determined on LB agar. Data represents the mean of two independent experiments.

6.4 Discussion

Experimental work demonstrated some VTEC strains are heat- and pressure resistant and resistance is independent of serotype or phylotype. More than one third of tested strains had reduction of colony counts of 3 logs under the current commercial HHP unit processing parameters (600 MPa for 3 min), indicating that HHP alone is not an effective method to kill *E. coli*. In addition, there was a large variation in HHP resistance of VTEC and the resistance was both strain and food matrix dependent (see Figure 4-1 to 4-4, Table 4-4).

Other potential treatment processes that could be used to kill *E. coli* include phenolic compounds and flavonoids. The antimicrobial properties of these compounds are well documented [1,12,15]. The mechanism of action includes inhibition of nucleic acid synthesis, cytoplasmic membrane damage and bacterial species- and strain dependent energy metabolism interference [3,6,10,11,13,14].

In conclusion, this research demonstrated that high pressure alone was insufficient to kill all strains of *E. coli* in meat and bacteriocins, lactic acid, and chitosan as additional hurdles can reduce *E. coli* from some food products. The lethal effect was strongly dependent on food matrix and preservation method. This research provided a comprehensive list of VTEC colony counts reduction for both heat and HHP treatments by strain and serotype. The data generated from the research increased our knowledge on VTEC resistance to common thermal preservation and HHP. The study on chitosan and bacteriocins provides valuable information on applying different antimicrobial treatments in conjunction with heat or pressure to

reduce *E. coli* from food and provides food industry new alternatives for producing safer food products.

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