Effect of chitosan and other antimicrobial hurdles on the survival of foodborne pathogens and heat-resistant *E. coli* on meat

by

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ABSTRACT

Listeria monocytogenes, *Salmonella* and Shiga-toxin producing *E. coli* (STEC) continue to cause disease outbreaks associated with meat. *E. coli* and *Salmonella*surviving the recommended cooking procedures present an additional risk to meat safety. The objectives of this thesis were to explore the single and combined effects of chitosan and other antimicrobial hurdles on the survival of pathogens and heat-resistant strains on meat.

Inhibitory effects of chitosan and bacteriocins in media were determined by a critical dilution assay; the antimicrobial activity of chitosan and a bacteriocin-producing strain of *Carnobacterium maltaromaticum* or the bacteriocins produced by this strain was evaluated on beef. Surface application of chitosan solution inactivated *E. coli* AW1.7 and *S.* Typhimurium by 1 log (CFU/g) on raw beef. Chitosan and purified bacteriocins acted synergistically in media but not on meat.

The effects of NaCl, chitosan and other additives on the heat resistance of *E. coli* was evaluated in ground beef that was grilled to a core temperature of 71 °C immediately after inoculation or after storage for two days at 4 °C. Addition of 3% NaCl increased survival of heat-sensitive *E. coli*, while the protective effect of NaCl was not observed if cells were cooled to 4 °C before mixing with cold meat and NaCl. Chitosan enhanced the thermal destruction of LHR-positive *E. coli* in ground beef stored at 4 °C for 2 days, while marinade, carvacrol, or potassium lactate had no such effect.

The combined lethality of chitosan and pressure was assessed with heat and pressure resistant strains of *E. coli* and *S.* Senftenberg in buffer and ground beef. Chitosan exhibited a bactericidal effect in both buffer and meat. Chitosan acted synergistically with treatment at 400 MPa in buffer but not in ground beef.

To assess the effects of chitosan and other antimicrobial hurdles on the survival of *L. monocytogenes* on ham, ham formulated with chitosan or other preservatives were treated at 600 MPa at 5 °C for 3 min. Surviving cells were differentially enumerated after pressure treatment and after 4 weeks of refrigerated storage. The single or combined use of chitosan inhibited the growth of *L. monocytogenes* on ham, and chitosan exhibited higher inhibitory effect than sodium diacetate/sodium lactate.

In conclusion, chitosan exhibits antimicrobial activity against *E. coli*, *Salmonella* and *L. monocytogenes* on meat, and is a useful hurdle concept for improving meat safety.

PREFACE

This thesis is an original work by Ziyi Hu.

Chapter 2 of this thesis is a literature review and a version has been accepted for publication as Hu, Z. and Gänzle, M.G., 2018. Challenges and opportunities related to the use of chitosan as food preservative. Journal of Applied Microbiology. I was responsible for writing the manuscript, and Dr. Michael Gänzle contributed to concept formation and manuscript revision.

A version of Chapter 3 of this thesis has been published as Hu, Z.Y., Balay, D., Hu, Y., McMullen, L.M. and Gänzle, M.G., 2018. Effect of chitosan, and bacteriocin–producing *Carnobacterium maltaromaticum* on survival of *Escherichia coli* and *Salmonella* Typhimurium on beef. International Journal of Food Microbiology. 290, 68-75. I was responsible for conducting the experiments and writing the manuscript. Danielle Balay and Dr. Ying Hu contributed to the development of methods in bacteriocin purification and manuscript revision. Dr. Michael Gänzle contributed to the hypothesis development, experimental design, manuscript composition and revision. Dr. Lynn McMullen contributed to manuscript revision.

Part of the experimental work in Chapter 4, including Figure 4-3, Figure 4-5, were performed by Alina Rohde and Jiayue Chen under the supervision of Dr. Michael Gänzle. The other parts were performed by Ziyi Hu under the supervision of Dr. Michael Gänzle. Dr. Michael Gänzle and Dr. Lynn McMullen contributed to the hypothesis development, experimental design and manuscript revision.

All the experimental work in Chapter 5 was performed by Ziyi Hu under the supervision of Dr. Michael Gänzle. I was responsible for conducting the experiments and writing the

manuscript. Dr. Michael Gänzle and Dr. Lynn McMullen contributed to the hypothesis development, experimental design and manuscript revision.

Ham preparations in Chapter 6 were performed by Danielle Balay and Dr. Januana Teixeira under the supervision of Dr. Michael Gänzle and Dr. Lynn McMullen. The other parts were performed by Ziyi Hu under the supervision of Dr. Michael Gänzle. Dr. Michael Gänzle and Dr. Lynn McMullen contributed to the hypothesis development, experimental design and manuscript revision.

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Chapter 1 **Introduction and Objectives**

Chitosan, a linear polysaccharide consisting of β-(1→4)-linked glucosamine and N-acetyl-D-glucosamine, has been considered as a wholesome food constituent (EFSA, 2011) and exerts antimicrobial activity against a wide range of microorganisms (Devlieghere et al., 2004; Kanatt et al., 2013; Younes et al., 2014). Chitosan is produced from by-products of crustaceans, insects and fungi (Ma et al., 2017; Muxika et al., 2017). Treatment of the shells of crustaceans with HCl and dilute NaOH yields purified chitin. Further treatment with 12.5 mol/L NaOH at more than 80 °C deacetylates chitin into chitosan (Arbia et al., 2013; Kumari et al., 2015; Puvvada et al., 2012). Chitosan has antimicrobial activity only if it is in the polycationic form at pH values below its pKa of 6.2-7.0 (Tsai and Su, 1999). Chitosan with higher degree of deacetylation (DD) and molecular weight (MW) has a higher positive charge density and thus exhibits an enhanced antimicrobial activity (Chien et al., 2016; Chung et al., 2004; Mellegård et al., 2011; Younes et al., 2014). Food components, including NaCl and proteins, adversely affect chitosan activity, presumably through shielding positive charges of chitosan (Devlieghere et al., 2004). Accordingly, chitosan is bacteriostatic or bactericidal at 0.010~1 g/L in vitro (Mellegård et al., 2011; Younes et al., 2014), while the minimum concentration of chitosan for observation of antimicrobial activity in milk, meat and seafood, reaches up to 5 to 20 g/kg (w/w) (Fernandes et al., 2008; Kanatt et al., 2013; Vardaka et al., 2016). Even though chitosan has been extensively studied for extending the shelf life of different meat products, few studies assessed chitosan lethality against pathogens. Surface application of chitosan solution at 1-2% (w/w) inactivated *E. coli* and *Salmonella* by 1-2 log (CFU/g) on raw meat (Kanatt et al., 2013; Vardaka et al., 2016), and chitosan-based film with the chitosan level of 0.389 mg/cm^2 exhibited listericidal effect by 0.8 log (CFU/g) on ready-to-eat turkey

meat (Guo et al., 2014). Therefore, a critical appraisal of the use of chitosan for improving meat safety requires additional studies validating the antimicrobial activity of chitosan against pathogens on meat.

Listeria monocytogenes, *Salmonella enterica* and virulent strains of *Escherichia coli*, especially Shiga-toxin producing *E. coli* (STEC) are important foodborne pathogens associated with outbreaks worldwide (EFSA, 2016). Cattle are an important reservoir of STEC (Nguyen and Sperandio, 2012) and consumption of beef contributes to foodborne STEC infections (Greig and Ravel, 2009; WHO, 2018). STEC contamination of muscle tissues originates from fecal contamination of hides (Low et al., 2005) and primarily occurs during carcass dressing and breaking (Aslam et al., 2004; Gill, 2009). *Salmonella* colonizes the gastrointestinal tract or lymphoid tissues of poultry, swine and cattle, and is easily transferred to meat during slaughtering and processing (Antunes et al., 2016). Accordingly, poultry, pork and beef products, especially ground meat, constitute the main vehicles of *Salmonella* infection (Chaves et al., 2017; EFSA, 2016; Webb et al., 2017). *Listeria monocytogenes* is the most significant pathogen associated with ready-to-eat (RTE) meat due to its persistence in food processing environment and ability to grow at refrigerated temperature (Gómez et al., 2014; Walker et al., 1990). Despite current intervention technologies, these pathogens continue to cause outbreaks associated with meat (CDC, 2018). Moreover, a food isolate with exceptional heat resistance, *E. coli* AW1.7, resists the thermal interventions recommended for eliminating foodborne pathogens (Dlusskaya et al., 2011; Mercer et al., 2017). The extreme heat resistance of *E. coli* is mediated by the locus of heat resistance (LHR), a 14 kb genomic island that encodes several putative stress proteins and is flanked by transposable elements (Boll et al., 2017; Lee et al., 2015; Lee et

al., 2016; Mercer et al., 2015). Acquisition of LHR also increases the heat resistance in *Salmonella enterica* and *Enterobacter cloacae* (Mercer et al., 2017). Diverse species possess LHR by horizontal gene transfer (Mercer et al., 2015; Boll et al., 2017), and two Shiga-toxin producing *E. coli* (STEC) isolates have already been identified as LHR positive (Ma and Chui, 2017). Remarkably, chlorine, pressure and certain essential oils, including carvacrol, thymol, thiol-reactive allyl-isothiocyanate (AITC) and cinnamaldehyde are also not effective in inactivation of LHR positive *E. coli* strains (Li and Gänzle, 2016; Zhi et al., 2016). All these highlight that LHR positive strains present an additional risk to food safety.

Taken together, the continuing burden of foodborne disease and the additional risk posed by LHR positive strains necessitate improved antimicrobial hurdles to ensure the meat safety. Chitosan induces a compromised integrity of the cell envelope and thus has the potential to sensitize the bacteria to commercial intervention technologies, such as heat and high hydrostatic pressure (Malinowska-Pańczyk et al., 2009; Surendran Nair et al., 2016). Bacteriocins produced by lactic acid bacteria (LAB) or bacteriocin-producing LAB cultures exhibit antimicrobial activity in foods and their applications also meet the consumers' demand for "natural preservatives" (Barbosa et al., 2017; Drider et al., 2006; Perez et al., 2014). However, the single or combined activity of chitosan and other antimicrobial hurdles, including heat, pressure and bacteriocins, against foodborne pathogens and LHR positive strains on meat still need to be validated.

Therefore, the objectives of this thesis were to test the following hypothesis:

Chitosan has only a limited preservative effect in meat and meat products when used alone but increases the efficacy of current pathogen intervention technologies used in meat production.

Objectives:

1) To critically review current studies on the use of chitosan as a food preservative (Chapter 2).

2) To investigate the combined use of chitosan and bacteriocins for control of *E. coli* and *Salmonella* on fresh meat (Chapter 3).

3) To determine the effect of chitosan on survival of heat-resistant and Shiga-toxin producing *E. coli* during cooking of burgers with or without NaCl (Chapter 4).

4) To determine the role of chitosan on the bactericidal effect of high pressure on pressure-resistant strains of *E. coli* and *Salmonella* (Chapter 5).

5) To assess the efficacy of chitosan alone or in combination with high pressure for control of *Listeria* on ready-to-eat ham (Chapter 6).

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Chapter 2 Literature Review

2.1 Introduction

Food safety and quality are fundamental concerns for consumers and the food industry. Current intervention and preservation technologies, however, do not prevent outbreaks of foodborne bacterial disease, or food spoilage and food waste (Hussain, 2013). Moreover, the negative public perception of commercial preservatives prompts an increasing preference of consumers for replacement of chemical preservatives by "natural" alternatives that are derived from biological systems (Amit et al., 2017; Román et al., 2017). To meet the consumers' demand for "natural preservatives", essential oils extracted from plants (Pandey et al., 2017; Sanchez-Maldonado et al., 2015), bacteriocins from lactic acid bacteria (LAB) such as nisin or pediocin PAß1-AcH and bacteriocin-producing protective cultures such as *Carnobacterium maltaromaticum* UAL307 (Micocin ® (Barbosa et al., 2017; Liu, 2014) are used commercially as food preservatives. Further improvement of food safety and quality, however, necessitate the development of other antimicrobials from natural resources.

Chitosan is a linear polysaccharide consisting of β-(1→4)-linked glucosamine and N-acetyl-D-glucosamine that has been proposed for use as food preservative. Chitosan is prepared by deacetylation of chitin, which is present in the exoskeleton of crustaceans and insects and in the cell walls of most fungi and some algae (Ma et al., 2017; Muxika et al., 2017). When the proportion of glucosamine exceeds the proportion of N-acetyl glucosamine, corresponding to a degree of deacetylation (DD) of more than 50%, the polymer is termed chitosan (Khor and Lim, 2003; Ramírez et al., 2010). Owing to its positive charge and unique functional groups, including the amino/acetamido groups at the C-2 position, and hydroxyl groups at the C-3 and C-6 positions, chitosan is a versatile

biopolymer with applications in the biomedical field, in wastewater treatment, agriculture, food protection, cosmetics, papermaking, and the textile industry (Ma et al., 2017; Muxika et al., 2017). While several reviews indicate the potential applications of chitosan as food preservative, challenge studies in food often report only a limited effect of chitosan on pathogens or spoilage organisms. This review aims to provide a critical appraisal of the challenges to food applications of chitosan that are imposed by the molecular structure of chitosan and its interactions with the food matrix, but also outline opportunities of the use of chitosan as food preservative.

2.2 Preparation of chitosan

Chitosan is prepared by purification, and deacetylation of chitin. Further enzymatic or chemical depolymerisation of chitosan yields water soluble chitosan-oligosaccharides (COS). To purify chitin from the shells of crustaceans, the shells are ground (Abdou et al., 2008), processed with HCl to achieve demineralisation, and boiled in dilute NaOH to remove proteins (Arbia et al., 2013; Kumari et al., 2015; Puvvada et al., 2012). Deacetylation of chitin is achieved through alkaline treatment at more than 80 °C. The degree of deacetylation (DD) is dependent on the reaction conditions (Teng, 2011; Yuan et al., 2011). Treatment with 12.5 mol/L NaOH at 95–100 °C deacetylates chitin within 2 h, yielding chitosan with DD of 87-90% and average MW of 160 -1600 kDa (Puvvada et al., 2012).

Generally, chitosan is acid soluble and has antimicrobial activity only when the ambient pH is lower than its pKa, which ranges from 6.2 to 7.0 (Devlieghere et al., 2004; Helander et al., 2001; Tsai and Su, 1999). For food applications, chitosan is either dissolved in acetic acid to a concentration of 1 – 2%, or applied as chitosan-based packaging film (Jovanović

et al., 2016; Muxika et al., 2017; Zhao et al., 2018). Chitosan has also been converted to chitosan nanoparticles or microparticles (CN/CM) through ionic crosslinking with polyanionic sodium triphosphate (TPP) (Chávez et al., 2011; Zhao et al., 2011). CN/CM were reported to be effective food preservatives (Chouljenko et al., 2017; Fang et al., 2015; Paomephan et al., 2018; Pilon et al., 2015), however, there is no evidence that CN/CM have superior antimicrobial activity when compared to chitosan solutions. Chitosan can also be depolymerized by chitosanases and chitinases (Aam et al., 2010). COS have higher solubility and lower antimicrobial activity when compared to high molecular weight chitosan (Fernandes et al., 2008; Mellegård et al., 2011).

2.3 Mode of action and factors affecting the antimicrobial activity of chitosan

Chitosan, exhibits bacteriostatic or bactericidal effects against a wide range of microorganism (Devlieghere et al., 2004). The mode of action of chitosan relates to alterations of the cell envelope and a compromised integrity of the cytoplasmic membrane. The mode of action of chitosan against Gram negative and Gram positive bacteria is depicted in Figure 1 and described in more detail below.

Polycationic chitosan disrupts the integrity of the Gram-negative outer membrane (**Figure 2-1A**). Outer membrane damage caused by chitosan was demonstrated through use of the fluorescent dye N-phenyl-1-naphthylamine (NPN), which is solubilized in membrane of Gram-negative bacteria only when the outer membrane is damaged (Loh et al., 1984; Träuble and Overath, 1973). Chitosan at the concentration of 0.01 to 5 g/L increased in NPN fluorescence in *E. coli*, indicating permeabilization of the outer membrane (Liu et al., 2004; Mellegård et al., 2011). Similar chitosan-induced permeabilization of the outer membrane was also observed in *Salmonella* (Helander et al., 2001).

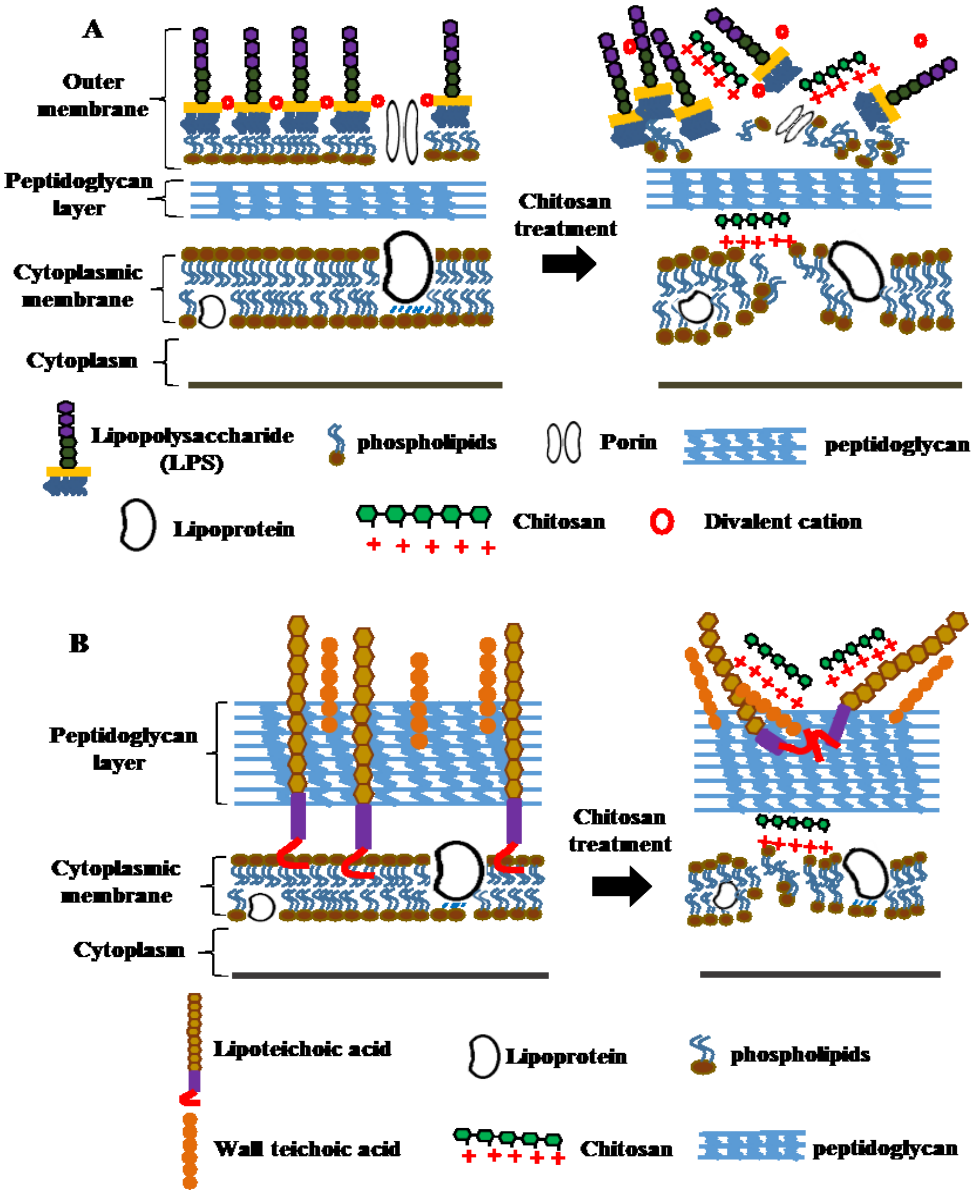

Figure 2-1 Mode of action of chitosan against Gram negative bacteria (Panel A) and Gram positive bacteria (Panel B): When the ambient pH is lower than pKa of chitosan, chitosan is polycationic chitosan molecules, which enables electrostatic interactions with negatively charged structures of the cell envelope, including the lipopolysaccharide (LPS) in the outer membrane of Gram negative bacteria (A), lipoteichoic acid and wall teichoic acids of Gram positive bacteria (B), and the cytoplasmic membrane. These electrostatic interactions can disrupt the integrity of cell envelope, subsequently cause dissipation of membrane potential, leakage of cells, leading to cell death (Helander et al. 2001; Liu et al. 2004; Mellegård et al. 2011; Raafat et al. 2008).

Chitosan also permeabilizes cytoplasmic membrane (**Figure 2-1A** and **B**). Quantification of the transmembrane potential with the lipophilic dye [3H] tetraphenylphosphonium

bromide ([3H]TPP^+) demonstrated that addition of 10 mg/L chitosan to suspensions of *Staphylococcus simulans* reduced the membrane potential from 110 mV to 30 mV, indicating dissipation of membrane potential and perturbation of membrane integrity (Raafat et al., 2008). In addition, chitosan also initiated a progressive efflux of K+ and UV-absorbing cellular components in *S. simulans*, *S. aureus*, *E. coli* and *Bacillus cereus*, further supporting an increased permeability of cytoplasmic membrane (Helander et al., 2001; Liu et al., 2004; Mellegård et al., 2011; Raafat et al., 2008).

A *pmrA* negative mutant of *Salmonella* Typhimurium with a more positively charged lipopolysaccharide (LPS) was more resistant to chitosan than its parent strain (Helander et al., 2001), and *S. aureus* mutants lacking teichoic acids (TA) or lipoteichoic acid (LTA) were also more resistant to chitosan than wild type *S. aureus* (Raafat et al., 2008). These finding suggest that the electrostatic interactions between positively charged chitosan and negatively charged LPS (**Figure 2-1A**), TA or LTA (**Figure 2-1B**) contribute considerably to the chitosan-mediated cell death and injury.

The degree of acetylation and the molecular weight impact antimicrobial activity of chitosan through altering the charge density of chitosan. Chitosan with higher degree of deacetylation has a higher positive charge density, allowing for a stronger electrostatic interaction with negative charged cell surface and leading to an enhanced antimicrobial activity (Chien et al., 2016; Chung et al., 2004; Mellegård et al., 2011; Younes et al., 2014). The minimum molecular weight of chitosan with DD of 84% for observation of antimicrobial activity was 2.3 kDa and the activity increased with increasing molecular weight. With chitosan of a DD of 52%, antimicrobial activity was observed only at a molecular weight of 11.9 kDa and higher (Mellegård et al., 2011). The higher antimicrobial

activity of chitosan with higher DD and molecular weight may be attributed to the higher positive charge density and the more intensive interaction with cell envelope. In food application, COS with MW of <5 kDa has no antibacterial activity while chitosan with MW of >80 kDa at a concentration of 0.5 % (w/v) was bactericidal in milk and bacteriostatic in cheese. Compared with chitosan, the higher reactivity and stronger interaction of COS with food components, such as protein and lipid, account for the loss of COS in food systems (Ausar et al., 2002; Fernandes et al., 2008).

The ambient conditions, including pH, temperature, divalent metal ions also affect antimicrobial activity of chitosan. A low pH favors protonation of chitosan and thus increases its antimicrobial activity (Devlieghere et al., 2004; Helander et al., 2001; Tsai and Su, 1999). Divalent metal ions, including Zn^{2+}, Ba^{2+}, Ca^{2+}, Mg^{2+}, at a concentration of 25 mmol/L in medium weaken the inhibitory activity of chitosan, probably through shielding of negative charges on the cell envelope (Chung et al., 2003; Tsai and Su, 1999). The ingredients present in different food products, including NaCl and proteins, may also decrease chitosan activity by shielding positive charges of chitosan (Devlieghere et al., 2004).

Antimicrobial activity of chitosan is also dependent on the target microorganisms. Since media composition highly influences the *in vitro* activity of chitosan, it is not possible to conclude on differences in resistance between microorganisms unless the target strains were assessed in the same medium. Few studies indicated certain Gram-negative bacteria, including *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Salmonella* Typhi, were more susceptible to chitosan than certain Gram-positive bacteria, including *S. aureus*, *B. cereus*, *Enterococcus faecalis* and *Micrococcus luteus* (Younes et al., 2014). Similarly,

chitosan also exhibited a higher activity against *E. coli* when compared to *B. cereus* (Mellegård et al., 2011). When cells were suspended in buffer containing 0.5% chitosan at pH 5.4, the decrease of cell counts of *E. coli* induced by chitosan was more than 3 log(CFU/mL) higher when compared to *S. aureus* (Liu et al., 2004). The reasons for these species-specific differences in resistance to chitosan are still unclear. The loss of teichoic acids (TA) and modification of LPS altered the susceptibility to chitosan in *S. aureus* and *S.* Typhimurium, respectively (Helander et al., 2001; Mellegård et al., 2011; Raafat et al., 2008). These studies highlight that the difference in charge distribution on the cell surface may account for the species- and strain specific differences in resistance to chitosan.

2.4 Challenge studies with pathogens to evaluate the use of chitosan as food preservative

A summary of challenge studies with chitosan, chitosan nanoparticles or chitosan-based films in food is provided in Table 1. In most cases, the lethality of chitosan is limited to a 2.5 log (CFU/g) decrease of cell counts irrespective of the food matrix and the form of application (**Table 2-1**). A reduction of more than 5 log (CFU/g) of *Listeria monocytogenes* was observed on apples and grapes coated with 2% w/v chitosan solution (Anacarso et al., 2011). This high antilisterial activity may be attributed to the smooth surface of apples and grapes, resulting in a high local concentration of chitosan and an intense interaction of bacterial cells with chitosan. Other studies observed bacteriostatic rather than bactericidal effects of chitosan in artificially contaminated food. Coating eggs with 2% chitosan solution was not lethal to *Salmonella* Enteritidis when chitosan solution was applied on egg shells and dried prior to the inoculation of bacterial cells, but offered a protective barrier reducing the penetration of *Salmonella* (Leleu et al., 2011). Similarly, chitosan films

were not bactericidal but delayed the growth of *Listeria monocytogenes* on slices of ready-to-eat sausages (Moradi et al., 2011). Incorporation of chitosan powder into bread at 0.6% w/w inhibited the growth of *B. cereus* and rope formation during storage at 30 °C for 3 days (Lafarga et al., 2013). Taken together, the disparity in lethality of chitosan shown among different reports may be attributed to the variation in chitosan property, food matrix and approaches of chitosan application.

Surface application of chitosan is the most frequent form of application (**Table 2-1**); only few studies directly compared the efficacy of chitosan solutions to nanoparticles or packing films. Chitosan solution exhibited stronger bactericidal activity against *L. monocytogenes* on black radish when compared to a chitosan packaging film (Jovanović et al., 2016). After coating of chitosan solution, samples are often drained or dried (Jovanović et al., 2016; Kanatt et al., 2013). With water evaporation, chitosan becomes more concentrated than the original chitosan solution, resulting in a higher local concentration of chitosan on the sample surface and a more intensive interaction with target cells.

Table 2-1 Bactericidal effect of different forms of chitosan on artificially contaminated foods

Chitosan preparation and application		Lethality ($logN_0/N$)	Product (reference)
Meat products			
Surface application	0.5% w/v**;** 350 kDa;	0.5 (*S.* Typhimurium)	Chicken skin[1]
	2% w/v	2 (*S. aureus*) 2.5 for *B. cereus*; 1 for *E. coli*; 0.5 for *P. fluorescens*	Chicken or mutton seekh kabab[2]
	2% w/v; 340 kDa	2 (*E. coli* O157:H7); 1 (*Salmonella*)	Fresh turkey meat[3]
Packaging film	0.389 mg chitosan/cm^2; 150 kDa	0.8 (*Listeria innocua*)	Ready-to-eat turkey meat[4]
	150 mg chitosan/g starch; 190–310 kDa	1 (spoilage bacteria cocktail of *Brochothrix thermosphacta*, *Carnobacterium maltaromaticum*, *Leuconostoc gelidum* and *Lactobacillus sakei*)	Ham[5]
Seafood			
Microoparticles (CM)	Surface application of 0.5% w/v CMs solution from chitosan with 50-190 kDa	1.9-3.9 (*V. vulnificus*); 1.9-2.6 (*V. parahaemolyticus*)	Live oysters[6]
Vegetables and fruits			
Nanoparticles (CN)	Washing samples with 800 mg/L CNs solution, which was produced from chitosan with 30 or 2100 kDa	1 (*E. coli*) 1 (*S.* Typhimurium)	Fresh vegetables[7]
Solution	2% w/v; 150 kDa	1.5 on zucchini, corn and radishes; 2 on mixed salad, carrots and zucchini; > 5 on apples and grapes (*L. monocytogenes*)	Zucchini, corn and radishes; mixed salad, carrots and zucchini; apples and grapes[8]
	2% w/v; 150 kDa;	1 (*Salmonella*)	Cantaloupe[9]
	1% w/v; 1600 kDa	0.5 (*L. monocytogenes*)	Broccoli florets[10]
Solution coating or packaging film	Solution: 1% w/v; Film: 0.5% w/w; 190-310 kDa.	2.5 (*L. monocytogenes*) with solution; 1.0 (*L. monocytogenes*) with packaging film	Black radish[11]

Lethality: Reduction of log (CFU/g) or log (CFU/mL); MW: Molecular weight; the degree of deacetylation was > 75% for all studies included in this table.

[1] Menconi et al. 2013 [2] Kanatt et al. 2013 [3] Vardaka et al. 2016 [4] Guo et al. 2014 [5] Zhao et al. 2018 [6] Fang et al. 2015 [7] Paomephan et al. 2018 [8] Anacarso et al. 2011 [9] Chen et al. 2012 [10] Severino et al. 2014 [11] Jovanović et al. 2016

2.5 Application of chitosan as food preservatives to control spoilage organisms

Studies that monitored the development of the non-pathogenic microbiota of food, including aerobic mesophilic bacteria, psychrotrophic bacteria, lactic acid bacteria, *Brochothrix*, *Pseudomonas* spp, Enterobacteriaceae, or yeast and molds are summarized in Table 2. In these cases, un-inoculated food samples were treated with chitosan solution, chitosan nanoparticles, or with chitosan-based films, followed by refrigerated storage and microbiological analysis during storage. Bacteriostatic effect of chitosan ranged from 1 to 6 log (CFU/g), depending on dosage and intrinsic characteristics of chitosan food matrix and storage condition (**Table 2-2**). In addition to the enumeration of microbial populations, the observation of microbial spoilage of vegetables and fruits allows assessment of the effectiveness of chitosan. Coating treatment with 1% (w/v) chitosan solution reduced the decay of sweet pepper by 20% after storage at 8 °C (Xing et al., 2011). Pre-harvest spray with 0.1% (w/v) chitosan solution or post-harvest coating with 1% (w/v) chitosan solution significantly reduced the decay index of chitosan-treated grape fruits after storage for 16 d at 20 °C or 42 d at 0 °C (Meng et al., 2008). To investigate the mechanisms of chitosan-mediated reduction of spoilage of fruits and vegetables, artificially wounded fruits were first coated with chitosan solution then inoculated with indicator fungal strains (Chien et al., 2007), or artificially wounded samples, inoculated, and then coated with chitosan (Shao et al., 2015). Independent of the sequence of inoculation with fungi and chitosan application, chitosan treated samples reduced the incidence of decay when compared to controls (Chien et al., 2007; Shao et al., 2015). Chitosan also inhibited spore germination, germ tube elongation and mycelial growth of many phytopathogens (Ben-Shalom et al.,

2003; Liu et al., 2007). The antifungal activity of chitosan in combination with the mechanical barrier provided by a chitosan coating probably contribute to the decreased decay the incidence of decay by inhibiting growth of indigenous microorganisms and protecting samples from exogenous contamination.

Table 2-2 Effect of chitosan on the microbial quality of food

Chitosan preparation and application		Effect of chitosan	Products (reference)
Meat products			
Surface application	0.5% w/v; 350 kDa	Psychrotrophic spoilage bacteria in samples treated with chitosan remained below detectable levels during storage at 4 °C.	Chicken skin[1]
	1.0% w/v	Cell counts of mesophilic and psychrotrophic bacteria, lactic acid bacteria, and yeast and mold were lower than controls after storage at 4 °C for 60 d by 3 – 6 log (cfu/g).	Sausage[2]
	1.5% w/v; 340 kDa	Total plate counts and cell counts of spoilage organisms including *Pseudomonas* spp., Lactic Acid Bacteria, *Brochothrix thermosphacta*, coliforms and yeasts-moulds, were lower than controls by 1-2 log (cfu/g) after storage at 4 °C for 12 days, extending the microbial shelf-life by more than 9 days.	Chicken breast meat[3] Turkey meat[4] Ready to cook chicken product [5]
	1 % w/v; 800 kDa	Cell counts of pseudomonads, lactic acid bacteria, and coliforms were lower than controls after 6 d of storage at 4 °C by 3.9-4.9 log (cfu/g).	Chicken breast fillets[6]
	2% w/v; 897 kDa	Total viable count and cell counts of psychrotrophic bacteria were lower than controls by 1 log after storage at 4 °C for 25 days.	Cooked pork sausages[7]
Integration of chitosan to product formula	Chitosan (1674 kDa) at 2 mg g^{-1} in minced pork	Total bacterial count and psychrotrophic counts were lower than controls by 1 log (cfu/g) after storage of minced pork at 5 °C for 8 days	Minced Pork[8]
	Chitosan (490 kDa) at 1% w/w in pork sausage.	Total viable counts, and cell counts of Lactic acid bacteria, *Pseudomonas* spp., *Brochothrix thermosphacta*, *Enterobacteriaceae*, yeasts and moulds were lower than controls by 0.5-1 log	Fresh pork sausages[9]

		(cfu/g) after storage at 4 °C for 28 days.	
Packaging film	Prepared from 2% w/v chitosan (100 kDa)	Total viable cell counts, cell counts of lactic acid bacteria, and yeasts and molds were lower than controls by 1.5-5 log (cfu/g) after storage at 4 °C for 20 days.	Cooked pork sausages[10]
	Prepared from 2% w/v chitosan	Total viable cell counts were lower than controls by 1 log (cfu/g) after storage at 4 °C for 12 days.	Pork meat patties[11]
		Seafood	
Surface application	1% w/v; 320 kDa	Inhibition of H_2S-producing organisms during storage at 4 °C.	Shrimp[12]
	1% w/v; 25 kDa	Total aerobic plate counts were lower than controls by 2 log (cfu/g) after 10 days of iced storage.	Pacific white shrimp[13]
	2% w/v; 450 kDa	Total viable counts and psychrotrophic counts were lower than controls by 1-3 log (cfu/g) after storage at 4 °C for 16 days.	Rainbow trout[14]
	3% w/v;	Total viable cells and cell counts of psychrotrophic bacteria were lower than controls by 1 log (cfu/g) after storage at 4 °C for 12 days.	Ready-to-eat peeled Shrimps[15]
	3% w/v; 149 kDa	Total plate counts were lower than controls by 4 log (cfu/g) after vacuum or modified atmosphere packaging storage at 2 °C for 14 days.	Lingcod (*Ophiodon elongates*) fillets[16]
	1.0% w/v; 1800, 960 or 660 kDa	Total viable counts were lower than controls by 2 log (cfu/g) after storage for 12 days at 4 ± 1 °C.	Herring and Atlantic cod[17]
Incorporation	Chitosan (10 kDa) insurimi at 2% w/w.	Aerobic plate counts were lower than controls by 1 log (cfu/g) after storage at 4 °C for 12 days.	Surimi gel made from African catfish (Clarias gariepinus)[18]
Coating with solution or nanoparticles	Solution: 1% w/v; 300 kDa; DD 65 %; Nanoparticles: 1% w/v; DD 20%	Cell counts of Aerobic bacteria were lower than controls by more than 1 log (cfu/g) after storage at 4 °C for 24 days. Conventional solution was more bacteriostatic than nanoparticles solution.	Shrimp Muscle[19]
		Vegetables, fruits and juice	
Surface application	1.5%w/v;	Total viable counts and cell counts of yeast and mold were lower than controls by 0.5-1 log (cfu/g) after storage at 4 °C for 7 days.	Pears[20]

	1% w/v; 190 to 310 kDa;	Cell counts of mesophilic aerobic bacteria, yeast and molds were lower than controls by 1 log (cfu/g) after storage at 10 °C for 7 days.	Fresh Blueberries[21]
	1.0% w/v	Lower decay incidence by 20% after at 8 °C for 35 days.	Sweet pepper (*Capsicum annuum L.*)[22]
	Pre-harvest spray with 0.1% w/v or coating with 1% w/v solution	Lower decay index after storage for 16 days at 20 °C or 42 days at 0 °C.	Grape fruit[23]
Incorporation	Solution (0.4% w/v; 1674 kDa) in apple juice at 2 g/L.	Total bacterial counts, cell counts of psychrotrophic bacteria, yeast and mould were lower than controls by 0.5-3.0 log (cfu g^{-1}) after storage at 5 °C for 15 days.	Apple juice[24]
Coating with solution or nanoparticles	0.2% w/v; 71 kDa	Cell counts of mesophilic and psychrotrophic bacteria were lower than controls by 3 log (cfu g^{-1}) after storage at 5 °C for 10 days. Solution and nanoparticles exhibited comparable bacteriostatic effect.	Fresh-cut apples[25]
		Bakery products	
Incorporation	Chitin(124±10 kDa; DD 19%) in bread at 1%.	Delay of mold growth in bread during storage of 3 days at 30 °C.	Bread[26]
Packaging film:	Prepared form 1.5% w/v chitosan	Delay of time to visible mould growth by 3 days and cell counts of mould were lower than controls by 2 log (cfu/g) after storage for 8 days at room temperature (about 25 °C).	Butter cake [27]
		Eggs	
Surface application	1 % w/v	Total aerobic cell counts chitosan-coated eggs were under detection limit while those of non-coated eggs increased to 20 cfu/ml after 5-weeks of storage at 22±1 or 32±1 °C.	Eggs[28]

The degree of deacetylation of chitosan was higher than 75% unless otherwise noted.
[1] Menconi et al. 2013 [2] Bostan and Mahan 2011 [3] Petrou et al. 2012 [4] Vasilatos and Savvaidis 2013 [5] Giatrakou et al. 2010 [6] Latou et al. 2014 [7] Lekjing 2016 [8] Malinowska-Pańczyk et al. 2009 [9] Soultos et al. 2008 [10] Siripatrawan and Noipha. 2012 [11] Qin et al. 2013 [12] Arancibia et al. 2015 [13] Yuan et al. 2016 [14] Ojagh et al. 2010 [15] Carrión-Granda et al. 2016 [16] Duan et al. 2010 [17] Jeon et al. 2002 [18] Amiza and Kang 2013 [19] Chouljenko et al. 2017 [20] Cé et al. 2012 [21] Sun et al. 2014 [22] Xing et al. 2011 [23] Meng et al. 2008 [24] Malinowska-Pańczyk et al. 2009 [25] Pilon et al. 2015 [26] Lafarga et al. 2013 [27] Sangsuwan et al. 2015 [28] Suresh et al. 2015

2.6 Use of chitosan to enhance the efficacy of other antimicrobial hurdles.

Chitosan potentiates the efficacy of commercial intervention technologies, such as heat and high hydrostatic pressure. Chitosan is generally applied as dilute solution in acetic acid. Those studies that used a solvent control demonstrated, however, that the carry-over of acetic acid or acetate, 1 – 20 mg/kg, does not impact the antimicrobial activity of chitosan (**Table 2-1** and **2-2**). Addition of chitosan to a concentration of 0.01%w/w enhanced the thermal inactivation of *E. coli* O157:H7 (EHEC) in ground beef by 1.5 log (CFU/g) (Surendran Nair et al., 2016). Chitosan at a concentration of 0.1% (w/v) acted synergistically with pressure treatment of apple juice to inactivate *E. coli* (Kumar et al., 2009). The combined application of chitosan and pressure demonstrated synergistic effects in elimination of *S. aureus* and *E. coli* in buffer, and in controlling bacterial growth in apple juice and minced pork during refrigerated storage (Malinowska-Pańczyk et al., 2009).

2.7 Application of chitosan to improve quality of food products.

Chitosan also exerts other beneficial effects on food quality that are independent of its antimicrobial activity and include retardation of lipid oxidation, retention of color, freshness, taste, odor and nutrients. The effects on food quality are dependent on the food matrix and are summarized in **Table 2-3**.

Table 2-3 Effect of chitosan on food quality

Chitosan preparation and application		Effect of chitosan	Products (reference)
		Meat products	
Surface application	1.0% w/v	Brighter and more attractive color.	Sausage[1]
	1.5% w/v; 340 kDa	Improvement in taste and odor.	Chicken breast meat[2] Turkey meat [3] Chicken product[4]
	1 % w/v; 800 kDa	Retardation of decline in odor and taste scores.	Chicken breast fillets[5]
	2% w/v; MW: 897 kDa	Retardation of lipid oxidation and change in color.	Cooked pork sausages[6]
Packaging film:	Prepared from 2% w/v chitosan (100 kDa) solution.	Retardation of lipid oxidation, changes in color, texture, and odor.	Cooked pork sausages[7]
	Prepared from 2% w/v chitosan solution	Retardation of lipid oxidation, increase in MetMb content, and decrease in color and odor scores.	Pork meat patties[8]
Incorporation	Chitosan **(**490 kDa) in sausages at 1% w/w	Retardation of lipid oxidation	Fresh pork sausages[9]
		Seafood	
Surface application	1% w/v; 25 kDa	Retardation of increase in melanosis and improvement in the texture parameters.	Pacific white shrimp[10]
	2% w/v; 450 kDa	Retardation of increase in peroxide value and total volatile base nitrogen.	Rainbow trout[11]
	2% w/v;	Retardation of lipid oxidation and improvement inodor, texture, color and taste.	Fresh *Channa Argus*[12]
	3% w/v; 149 kDa	Retardation of lipid oxidation under vacuum or modified atmosphere packaging.	Lingcod (Ophiodon elongates) fillets[13]
	1.5% w/w	Retardation of increase in melanosis and loss in freshness.	Whiteleg shrimp (*Litopenaeus vannamei*) [14]
	1.0% w/v of chitosan with 1800, 960, or 660 kDa	Retardation of lipid oxidation.	Herring and Atlantic cod[15]

Chitosan preparation and application		Effect of chitosan	Products (reference)
Incorporation	Chitosan (10 kDa) in surimi at 2% w/w.	Retardation of lipid oxidation, extension of shelf life by 4 days.	Surimi gel made from African catfish (Clarias gariepinus)[16]
		Vegetables and fruits	
Surface application	1.0% w/v	Reduction of cell injury in plant tissue, retention of vitamin C content, and enhancement of self-defence system.	Sweet pepper (*Capsicum annuum* L.)[17]
	1% w/v	Retardation of loss in weight	Grape fruits[18]
	0.5, 1.0 or 2.0% w/v; 50–190 kDa	Retardation of loss in firmness, weight, chlorophyll and vitamin C, as well as reduction of cell injury in plant tissue and enhancement of self-defence system.	Guava (*Psidium guajava* L.)[19]
	1.0%, 1.5% or 2.0% (w/v)	Retardation of loss in weight, firmness and changes in the peel colour.	Papaya[20]
		Sauce	
Incorporation	Chitosan (310 or 123 kDa) in mayonnaise at 100 mg/kg.	Improvement in odor and taste attributes, and retardation of lipid oxidation.	Mayonnaise[21]
		Eggs	
Surface application	1 % w/v	Retardation of loss in weight, increase in air space, and decline in Haugh Unit value, yolk index, shell strength and quality grade.	Eggs[22]
	3% w/v	Retardation of loss in weight, decline in Haugh unit and yolk index.	Eggs[23]
	1% w/v; 1110 kDa.	Retardation of loss in weight and decline in Haugh unit.	Eggs[24]

The degree of deacetylation of chitosan was higher than 75% unless otherwise noted.
[1] Bostan and Mahan 2011 [2] Petrou et al. 2012 [3] Vasilatos and Savvaidis 2013 [4] Giatrakou et al. 2010 [5] Latou et al. 2014 [6] Lekjing 2016 [7] Siripatrawan and Noipha 2012 [8] Qin et al. 2013 [9] Soultos et al. 2008 [10] Yuan et al. 2016 [11] Ojagh et al. 2010 [12] Yang et al. 2015 [13] Duan et al. 2010 [14] Huang et al. 2012 [15] Jeon et al. 2002 [16] Amiza and Kang 2013 [17] Xing et al. 2011 [18] Meng et al, 2008 [19] Hong et al. 2012 [20] Ali et al. 2011 [21] García et al. 2014 [22] Suresh et al. 2015 [23] Caner and Cansiz 2007 [24] Wardy et al. 2014

Meat and seafoods. Application of chitosan significantly reduced the rate of lipid oxidation, which is usually indicated by thiobarbituric acid reactive substances and peroxide value of meat and seafood (**Table 2-3**). The ability of chitosan to control lipid

oxidation relate to the scavenging of reactive radicals (Kim and Thomas, 2007; Wan et al., 2013), the formation of stable complex with volatile aldehydes derived from decomposition of lipid (Shahidi et al., 1999), and the provision of a barrier to oxygen diffusion (Sathivel et al., 2007).

The color of specific foods strongly affects purchasing decisions of consumers (Gao et al., 2013). Chitosan treatments in different forms retarded the color alteration in sausage, pork meat patties, and pacific white shrimp (**Table 2-3**). Metmyoglobin (MetMb) is the major factor causing the browning of fresh meat (Bekhit et al., 2007). The color retention caused by chitosan was achieved through decreasing MetMb concentration, and may also relate to the anti-oxidative activity of chitosan (Qin et al., 2013).

Melanosis is a type of spoilage specific for crustaceans. During post-mortem storage of crustaceans, microbial compounds, including peptidoglycan binging protein (PGBP) produced by Gram positive bacteria, lipopolysacharide and β-(1→3)-glucan binding protein (LGBP) produced by Gram negative bacteria, and β-(1→3)-glucan binding protein (BGBP) produced by fungi, accumulate and activate polyphenoloxidase (PPO). PPO oxidizes monophenols, particularly tyrosine, into quinones, followed by non-enzymatic polymerization of quinones to form dark pigments called melanin. The accumulation of melanin incurs the formation of black spots on carapace, namely, melanosis, thus substantially decreasing the commercial value of crustacean products (Amparyup et al., 2013; Garcia-Molina et al., 2005; Gonçalves et al., 2016). Coating shrimps with 1-1.5% chitosan solution significantly retarded melanosis in shrimps (Huang et al., 2012; Yuan et al., 2016), and the protective effect against melanosis likely relates to its anti-oxidative activity and antimicrobial activity (Huang et al., 2012).

The texture profile is a widely used freshness indicator for seafood products (Cheng et al., 2014). Myofibrillar and connective tissue proteins are the major elements maintaining the textural properties of shrimps and fish. Microbial and endogenous proteases lead to softening of the texture during storage (Hultmann and Rustad, 2004; Yuan et al., 2016). In some cases, surface application of chitosan solution retarded the softening during storage of fish, presumably through inhibition of microbial spoilage or interactions with myofibrillar proteins to form the compact structure (Huang et al., 2012; Yang et al., 2015; Yuan et al., 2016).

Eggs. Coating treatment with chitosan solutions also preserved the freshness and enhanced the commercial value of eggs (**Table 2-3**). The protective barrier formed by chitosan coating on eggshell surface may offer all these benefits by decreasing transfer of carbon dioxide and water vapor through the eggshell pores, eventually enhancing storability of eggs (Robinson, 1987; Suresh et al., 2015; Wardy et al., 2014; Williams, 1992).

Vegetables and fruits. During the storage of vegetables and fruits, metabolism and respiration of plant tissue leads to weight loss, oxidation of vitamin C, and a continual decline in fruit firmness (Ali et al., 2011; Han, 2014; Hong et al., 2012; Lazan and Ali, 1993; Xing et al., 2011; Zhu et al., 2008). Coating with chitosan solution significantly reduced the rate of vitamin C loss in Guava and sweet pepper (Hong et al., 2012; Xing et al., 2011). Vitamin C loss is favoured by the presence of O_2 (Ayranci and Tunc, 2004) and coating of fruits with chitosan solution significantly reduced O_2 diffusion into plant tissue (Ali et al., 2011). Chitosan coatings delayed the ripening process and tissue softening of

guava (Hong et al., 2012), litchi fruit (Dong et al., 2004), papaya (Ali et al., 2011) and grapes (Meng et al., 2008).

In addition to performing direct protective effect, coating treatment with chitosan solution also enhanced the activities of peroxidase (POD) and superoxide dismutase (SOD), plant defensive-enzymes that aid self-detoxification under stress (Jahnke et al., 1991; Meng et al., 2008; Xu et al., 2009), in sweet pepper and guava fruits, concomitantly resulting in a decreased membrane injury (Hong et al., 2012; Xing et al., 2011). These findings suggest that chitosan can also promote protection of vegetables and fruits through acting as a defensive-enzyme enhancer (Hong et al., 2012; Xing et al., 2011).

2.8 Concluding remarks

Chitosan has antimicrobial activity only if it is in the polycationic form at pH values below its pKa. The antimicrobial activity of chitosan depends on the electrostatic interactions between polycationic chitosan molecules and negatively charged cell envelopes. Food components, including NaCl, proteins and starch, adversely affect chitosan activity if the positive charge of chitosan is neutralized. Therefore, inactivation of pathogens by chitosan on food is typically limited to a decrease of 1 - 2 log (CFU/g), which provides a significant challenge to the application of chitosan as general food preservative. In specific applications, however, provide opportunities for the use of chitosan as effective preservative. First, surface application of chitosan on smooth fruits and vegetables concentrates chitosan and allows effective microbiocidal activity. Second, chitosan can potentiate the efficacy of other intervention technologies, including heat and pressure treatments, to become part of an effective hurdle concept. Third, chitosan improves food quality independent of its antimicrobial activity in some cases, e.g. by retardation of lipid

oxidation, plant metabolism, or melanosis, which may favour chitosan applications even if the antimicrobial effect is limited. Chitosan is thus a promising food preservative in specific applications.

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Chapter 3 **Effect of chitosan, and bacteriocin- producing *Carnobacterium maltaromaticum* on survival of *Escherichia coli* and *Salmonella* Typhimurium on beef.**

3.1 Introduction

Salmonella enterica and virulent strains of *Escherichia coli*, especially Shiga-toxin producing *E. coli* (STEC), are foodborne zoonotic agents associated with outbreaks worldwide and pose a threat to public health (EFSA, 2010; Nguyen and Sperandio, 2012). Cattle are a main vehicle for transmission of STEC but they also transmit *Salmonella* (Nguyen and Sperandio, 2012; Wingstrand and Aabo, 2014). Contamination of muscle tissues occurs primarily with the dehiding and evisceration steps during the beef slaughter process (Aslam et al., 2004; Barkocy-Gallagher et al., 2001). In North America, beef carcasses are routinely decontaminated by pasteurization with steam or hot water, and by spraying with lactic acid and/or peroxyacetic acid (Gill, 2009). Despite multiple pathogen intervention technologies *E. coli* and *Salmonella* continue to cause outbreaks associated with beef (CDC, 2014). The continued presence of *Salmonella* and STEC on fresh beef may relate to recontamination of carcasses during handling and cutting (Gill, 2009), or to strain-to-strain variation of the resistance of *E. coli* and *Salmonella* to heat and acid (Dlusskaya et al., 2011; Foster, 2004; Liu et al., 2015, Mercer et al., 2017). The burden of foodborne disease caused by STEC and *Salmonella* necessitates novel tools to ensure the safety of beef and beef products.

Chitosan, poly-(β-(1→4)-glucosamine, is a partially or fully deacetylated derivative of chitin and exhibits antimicrobial activity when the amino group is protonated, i.e. at a pH below the pK_A of 6.2–7.0 (Devlieghere et al., 2004; Tsai and Su, 1999). The antimicrobial activity of chitosan relates to its polycationic properties, which enable electrostatic

interactions with negatively charged structures of the cell envelope, including the cytoplasmic membrane and the lipopolysaccharide (LPS) in the outer membrane of Gram negative organisms (Devlieghere et al., 2004; Helander et al., 2001; Mellegard et al., 2011). Chitosan has GRAS approval in the U.S.A. (FDA, 2011) and is an effective preservative in meat or meat products when applied at concentrations of 1 – 10 g/L (Kanatt et al., 2013; Sagoo et al., 2002; Surendran-Nair et al., 2016). Chitosan seems particularly effective when used in combination with other preservative agents including heat, antimicrobial phenolic compounds (Surendran-Nair et al., 2016), or citrus extracts (Vardaka et al., 2016). The outer-membrane permabilizing activity of chitosan may also support synergistic activity of chitosan with bacteriocins of lactic acid bacteria.

Bacteriocins produced by lactic acid bacteria (LAB) are ribosomally synthesized peptides that have antimicrobial activity in nanomolar concentrations (Drider et al., 2006). Bacteriocins are classified into Class I peptides, which undergo post-translational modifications, and unmodified Class II peptides (Alvarez-Sieiro et al., 2016). Class I bacteriocins include lantibiotics, e.g. nisin, and cyclic bacteriocins, e.g. carnocyclin A; Class II bacteriocins include the pediocin-like bacteriocins that exhibit activity against *Listeria monocytogenes* (Alvarez-Sieiro et al., 2016). Food applications of purified compounds or food-grade bacteriocin producing protective cultures inhibit foodborne pathogens as well as spoilage organisms (Drider et al., 2006; Perez et al., 2014). However, bacteriocins of lactic acid bacteria are inactive against Gram-negative bacteria because the outer membrane prevents access to the cellular target, the cytoplasmic membrane (Gänzle et al., 1999a; Stevens et al., 1991). Chemical or physical treatments that disrupt the outer membrane may allow the use of bacteriocins for control of Gram-negative pathogens in

food (Cutter et al., 1995; Martin-Visscher et al., 2011). The outer-membrane permeabilizing activity of chitosan sensitises *E. coli* and *Salmonella* to nisin (Cai et al., 2010); however, this synergistic effect has not been validated in food applications, and was not verified for bacteriocins other than nisin.

The aim of this study was to determine the single and combined antimicrobial activity of chitosan and bacteriocins in media, and to verify the activity in a model meat system mimicking pathogen intervention technologies that used in beef processing. The heat resistant *E. coli* AW1.7 and *Salmonella enterica* Typhimurium TA2442 were used as target organisms; nisin and bacteriocin cocktails purified from two strains of *Carnobacterium maltaromaticum* were evaluated to represent Class I and Class II bacteriocins.

3.2 Material and methods

3.2.1 Bacterial strains and culture conditions.

Escherichia coli AW1.7, a heat resistant beef isolate (Dlusskaya et al. 2011) and *Salmonella. enterica* Typhimurium TA2442, obtained from the *Salmonella* genetic stock centre (Calgary, AB, Canada) were aerobically grown in Luria-Bertani broth (LB; Difco; Becton, Dickinson and Company, Sparks, MD, USA) at 37 °C for 18 h. *E. coli* AW1.7 and *S.* Typhimurium were enumerated on LB agar (Difco) to detect all viable cells, or on violet red bile agar (VRBA, Difco) to enumerate cells of *E. coli* AW1.7 and *S.* Typhimurium cells without sublethal injury. *Carnobacterium divergens* LV13, a bacteriocin sensitive indicator strain, *C. maltaromaticum* UAL307, a strain used in commercial biopreservatives and producing piscicolin 126, carnobacteriocin BM1, and carnocyclin A (Martin-Visscher et al., 2011), and *C. maltaromaticum* UAL8 producing carnobacteriocin A, BM1 and B2 (Allison *et al.,*1995) were routinely grown in All Purpose Tween (APT) broth (Difco) at

25°C. APT agar was used to enumerate viable carnobacteria. For purification of bacteriocins from cultures of *C. maltaromaticum* UAL307, the strain was cultured in Casamino Acid (CAA) medium containing the following per litre: 15 g casamino acid; 5 g yeast extract; 2 g K_2HPO_4; 2 g $C_6H_{14}N_2O_7$; 0.1 g $MgSO_4$; 0.05 g $MnSO_4$; pH=6.5 at 25°C for 21 to 24 h.

3.2.2 Chemicals and preparation.

High molecular weight chitosan (HMWC) was supplied by Yuhan Ocean Biochemistry Co. Ltd. (Tauzhou, China). The degree of deacetylation and molecular weight of HMWC were 92% and 210 kDa, respectively. Water soluble chitosan (WSC) was prepared by enzymatic hydrolysis of HMWC with neutral protease from Ningxia Xiasheng Industry Co. Ltd. (Ningxia, China). The degree of deacetylation (DD) of WSC was 92% as determined by titration (Tolaimate et al., 2000). The degree of polymerization (DP) as determined by size exclusion chromatography on a Superdex Peptide column (GE Healthcare) ranged from 4- to 50 units. Chitosan oligosaccharides (COS) with a degree of deacetylation of 100% and a DP of 2-6 were obtained from GlycoBio (Dalian, China). HMWC, WSC or COS were dissolved in 1% (w/v) acetic acid (Fisher Scientific, Canada), the pH was adjusted to 5.4 with 10 M NaOH, and the concentration was adjusted to 1% (w/v). HMWC stock solution with pH 5.4 was stored at 4 ℃ for use within one week; WSC or COS stock solutions were prepared on the day or use.

A nisin preparation containing 2.5% nisin and 97.5% NaCl and milk solids was obtained from MP Biomedicals (Montreal, Canada). A nisin stock solution containing 125 mg/L nisin was prepared by dissolving 25 mg commercial nisin preparation and 37.5 mg NaCl in 4.8-4.85 mL 0.02 M HCl (Sigma-Aldrich, USA), followed by adjustment of the pH to

5.4 with NaOH solution and adjustment of the total volume to 5 mL with water. The nisin solution was sterilized by filtration.

3.2.3 Partial purification of bacteriocins and determination of bacteriocin activity.

The bacteriocins produced by *C. maltaromaticum* UAL307 were purified as described (Balay et al., 2017) with some modifications. *C. maltaromaticum* UAL307 was grown in 1 liter of Casamino Acid (CAA) medium. After 21 to 24 h of incubation, the culture including cells and supernatant was applied to a column (2.5×50 cm) containing 60 g/L of Amberlite XAD-16 N resin (Sigma-Aldrich®, Saint Louis, MO, USA), equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA), at a flow rate of 5 mL/min at 6°C. The column was successively washed with 500 mL of H_2O, 500 mL of 20% (v/v) ethanol, and 500 mL of 40% (v/v) ethanol all at 10 mL/min. Bacteriocins were eluted with 1 liter of 70% isopropyl alcohol, acidified to pH 2 at 5 mL/min. This fraction was concentrated to around 24 mL using a Buchi® rotary evaporator (Brinkman Instruments, Westbury, NY, USA) at 30°C under vacuum and loaded onto three Water-Pak 12 cc C18 cartridges. The three cartridges were each washed with 20 mL H_2O, 20 mL 30% (v/v) ethanol, 20 mL 20% (v/v) isopropanol at a flow rate of 5 mL/min. Bacteriocins were eluted from each cartridge with 40 mL of 70% (v/v) isopropanol, pH 2. The active fractions collected from each of the 3 cartridges were combined and concentrated under vacuum to a volume of about 5 mL. All fractions were assayed for antimicrobial activity with *C. divergens* LV13 as the indicator strain. The activity was determined by a critical dilution assay (Eloff, 1998) with some modification. In brief, serial two-fold dilutions of each fraction with APT broth were prepared on 96-well microtiter plates (Corning, USA). Overnight cultures of *C. divergens* LV13 in APT broth were subcultured and incubated at 25 °C for 12 h, diluted ten-fold and

used to inoculate the microtiter plates. After incubation of the plates for 18 h, 40 µl of a 0.2 g/L p-iodonitrotetrazolium violet (INT) (Sigma-Aldrich) solution in water was added to each well and the plate was incubated for 3 h at 25 °C. The wells without bacterial growth remained colorless; one activity unit (AU) was defined as the highest dilution of each fraction that inhibited growth of *C. divergens*.

3.2.4 Determination of inhibitory activity of different antimicrobials against *E. coli* AW1.7 and *S.* Typhimurium.

The inhibitory effects of chitosan, nisin, or purified bacteriocins against *E. coli* AW1.7 and *S.* Typhimurium were determined by a critical dilution assay as described (Gänzle et al., 1999a) with some modifications. In brief, two-fold serial dilutions of HMWC WSC, or COS were prepared with MES-buffered nutrient broth (NB-MES) in 96-well microtiter plates (Corning, USA); 2D "checkerboard" dilutions to determine the combined activity of chitosan and bacteriocins were prepared as described (Gänzle et al., 1999a). *E. coli* AW1.7 and *S.* Typhimurium were sub-cultured twice in nutrient broth (NB) and incubated at 37 °C for 8-10 h and 12 h, respectively. The cultures were diluted ten-fold with NB-MES, and 50 µl of these diluted cultures were added to the microtitre plates. The plates were incubated for 16–20 h at 37 °C, the optical density was measured at 630 nm using a microtiter reader (Varioskan Flash, Thermo Electron Corporation, Canada), and the MIC of chitosan, nisin, or purified bacteriocins was assessed as concentration in mg/L or AU/mL inhibiting growth of the indicator strains by 50%.

3.2.5 Preparation of meat samples

Frozen lean beef was obtained as vacuum packaged and frozen bulk product. To obtain aseptic cuts of beef, frozen beef was tempered at 4°C for 12 h and cut into 2.5 cm and 7.5

cm steaks. These steaks were flamed with ethanol to sterilize the surface, triple wrapped in plastic bags and stored at -20°C. To prepare meat cylinders, frozen steaks were tempered at room temperature for 1 to 2 h. A sterilized circular corer with a diameter of 2.0 cm (surface area of 3.14 cm^2) was hammered into the partially frozen meat. The core of meat was aseptically sliced into cylinders around 5 mm thick. Meat cylinders were stored at -20°C until use. Total cell counts and coliform cell counts of the meat cylinders were enumerated on LB agar and VRBA; both cell counts were below the detection limit of 100 CFU/g.

3.2.6 Establishment of bench-top steaming apparatus and steaming procedures

The steaming apparatus (**Figure 3-1**) consisted of a glass flat bottom flask that was placed on a magnetic heater to generate stream. A foil-insulated custom-made glass nozzle conducted the stream to the meat sample. The distance between the steam outlet and the surface of the meat samples was 2.2 cm.

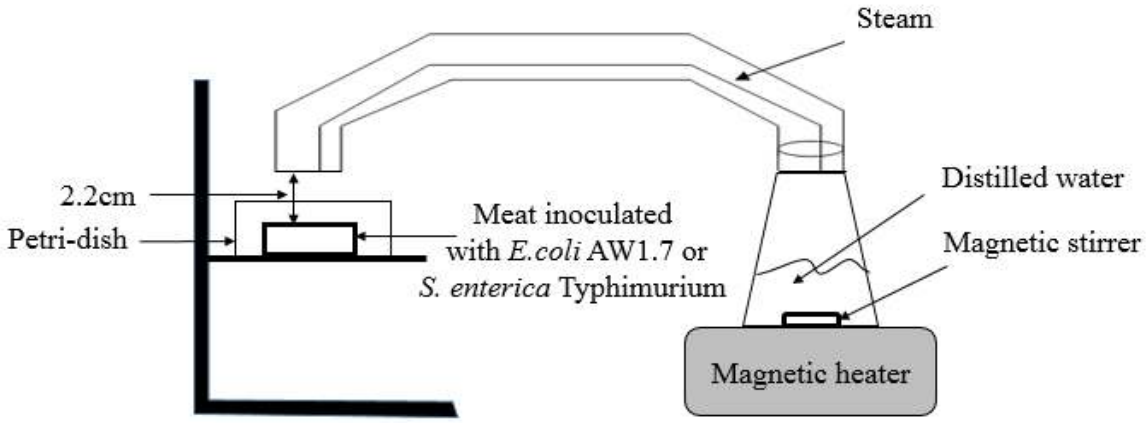

Figure 3-1 Schematic diagram of the bench-top steaming apparatus in this work

3.2.7 Different treatments and microbiological analysis of samples

Meat cylinders were thawed at room temperature for 1 h. The meat surface was inoculated with 100 µl of cultures of *E. coli* AW1.7 or *S.* Typhimurium and the surface was air dried 20 °C for 15 min; uninoculated samples without treatment were used as negative control. Positive controls were inoculated but did not receive any treatment; other samples were steamed for 8 s. Steamed samples were also treated by adding 200 µL of one or two of the following solutions or organisms: 8% lactic acid, 1% acetic acid, 1% HMWC solution in 1% acetic acid, bacteriocins partly purified from cultures of *C. maltaromaticum* UAL307, culture of *C. maltaromaticum* UAL8 culture, or culture of *C. maltaromaticum* UAL307. When combination treatments of two solutions were used, 100µL of each of the two solutions was added. After treatment, samples were air dried and incubated for 4 h. Total cell counts, cell counts of coliform bacteria, and cell counts of carnobacteria were determined by surface plating of appropriate dilutions on LB agar, VRBA, and APT agar, respectively. Observation of a uniform colony morphology verified that the colony morphology of carnobacteria enumerated after refrigerated storage matched the colony morphology of the inocula.

3.2.8 Microbiological analysis of samples during vacuum-packaged and refrigerated storage.

A second experiment employed the most efficient treatments to observe the antimicrobial efficacy during 4 weeks of refrigerated storage. Samples inoculated with 100 µl of *E. coli* AW1.7 or *S. enterica* Typhimurium cultures (around 10^8 CFU/cm^2) were treated as described above, vacuum-packaged and stored for 32 days (d) at 4°C. Uninoculated and

untreated inoculated controls were also prepared as described above. The plate counts of samples were determined at 4 h and 1, 4, 8, 16, 24 and 32 d.

3.2.9 Statistical analysis.

Experiments were performed in biological duplicates or triplicates. All data are expressed as means ± SD. Differences among treatments were tested for significance by one-way or two-way ANOVA with Least Significant Difference (LSD) test using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) for Windows 8.1. Significance was assessed at an error probability of 5% ($p \leq 0.05$).

3.3 Results

3.3.1 Single and combined activity of bacteriocins or chitosan in media.

To assess the activity of bacteriocins, the MIC of nisin and a bacteriocin preparation from *C. maltaromaticum* UAL307 were determined with *E. coli* and *S.* Typhimurium as indicator strains. At pH 5.4, the MIC of nisin against *E. coli* AW1.7 was 10 mg/L whereas *S.* Typhimurium was resistant to nisin at a concentration of 20 mg/L. A single chromatographic step achieved partial purification of bactericoins produced by *C. maltaromaticum* UAL307 (Balay et al., 2017). Elution of the column with 70% isopropanol eluted peptides with antimicrobial activity while all other fractions obtained in the purification procedure exhibited no activity. The activity of the final bacteriocin preparation was 20480 AU/mL. Assaying the antimicrobial activity of the preparation against *E. coli* and *S.* Typhimurium demonstrated that these two Gram-negative organisms were about 100 times less sensitive than *C. divergens* (**Figure 3-3**). The MIC of chitosan oligosaccharides (COS), water soluble chitosan (WSC) and high molecular weight chitosan (HMWC) against *E. coli* ranged from 14 to 42 mg/L (**Figure 3-2** and **3-3**); the HMWC was

the most active of the three chitosan preparations. The MIC of COS, WSC and HMWC against *S.* Typhimurium ranged from 30 to 69 mg/L; again, again, HMWC was the most active compound (**Figure 3-2** and **3-3**).

The combined activity of bacteriocins and chitosan preparations is shown in Figures 2 and 3. Nisin did not increase the susceptibility of *E. coli* AW1.7 and *S.* Typhimurium to chitosan (**Figure 3-2**); however, a synergistic effect was observed for HMWC and bacteriocins from *C. maltaromaticum* UAL307; this synergistic effect was weaker or absent for the COS or WSC (**Figure 3-3**). These results indicate that high molecular weight chitosan permeabilizes the outer membrane of *E. coli* and *S.* Typhimurium to bacteriocins from *C. maltaromaticum* UAL7.

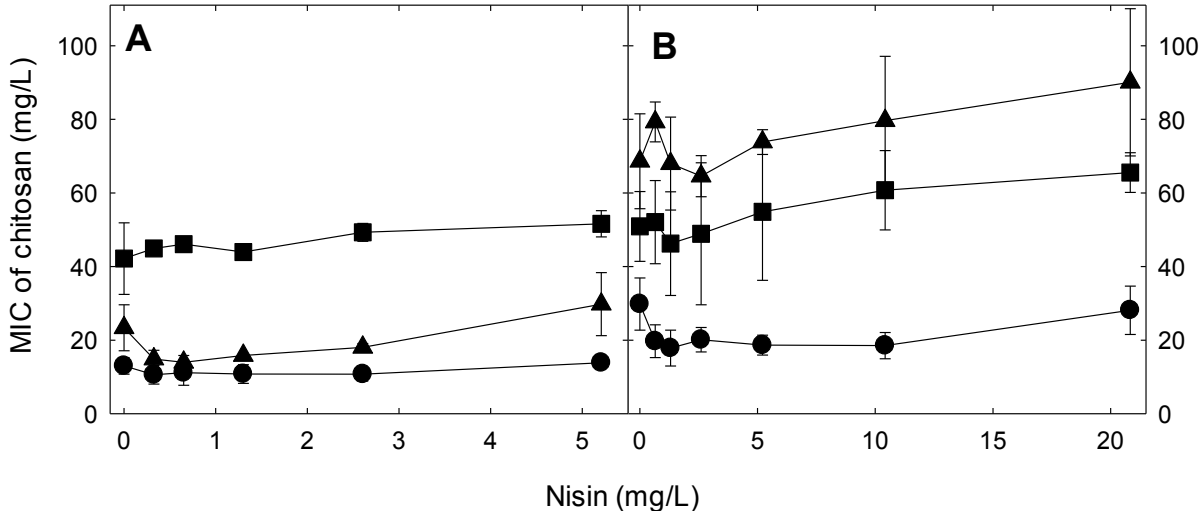

Figure 3-2 Effect of nisin on the activity of chitosan-oligosaccharides (▲), water-soluble chitosan (■) and high-molecular weight chitosan (●) against *E. coli* AW1.7 (A) and *S. enterica* Typhimurium (B) in media with pH 5.4. Error bars indicate the means ± standard deviation of triplicate independent experiments.

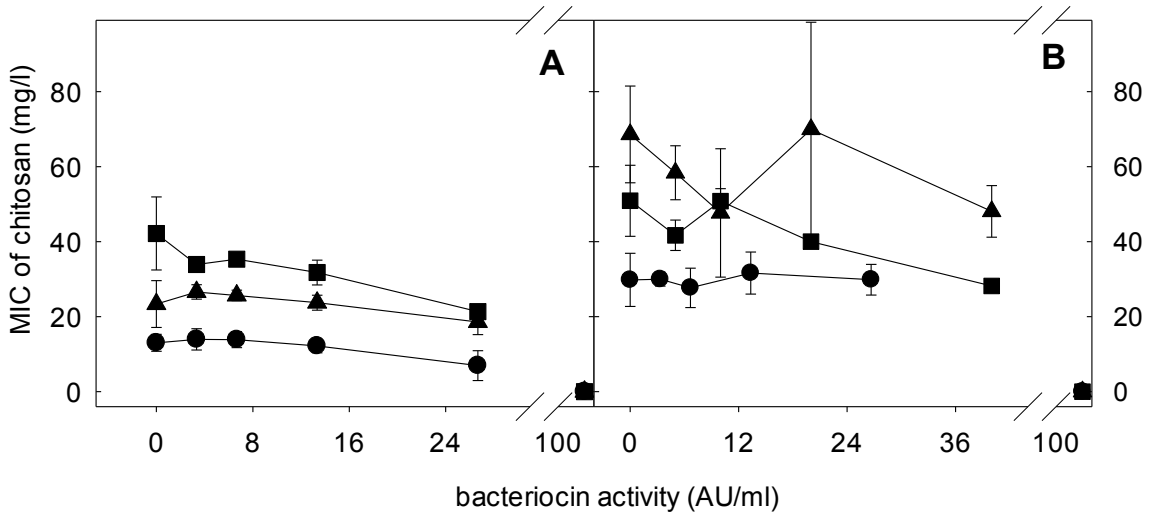

Figure 3-3 Effect of bacteriocins produced by *C. maltaromaticum* UAL 307 on the activity of chitosan-oligosaccharides (▲), water-soluble chitosan (■) and high-molecular weight chitosan (●) against *E. coli* AW1.7 (A) and *S. enterica* Typhimurium (B) in media with pH 5.4. Data to the right of the axis break indicate the MIC of bacteriocins in absence of any chitosan preparation. Data are shown as means ± standard deviation of triplicate independent experiments

3.3.2 Screening the efficient treatments in inactivating *E. coli* AW1.7 and *S. enterica* Typhimurium on fresh lean beef.

An initial experiment explored the effect of steam and lactic acid alone, in combination with chitosan, or in combination with chitosan and bacteriocin-producing carnobacteria or bacteriocins. Based on the *in vitro* screening, HMWC and bacteriocins from *C. maltaromaticum* UAL307 were selected to determine their single and combined antimicrobial effects on meat. Surviving cells of *E. coli* and *S.* Typhimurium were enumerated on LB agar and VRBA to quantify viable and sub lethally injured cells. After inoculation, cell counts on the surface of lean beef cylinders ranged from 6.2 to 6.9 log(CFU/cm^2) (**Figure 3-4**). Steaming reduced cell counts of *S.* Typhimurium by approximately 1 log(CFU/cm^2) (**Figure 3-4**) while no significant cell reduction of *E. coli*

was observed after steaming. Treatment with lactic acid after steaming had no additional antimicrobial effect (**Figure 3-4**). Likewise, treatments of meat with cultures of *C. maltaromaticum* or purified bacteriocins produced from *C. maltaromaticum* UAL307 were as effective as treatments with steam only (data not shown). Treatments of meat with chitosan after steaming additionally reduced cell counts of *E. coli* and *S.* Typhimurium by approximately 1 log(CFU/cm^2) (**Figure 3-4**). The antimicrobial effect of steam plus chitosan treatment was not increased by addition of bacteriocin-producing carnobacteria, or bacteriocins purified from *C. maltaromaticum* UAL307 (**Figure 3-4**). Different from *in vitro* results (**Figure 3-4**), chitosan and bacteriocins displayed no synergistic activity; however, chitosan addition to meat substantially enhanced the antimicrobial effect of steam treatment.

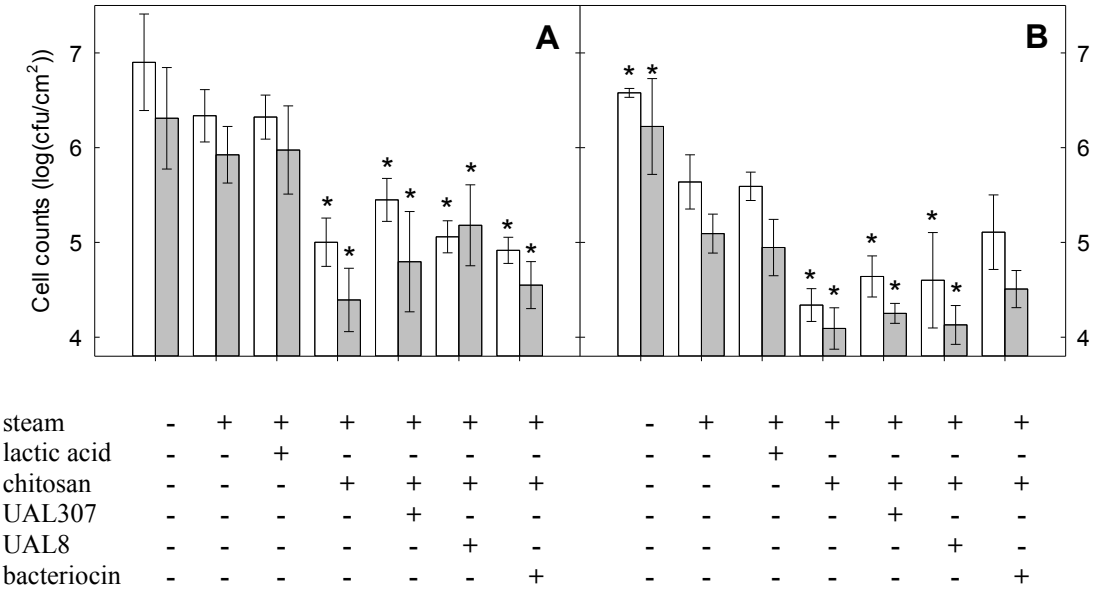

Figure 3-4 Cell counts of lean, aseptic beef cylinders inoculated with *E. coli* (Panel A) or *Salmonella* (PanelB) after different pathogen intervention treatments as indicated. Cell counts were enumerated on LB agar (white bars) and VRBA agar (grey bar). Steam, treatment for 8 sec, lactic acid, application of 8% lactic acid; chitosan, surface application of 1% high molecular weight chitosan in 1% acetic acid; UAL307, inoculation with *C. maltaromaticum* UAL307 after steaming; inoculation with *C. maltaromaticum* UAL8 after

steaming; Bacteriocin, purified bacteriocins produced by *C. maltaromaticum* UAL307 (1280 AU/mL). Data indicate means ± standard deviation of two or three independent experiments. Cell counts that are different from the cell counts of samples treated with only steam are indicated by an asterisk (*$P<0.05$)*.

3.3.3 Effect of treatment with steam and chitosan on meat microbiota during refrigerated storage.

Subsequent experiments aimed to determine the influence of intervention treatments with steam and chitosan on the viability of *E. coli* and *Salmonella* during refrigerated storage. Meat was additionally inoculated with carnobacteria to assess the impact of intervention treatments on non-pathogenic meat microbiota. Results obtained with *E. coli* AW1.7 are shown in **Figure 3-5**. Cell counts of *E. coli* were reduced by 1 – 2 log(CFU/cm^2) during refrigerated storage; this reduction was particularly apparent for cell counts on VRBA, which exclude sublethally injured cells (**Figure 3-5A** and **C**). The effect of streaming on cell counts of *E. coli* during storage was generally not significant; likewise, addition of acetic or lactic acids did not influence cell counts after treatment or after treatment and storage (**Figure 3-5A** and **C**). Treatment with chitosan reduced cell counts by 1 log(CFU/g) and this difference to the steam treated control remained throughout the 32 d of storage (**Figure 3-5A** and **C**). Inoculation of meat with carnobacteria did not affect cell counts of *E. coli* during refrigerated storage (**Figure 3-5B** and **D**); however, chitosan was also effective in presence of carnobacteria (**Figure 3-5B** and **D**). The overall reduction of cell counts that was achieved by steam and lactic acid intervention treatments, chitosan addition and refrigerated storage exceeded 3 log(CFU/cm^2) (**Figure 3-5**).

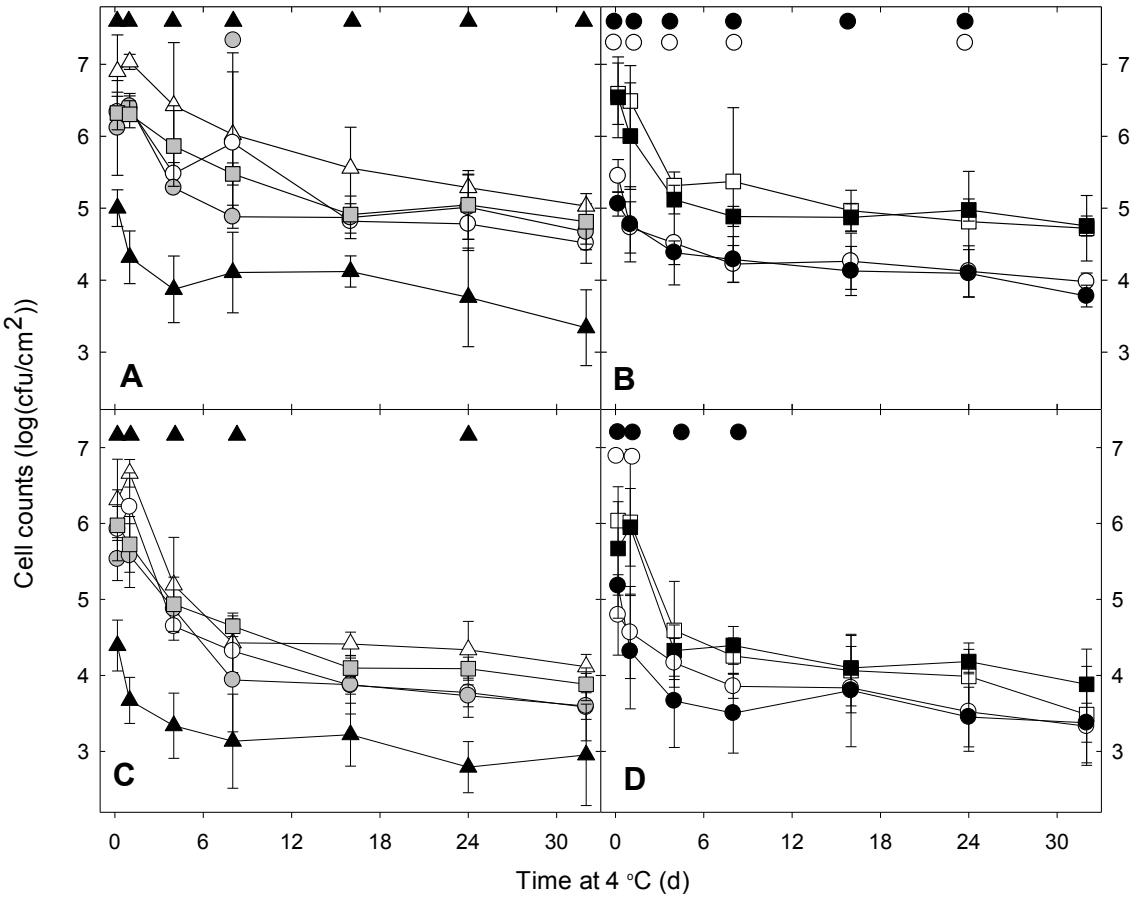

Figure 3-5 Cell counts of *E. coli* on vacuum packaged lean beef cylinders during storage at 4 °C.The counts of *E. coli* were enumerated on LB agar (Panels A and B) or on VRBA (Panels C and D).Beef cylinders shown in Panels A and C were inoculated only with *E. coli*; samples shown in panels B and D were inoculated with *C. maltaromaticum* UAL307 (□) or UAL8 (○) after steaming. Panels A, C: Before packaging, beef cylinders were not treated (control, Δ), or treated with steam for 8 sec (○) in combination with the following additions: acetic acid (●); lactic acid (■); or 1% HMWC (▲). Panels B, D: Treatment with steam for 8 sec, followed by inoculation with *C. maltaromaticum* UAL307 (□); *C. maltaromaticum* UAL8 (■); *C. maltaromaticum* UAL307 with 1% HMWC (○); or *C. maltaromaticum* UAL8 with 1% HMWC (●). Data indicate means ± standard deviation of two or three independent experiments. For treatments that were significantly more lethal than steam and storage for the same time ($P<0.05$), the corresponding symbol is indicated at the upper x-axis.

The cell counts of *S.* Typhimurium during refrigerated storage are shown in Figure 6.

Comparable to *E. coli*, chitosan reduced cell counts by about 1 log(CFU/cm^2) while

treatments with organic acids were ineffective (**Figure 3-6**). Different from *E. coli*, steam treatment significantly reduced cell counts of *Salmonella* by about 1 log(CFU/cm^2), and cell counts of *Salmonella* remained stable throughout refrigerated storage unless carnobacteria and chitosan were both present. In presence of chitosan and any of the two strains of *C. maltaromaticum*, cell counts were reduced by 1 – 2 log(CFU/cm^2) during refrigerated storage (**Figure 3-6B** and **D**). The overall reduction of cell counts achieved by steam treatment followed by addition of chitosan and carnobacteria exceeded 3 log(CFU/cm^2).

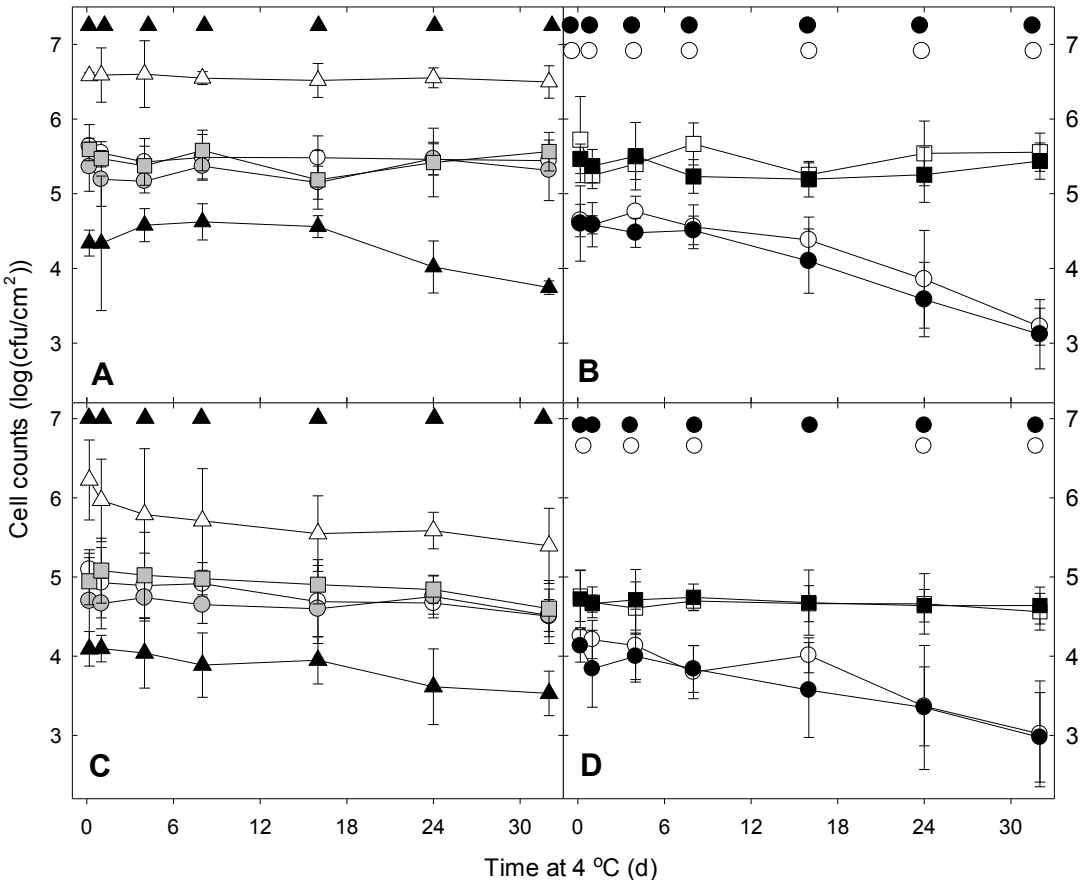

Figure 3-6 Cell counts of *S. enterica* on vacuum packaged lean beef cylinders during storage at 4 °C**.** The counts of *S. enterica* were enumerated on LB agar (Panels A and B) or on VRB agar (Panels C and D). Beef cylinders shown in Panels A and C were inoculated only with *S. enterica*; samples shown in panels B and D were inoculated with *C.*

maltaromaticum UAL307 (□) or UAL8 (○) after steaming. Panels A, C: Before packaging, beef cylinders were not treated (control, Δ), or treated with steam for 8 sec (○) in combination with the following additions: acetic acid (●); lactic acid (■); or 1% HMWC (▲). Panels B, D: Treatment with steam for 8 sec, followed by inoculation with *C. maltaromaticum* UAL307 (□); *C. maltaromaticum* UAL8 (■); *C. maltaromaticum* UAL307 with 1% HMWC (○); or *C. maltaromaticum* UAL8 with 1% HMWC (●).Data indicate means ± standard deviation of at least two independent experiments. For treatments that were significantly more lethal than steaming and storage for the same time (*P<0.05)*, the corresponding symbol is indicated at the upper x-axis.

Because the presence of carnobacteria influenced survival of *Salmonella* during refrigerated storage of beef when chitosan was present, cell counts of carnobacteria were additionally monitored during refrigerated storage. Cell counts of co-cultures with *Salmonella* are shown in **Figure 3-7**; cell counts of co-cultures with *E. coli* were essentially identical (data not shown). The two strains of *C. maltaromaticum* also showed a comparable response to treatment and refrigerated storage (**Figure 3-7** and data not shown). In the absence of chitosan, carnobacteria grew from about 6 log(CFU/cm^2) to 7 log(CFU/cm^2) (**Figure 3-7**). Chitosan initially reduced cell counts of carnobacteria by about 99%; however, during refrigerated storage, the surviving cells grew to high cell counts even in presence of chitosan.

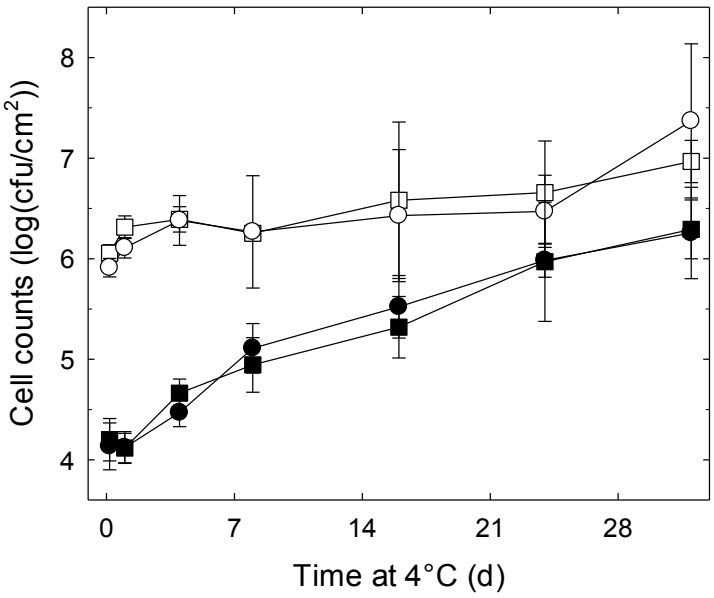

Figure 3-7 Cell counts of *Carnobacterium* on vacuum packaged lean beef cylinders inoculated with *S. enterica* and *C. maltaromaticum* UAL307 or *C. maltaromaticum* UAL8 during storage at 4 °C for 32 days. Carnobacteria were selectively enumerated on APT agar. Beef cylinders were inoculated with *S. enterica,* treated with HMWC (black symbols) or not (open symbols), steamed for 8 sec, followed by inoculation with *C. maltaromaticum* UAL307 (□,■) or *C. maltaromaticum* UAL8 (○,●). Data indicate means ± standard deviation of at least two independent experiments. Comparable cell counts of carnobacteria were obtained from beef cylinders inoculated with *E. coli* (data not shown).

3.4 Discussion

This study assessed the activity of chitosan in combination with steam pasteurization, acid interventions, and bacteriocins or bacteriocin producing cultures to reduce beef contamination with *Salmonella* and *E. coli*. The North American beef industry applies steam pasteurization or hot water washes in combination with application of lactic acid or peroxyacetic acid to reduce carcass contamination. Steam pasteurization reduces the numbers of *E. coli* on meat by 0.05 to 2 log (CFU/cm^2) (Corantin et al., 2005; Gill, 2009; McCann et al., 2006; Minihan et al., 2003). The variable effect of steam or hot water interventions may relate to variations in the intensity of thermal treatments, differences

between lean and adipose tissue, or to strain-to-strain variation of heat resistance (Dlusskaya et al., 2011). The variable effect of thermal interventions necessitates improved intervention technologies to reduce the burden of foodborne disease associated with beef. The present study implemented a lab-scale steam treatment to heat the surface of the meat to >95°C for several seconds, thus matching conditions that are typically employed in beef processing (Gill, 2009). *E. coli* AW1.7 is a heat resistant beef isolate (Dlusskaya et al., 2011) and heat resistance of the strain is mediated by the locus of heat resistance (LHR) (Mercer et al., 2015). LHR-mediated heat resistance is observed in approximately 2% of all *E. coli* and in 4% of *E. coli* isolated from beef processing plants (Mercer et al., 2015); LHR-mediated heat resistance also occurs in *Salmonella* but with a much lower frequency (Mercer et al., 2017). The bactericidal effect of steam treatment on *E. coli* AW1.7 and *S.* Typhimurium corresponded to the differential heat resistance of the two organisms. Steam treatment is effective only on the surface of the tissue, therefore, stream treatments reduced cell counts of the heat sensitive *Salmonella* by less than 2 log(CFU/cm^2) (**Figure 3-4** and **3-7**). Interventions with lactic or acetic acids had no effect on cell counts of *E. coli* or *Salmonella*, reflecting the acid resistance of these organisms (Foster, 2004) and the high buffering capacity of lean tissue.

Bacteriocins from lactic acid bacteria alone or in combination with chitosan may increase the bactericidal effect of pathogen intervention technologies in beef processing. Bacteriocins from *C. maltaromaticum* and nisin inhibited *E. coli* AW1.7 and *S. enterica* Typhimurium in media with pH 5.4, in keeping with prior observations that a low pH increases sensitivity of Gram-negative bacteria (Gänzle et al., 1999b; Martin-Visscher et al., 2011). High proton concentrations, corresponding to a low pH, displace divalent cations

from the LPS binding sites; the resulting increase in permeability of the outer membrane renders cells more susceptible to hydrophobic inhibitors including bacteriocins (Vaara, 1992, Gänzle et al., 1999b). The net charge density of chitosan and the intensity of electrostatic interactions between chitosan and cell surface are crucial to antibacterial activity; therefore, chitosan is active only when the ambient pH is below its pK_A of 6.5 (Gerasimenko et al., 2004; Kong et al., 2010; Mellegard et al., 2011; Zheng and Zhu, 2003). High molecular weight chitosan generally exhibits a higher antibacterial activity than chitosan oligosaccharides (Mellegard et al., 2011), which was confirmed in the present study. Chitosan with higher activity also leads to a more intense disruption of outer membrane (OM) of *E. coli*. (Mellegard et al., 2011). Perturbation of the outer membrane permeability barrier by chitosan (Eaton et al., 2008; Helander et al., 2001; Kong et al., 2010) may increase the sensitivity to outer-membrane impermeant inhibitors such as bacteriocins. Synergistic activity of chitosan and nisin has previously been described *in vitro* (Cai et al., 2010) but has not been employed to inhibit Gram-negative organisms in food. This study employed NB broth to determine the *in vitro* synergistic activity; the low protein content of this medium minimizes interactions of chitosan with media components. Synergistic activity of chitosan was observed with high molecular weight chitosan and bactericiocins from *C maltaromaticum*, in keeping with prior observation that outer membrane perburbation sensitizes *E. coli* to carnocyclin A (Martin-Visscher et al., 2011). However, inconsistent with prior reports (Cai et al., 2010), synergistic activity was not observed with nisin and chitosan. We employed commercial nisin containing 2.5 % nisin with NaCl and milk proteins. These ingredients may decrease chitosan activity by neutralizing the positive charges of chitosan (Devlieghere et al., 2004).

In this study, addition of HMWC after steaming reduced *E. coli* or *Salmonella* by around 1 log(CFU/cm^2) while treatments with lactic or acetic acids had no additional effect. The overall bactericidal effect of chitosan on meat, which reduced cell counts by 90%, matched the reduction of cell count of *Salmonella* in chicken skin by application of 0.5% chitosan (Menconi et al., 2013) and the effect of addition of 2% chitosan of cell counts of *E. coli* in kabab (Kanatt et al., 2013). Carnobacteria were more sensitive to chitosan application on meat than *E. coli* or *Salmonella* (**Figure 3-5**, **3-6**, and **3-7**); however, chitosan did not prevent growth of carnobacteria to high cell counts during refrigerated storage.

The application of chitosan in meat was particularly effective in hurdle applications that combined chitosan with heat and additional antimicrobial agents. Chitosan addition at a level of 0.1% did not affect survival of enterohaemorrhagic *E. coli* during refrigerated storage of ground beef; however, chitosan showed synergistic effects with rutin and resveratrol during cooking of beef patties (Surendran-Nair et al., 2016). The use of citrus extract in combination with low molecular weight chitosan showed an additive effect against *E. coli* and *S. enterica* populations in fresh turkey meat stored under vacuum at 4°C or 10°C (Vardaka et al., 2016). A potential synergistic effect of bacteriocins and chitosan on meat, however, remains unknown. Nisin in raw meat is inactivated by addition of glutathione (GSH) (Rose et al., 1999); moreover, nisin exhibited no synergistic activity with chitosan. Meat applications combining chitosan and bacteriocins thus focused on bacteriocins of *C. maltaromaticum* and application of bacteriocin-producing cultures on meat. Cell counts on LB and VRBA differed by less than 1 log(CFU/cm^2) after treatment of meat with steam and chitosan, indicating that outer membrane perturbation by chitosan, which was demonstrated *in vitro* (Helander et al., 2001), is not observed on meat.

Accordingly, the application of bacteriocins did not reduce cell counts of *E. coli* and *Salmonella*, and did not enhance the bactericidal effect of chitosan (**Figure 3-4**, **3-5**, and **3-7**).

The present study evaluated the use of the bacteriocin-producing cultures *C. maltaromaticum* UAL8 and UAL307 as an alternative strategy to control enteric pathogens in combination with chitosan. Application of 2% chitosan reduced cell counts of *E. coli* and *Salmonella* during refrigerated storage of vacuum packaged turkey meat (Vardaka et al., 2016), but the effect of spoilage microbiota was not considered. Remarkably, refrigerated storage differentially affected *E. coli* and *Salmonella*, chitosan, and protective cultures. Cell counts of *E. coli* were reduced during refrigerated storage; the reduction was irrespective of the presence of chitosan or carnobacteria. In contrast, cell counts of *Salmonella* remained stable during storage unless carnobacteria and chitosan were both present (Fig. 5, 6 and 7). In both cases the combined bactericidal effect of steam treatment, chitosan, and protective cultures reduced cell counts by 3 log(CFU/cm^2). This represents a substantial improvement to current or proposed intervention technologies (Gill, 2009; Surendran-Nair et al., 2016). It remains unknown whether the effect of carnobacteria relates to competition for nutrients and acid formation, or to a specific effect of the bacteriocins that are produced during storage (Holzapfel et al., 1995).

In conclusion, chitosan exhibited bactericidal activity against *Salmonella* and *E. coli* on beef. Chitosan exhibited no synergistic activity with bacteriocins on meat, however, chitosan together with bacteriocin-producing protective cultures reduced cell counts of *Salmonella*. The use of chitosan and protective cultures in addition to steam treatment was significantly more effective than the use of steam alone or in combination with lactic acid,

and thus may provide novel solutions for improved meat safety. The application is particularly promising for production of ground beef and mechanically tenderized beef, where internal contamination with pathogenic bacteria may occur (Gill et al., 2005; Phebus et al. 2000).

3.5 References

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Chapter 4 Effect of sodium chloride, chitosan and other additives on the heat resistance of Shiga-toxin producing *Escherichia coli* in ground beef

4.1 Introduction

Enterohemorrhagic *Escherichia coli* (EHEC), as one of the major foodborne pathogens, remain an unsolved problem for food safety. The serious hazard to human health posed by EHEC is characterized by an infectious dose of less than 10 cells (Paton et al., 1996; Tilden et al., 1996) and the hemolytic uremic syndrome (HUS) with substantial morbidity and mortality (Croxen et al., 2013). Cattle are a main reservoir of EHEC, and their fecal shedding are an important source of food and environmental contamination (Low et al., 2005). Pathogen intervention during beef processing aims to reduce meat contamination with pathogenic *E. coli*, however, despite these interventions, 0.5 – 2% of ground beef samples in North America is contaminated with EHEC (Aslam et al., 2004; Ferens and Hovde, 2011; Gill, 2009). Accordingly, EHEC food-borne infection is still linked to the consumption of undercooked ground beef patties (Rhee et al., 2003; WHO, 2018).

Domestic cooking of meat to an internal core temperature of 71 °C is currently referred to as a safe handling to eliminate food-borne pathogens in ground beef (Health Canada, 2015). However, the heat resistance of some *E. coli* strains questions the safety of this recommended cooking temperature (Dlusskaya et al., 2011; Jin et al., 2008; Liu, 2015). In *Escherichia coli* and related bacteria, the exceptional resistance to heat is attributed to a 14 or 19 kb genomic island termed locus of heat resistance (LHR) (Boll et al., 2017; Mercer et al., 2015). LHR-positive *E. coli* resisted cooking in beef patties to an internal temperature of 71 °C with a reduction of cell counts by less than 2 log (CFU/g) (Mercer et al., 2017). The LHR occurs in about 2% of strains of *E. coli* and is transferred between strains of *E. coli* (Boll et al., 2017; Mercer et al., 2015), including pathogenic strains, however, of 612

Shiga toxin-producing *E. coli* (STEC) isolates, only two (0.3%) isolates were positive for the LHR (Ma and Chui, 2017).

Addition of 2-4% NaCl can increase the heat resistance of *E. coli* irrespective of the presence of the LHR (Mercer et al., 2017; Pleitner et al., 2012). NaCl also increased the heat resistance of *E. coli* O157:H7 ground beef containing 2.7 or 2.7 % NaCl (Juneja et al., 2015). NaCl alone or in conjunction with marinades is applied to improve the taste and texture of meat products (Verbeke et al, 2010; Vlahova-Vangelova and Dragoev, 2014). However, effects of NaCl or marinade on the heat resistance of LHR-positive *E. coli* and LHR-negative *E. coli* in ground beef have not been compared. Additionally, improving the safety of ground beef products necessitates the development of more effective interventions. Carvacrol and chitosan are two membrane active compounds that are derived from biological systems, have GRAS approval in the U.S.A. (FDA, 2011, 2018) and enhanced thermal destruction of EHEC in hamburger patties (Juneja and Friedman, 2008; Surendran Nair et al., 2016). Potassium lactate is also an effective preservative in extending shelf life of meat products (Sofos and Geornaras, 2010). However, effects of these compounds on survival of heat resistant *E. coli* in beef patties cooked to an internal core temperature of 71 °C have not been validated.

Therefore, this study investigated the effect of NaCl and other different additives on heat resistance of LHR-positive and LHR-negative *E. coli* strains in ground beef, aiming at extendsion of knowledge about the survival of *E. coli* in ground beef after cooking and discovery of effective antimicrobials that can potentiate the heat inactivation of *E. coli* in ground beef.

4.2 Materials and methods

4.2.1 Bacterial strains and culture conditions.

Bacterial strains used in this study are listed in **Table 4-1**. *E. coli* AW1.7ΔpHR1 was obtained with *E. coli* AW1.7 through plasmid curing, which also eliminated the LHR (Mercer et al., 2015; Pleitner et al., 2012). Isogenic LHR-positive and LHR-negative derivatives of *E. coli* AW1.7ΔpHR1 were generated by transformation with pLHR or the control plasmid pRK767 (Mercer et al., 2015). Strains of *E. coli* were cultured at 37 °C in Luria-Bertani (LB, Difco™, Becton Dickinson, Sparks, MD, USA) media. *E. coli* AW1.7ΔpHR1(pLHR) and *E. coli* AW1.7ΔpHR1(pRK767) were cultured in LB with 15 mg/L tetracycline-HCl to ensure plasmid maintenance.

Table 4-1 *E. coli* strains used in this research

Strains	Description	Reference
AW1.7	LHR-positive wild type isolate from carcass	Dlusskaya et al. (2011)
AW1.7ΔpHR1	LHR-negative, heat sensitive derivative of AW1.7	Pleitner et al. (2012)
AW1.7ΔpHR1(pLHR)	Transgenic LHR-positive derivative of AW1.7	Mercer et al. (2015)
AW1.7ΔpHR1(pRK767)	Transgenic LHR-negative derivative of AW1.7	Mercer et al. (2015)
AW1.3	Wild type isolate from carcass	Aslam et al. (2003)
GM14.3	Wild type isolate from ground meat	Aslam et al. (2003)
O157:H7; C0283	EHEC isolate from cattle faces	Liu et al. (2012)
O145:NM; 03-6430	EHEC isolate from human	Liu et al. (2012)
O26:H11; 05-6544	EHEC isolate from human	Liu et al. (2012)
O121:H19; 03-2832	EHEC isolate from human	Liu et al. (2012)
O145:NM; PARC 449	EHEC isolate from unknown source	Liu et al. (2012)

4.2.2 Meat products, marinades and chemicals.

Lean ground beef (15% fat) was purchased from local supermarket and stored at -20 °C in portions of 200 g until use. Cell counts of un-inoculated ground beef were enumerated on All Purpose Tween (APT) Agar (Difco) and Violet Red Bile (VRB) agar (Difco, Sparks, MD US), respectively. The plates were incubated at 37 °C for 24 h. Cell counts on APT agar and VRB agar were 3.5 ± 0.9 log (CFU/g) and 2.3 ± 0.3 log (CFU/g), respectively.

Chitosan with high molecular weight (210kDa) was supplied by Yuhan Ocean Biochemistry Co. Ltd. (Tauzhou, China). The deacetylation degree as determined by titration (Tolaimate et al., 2000) was 92%. Chitosan was dissolved in 1% (w/v) acetic acid (Fisher Scientific, Canada) and pH of the chitosan solution was adjusted to 5.4 with 10 M NaOH. Chitosan solutions were prepared on the day of use.

Teriyaki marinade was provided by Griffith Foods and contained sugar, salt, soy sauce powder (soy sauce from wheat and soybeans, corn maltodextrin), sodium phosphates, flavor, caramel, garlic powder, onion powder, spices, xanthan gum, monounsaturated vegetable oil, sulphites. Carvacrol was purchased from Fisher Scientific (New Jersey, USA). Potassium L-lactate solution used in this study contains around 60% potassium L-lactate (Sigma-Aldrich, St. Louis, USA)

4.2.3 Protocols for inoculation and treatment to study the effect of NaCl on the heat resistance of *E. coli* in ground beef.

E. coli were streaked onto LB agar and incubated at 37 °C for 18 h, and sub-cultured on LB agar, inoculated in LB broth and incubated at 37 °C for 18 h with agitation (200 rpm). EHEC cocktails were created by mixing equal volumes of each single strain stationary cultures. Five different protocols were used to sequentially mix ground beef and NaCl with

bacterial cultures (**Figure 4-1**). Cells were washed by centrifugation of cultures at 5311 x g and re-suspension in 0.1% peptone water. Washing was performed at 20°C (protocol B) or at 4°C (protocols D and E). Mixing of cells with meat and NaCl was performed in a Stomacher without pre-cooling of cultures (A, B and C); in protocols D and E, NaCl was added after mixing of cultures with meat and 12 h of refrigerated storage (**Figure 4-1**). Beef patties were heated after mixing with cells and NaCl and holding for 30 min at ambient temperature (protocols A, B and D), or after storage for 2 d at 4°C (protocol C and E). The temperature of the meat after inoculation was monitored by insertion of a thermometer (Tinytag, Interworld Electronics Inc., Markham, ON, Canada) into the geometric centre of the beef patty.

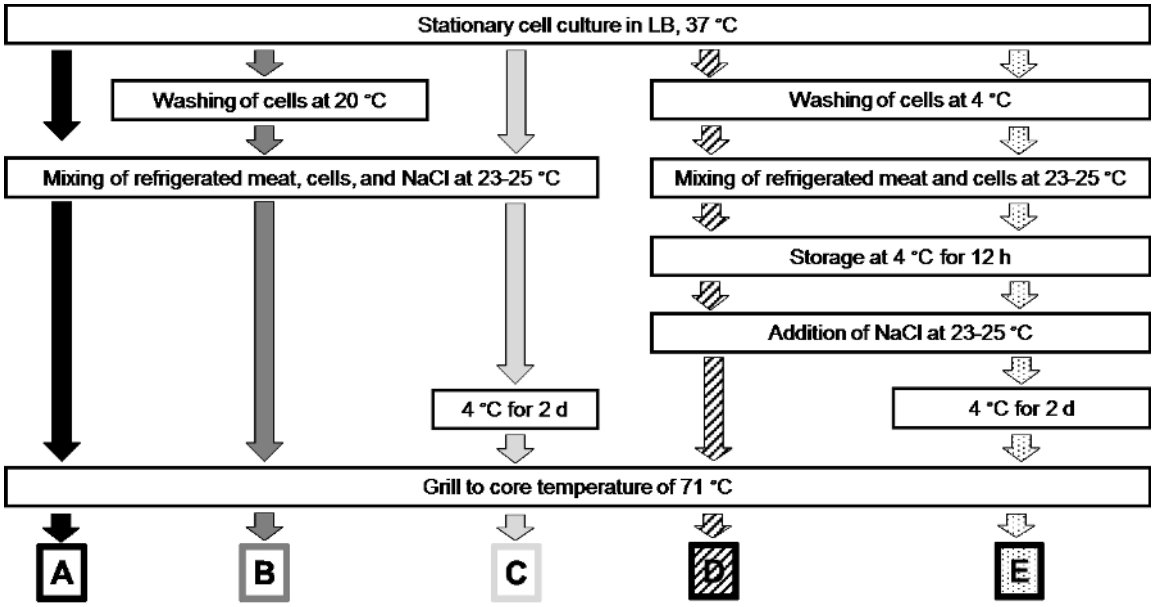

Figure 4-1 Different protocols (A, B, C, D, E) for inoculation and treatment to study the effect of NaCl on the heat resistance of *E. coli* in ground beef.

4.2.4 Effect of other additives on the heat resistance of *E. coli* in ground beef without storage.

The effects of other additives on the heat resistance of *E. coli* in ground beef were assessed with the protocol A (**Figure 4-1**). Teriyaki powder was first mixed with sterilized water at the ratio of 5.7:11 by weight to create a marinade solution; this marinade solution was mixed with 10 mL of stationary culture and 200 g ground beef. The composition of the final raw burger was 83.3% meat, 11% water, and 5.7% teriyaki powder by weight. The effect of carvacrol and potassium lactate was investigated by inoculation of 200 g ground beef with 10 mL of stationary culture. Carvacrol was dissolved in ethanol at the ratio of 1:1 by volume, dispersed in teriyaki marinade, and the carvacrol / teriyaki marinade then mixed with inoculated ground beef at a ratio of 16.7:83.3 (marinade solution: meat). The resulting final concentration of carvacrol was 0.10%. Potassium L-lactate solution was mixed with inoculated ground beef at a ratio of 3:100 (v/w). Chitosan solution (1%) was mixed with 200 g ground beef, followed by mixing with 10 mL of stationary culture. Mixing of ground beef, cells, and additives was achieved by stomaching for 2 min; after 35 minutes at 23-25 °C, beef patties were grilled as described below.

4.2.5 Effect of chitosan on survival of *E. coli* in ground beef during 2 d storage at 4 °C.

The effect of chitosan on heat resistance of *E. coli* in beef patties was additionally assessed with protocol E (**Figure 4-1**). Stationary cultures (20 mL) were washed with cold peptone water, mixed with 200 g ground beef, and stored at 4 °C for 12 h. After storage, the inoculated ground beef was mixed with 20 mL of 1% chitosan solution, stomached for 2

min at 23-25 °C, and stored at 4 °C for 48 h. After storage, a 10 g of raw meat was sampled and enumerated (cell counts at 2 day).

4.2.6 Sampling, grilling and enumeration of *E. coli*.

To determine the cell counts of samples, inoculated beef was sampled before storage (protocols C and E) and before grilling by sampling 10 g, followed by mixing with 20 mL of 0.1% peptone water in a stomacher. The homogenate was serially diluted, and viable cell counts were determined by plating appropriate dilutions on LB and VRB agars with a spiral plater, followed by incubation at 37 °C for 24 h.

Prior to grilling, ground beef was shaped into a ball, rolled onto the burger press covered by aluminium foil, and pressed to form a patty. Patties were cooked on a clamshell grill (Cuisinart 5-in-1 griddler, Woodbridge, ON, Canada) that was preheated to medium heat for at least 20 min. Monitoring of the internal temperature of the patty during the grilling process, sampling and enumeration after the complete grilling were conducted as follows: (1) In initial experiments to assess the effect of NaCl on survival of different heat resistant and heat sensitive *E. coli* strains, the internal temperature of the patty was monitored with one thermocouple (Tinytag, Interworld Electronics Inc., Markham, ON, Canada) inserted into the geometric centre of the patty. Once the core temperature reached 71 °C, the burger was placed in 200 mL of iced buffered peptone water and stomached for 2 min. The homogenate was serially diluted and the appropriate dilutions were plated on LB agar using a spiral plater and the plates were incubated overnight at 37 °C for 24 h. (2) Subsequent experiments to assess the effect of NaCl on survival of isogenic *E. coli* strains and EHEC in cooked beef patties, and to study the effect of other additives (teriyaki, carvacrol, potassium lactate and chitosan) on heat resistance of single isogenic *E. coli* strains in

ground beef were monitored with two thermocouples that were inserted approximately 1 cm to the left and to the right of the geometric centre of the patty. The temperature profile of beef patties monitored by two thermometers during grilling is shown in **Figure 4-2**. After both thermocouples indicated a temperature of 71 °C, a 10 g of meat between the two thermocouples was sampled with a corer. The cored meat was put into a filter stomacher bag with 20 mL of iced peptone water and stomached for 2 min; viable cell counts were determined by surface plating as indicated above.

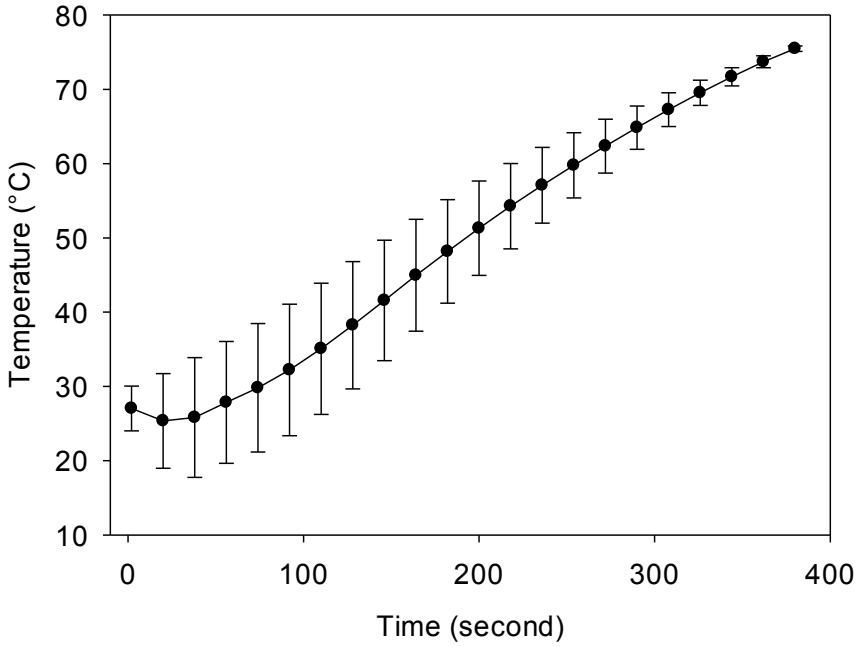

Figure 4-2 Core temperature profile of un-inoculated ground beef patties during grilling process

4.2.7 Statistical analysis.

Experiments were performed in biological triplicates. All data are expressed as means ± SD. Differences among variables were tested for significance by one-way or two-way ANOVA with Least Significant Difference (LSD) test using PASW Statistics 18 (SPSS

Inc., Chicago, IL, USA) for Windows 8.1. Differences at $P \leq 0.05$ were considered to be significant and $n \geq 2$.

4.3 Results

4.3.1 Effect of NaCl on survival of heat resistant and heat sensitive *E. coli* single strains in cooked beef patties

To assess the effect of NaCl on survival of *E. coli* after grilling of beef patties, NaCl and stationary cell culture were mixed with ground beef together, followed by cooking to a core temperature of 71 °C. The temperature was controlled by a single thermocouple inserted into the geometric centre of the patty. In burger patties without NaCl, the reduction of viable LHR-positive strains of *E. coli* was less than the reduction of LHR-negative and heat sensitive strains of *E. coli*. Addition of 3% NaCl did not alter the heat resistance of LHR-positive *E. coli* (**Figure 4-3A**), however, addition of NaCl increased the survival of LHR-negative *E. coli* and their survival in patties with 3% NaCl was equivalent to that of LHR-positive strains (**Figure 4-3B).**

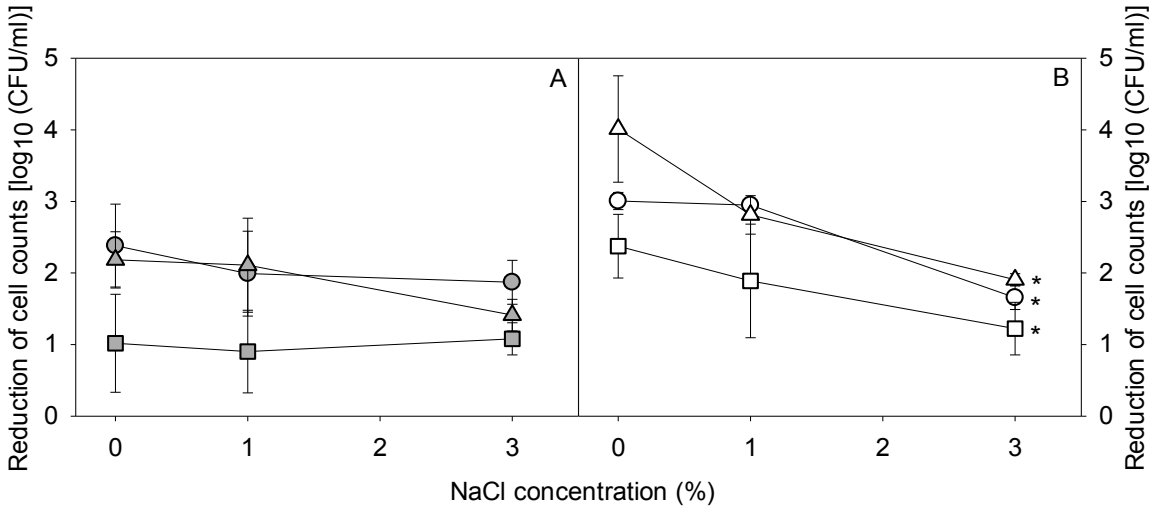

Figure 4-3 Reduction of heat resistant *E. coli* (Panel A) and heat sensitive *E. coli* (Panel B) after grilling the burger to 71 °C core temperature with 0% (control), 1% and 3% NaCl addition. Samples were prepared following protocol A, and the whole grilled burger was sampled for enumeration. Cell counts were enumerated on LB agar. Heat resistant strains in panel A: *E. coli* AW1.7 (grey dot), *E. coli* AW1.7ΔpHR1(pLHR) (black triangle), and

E. coli GM14.3 (grey square). Heat sensitive strains in panel B: *E. coli* AW1.7ΔpHR1 (white dot), *E. coli* AW1.7ΔpHR1(pRK767) (white triangle), and *E. coli* AW1.3 (white square). Error bars indicate the means ± standard deviation of triplicate independent experiments. Cell counts that are different from the cell counts of control group are indicated by an asterisk ($P<0.05$).

4.3.2 Factors affecting the effect of NaCl on survival of *E. coli* in cooked beef patties.

Initial experiments mixed cells of *E. coli* with NaCl and meat at a temperature of 15 – 20 °C, i.e. at a temperature that support a physiological response of *E. coli*. To determine whether an alteration of the sequence of the addition of NaCl and cooling or storage at 4 °C impacts heat resistance or the effect of NaCl on heat resistance, cells were prepared by different protocols as shown in **Figure 4-1**. Temperature profiles of meat handled with protocols B and D are shown in **Figure 4-4**. Simultaneous addition of cells and NaCl to refrigerated meat resulted in a temperature of about 20 °C (**Figure 4-4A**). In contrast, refrigeration of the meat after addition of *E. coli* allowed addition of NaCl at a temperature of 5 – 15 °C (**Figure 4-4B**). To minimize the temperature variation during grilling of patties, the temperature was monitored with two thermocouples and the meat between the two probes was used to enumerate surviving cells of *E. coli*. Grilling of burgers reduced cell counts of *E. coli* AW1.7ΔpHR1(pLHR) by around 3.5 (CFU/g) irrespective of the presence of NaCl or the protocol used for inoculation (**Table 4-2**). In contrast, cell counts of *E. coli* AW1.7ΔpHR1(pRK767) were not detectable after grilling of burgers without salt, regardless of the protocol used for inoculation. Addition of NaCl protected *E. coli* AW1.7ΔpHR1(pRK767) only in protocols A, B and C, i.e. when cells were kept at a temperature of more than 15°C during or after addition of NaCl (**Table 4-2**).

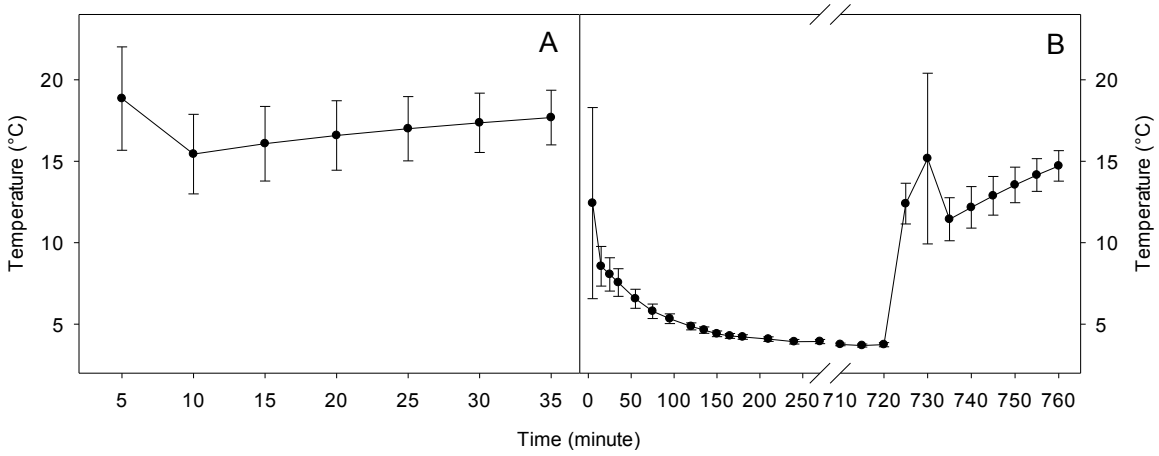

Figure 4-4 Temperature profile of raw samples treated with Protocol B (Panel A) or Protocol D (Panel B) shown in **Figure 4-1.** In Protocol B, *E. coli* cells that were washed at 23-25 °C, NaCl and raw ground beef were mixed together and stomached for 2 min, one thermal probe was inserted into the geometric centre of raw patty to monitor internal temperature of raw patty for 35 minutes at 23-25 °C. In Protocol D, raw ground beef was inoculated with *E. coli* cells washed at 4 °C, the raw patty was stored at 4 °C and one thermal probe was inserted into the geometric centre of raw patty to monitor internal temperature of raw patty for 720 min prior to the addition of NaCl (Panel B, from 5 min to 720 min). After refrigerated storage, NaCl was mixed with the cold patty and stomached for 2 min at 23-25 °C (Panel B, from 720 min to 725 min), and then the internal temperature of the patty was monitored as above for 35 min at 23-25 °C (Panel B, from 730 min to 760 min).

Table 4-2 Effect of different protocol on survival of *E. coli* AW1.7ΔpHR1(pLHR) and *E. coli* AW1.7ΔpHR1(pRK767) in burger with NaCl

	E. coli AW1.7ΔpHR1(pLHR)	*E. coli* AW1.7ΔpHR1(pRK767)
Treatment	Cell reductions after grilling [log10 (CFU/g)]	
Addition of cell culture and NaCl together (A)		
Control	3.4 ± 0.3	> 6.1
3% NaCl	3.7 ± 0.2	5.1±0.4
Addition of washed cell cultures and NaCl together (B)		
Control	3.5 ± 0.3	> 6.1
3% NaCl	3.4 ± 0.4	5.3 ± 0.7
Addition of cell culture and NaCl together, followed by storage at 4°C for 2 d (C)		
Control	ND	> 6.1
3% NaCl	ND	5.8 ± 0.6
Addition of washed cultures, followed by storage at 4°C for 12 h and addition of NaCl (D)		
Control	ND	> 6.1
3% NaCl	ND	> 6.1
Addition of washed cultures, followed by storage at 4°C for 12 h, addition of NaCl and storage at 4°C for 2 d (E)		
Control	3.7 ± 0.3	> 6.1
3% NaCl	3.8 ± 0.2	> 6.1

Detection limit: 1.6 log10 (CFU/g).
"ND" indicates "not determined"
Different protocols (A, B, C, D, E) for treatment indicated in this table are shown in **Figure 4-1** and elaborated in "Materials and methods" section 4.2.3. In each protocol, inoculated samples without NaCl addition were considered as control group, and core part of burger (10 g) was sampled for enumeration after core temperature of burger reached 71 °C. Cell reductions after grilling were determined on LB agar.

4.3.3 Effect of NaCl on survival of EHEC in grilled burger patties

To assess effect of NaCl on the heat resistance of EHEC in ground beef, a 5-strain cocktail of EHEC were inoculated into ground beef with 0% or 3% NaCl using protocol A (cells at ~ 20 °C at NaCl addition) or protocol D (cells at < 10 °C at NaCl addition) (Fig. 1 and 4). Similar to LHR-negative non-pathogenic *E. coli*, addition of 3% NaCl did not decrease the thermal inactivation of EHEC unless cells, NaCl and meat in were mixed at a temperature of about 20 °C (**Figure 4-5**). Cooling cells to 4 °C in raw meat for 12 h prior to NaCl

addition (**Figure 4-4B**) also eliminated the protective effect of NaCl against heat (**Figure 4-5**).

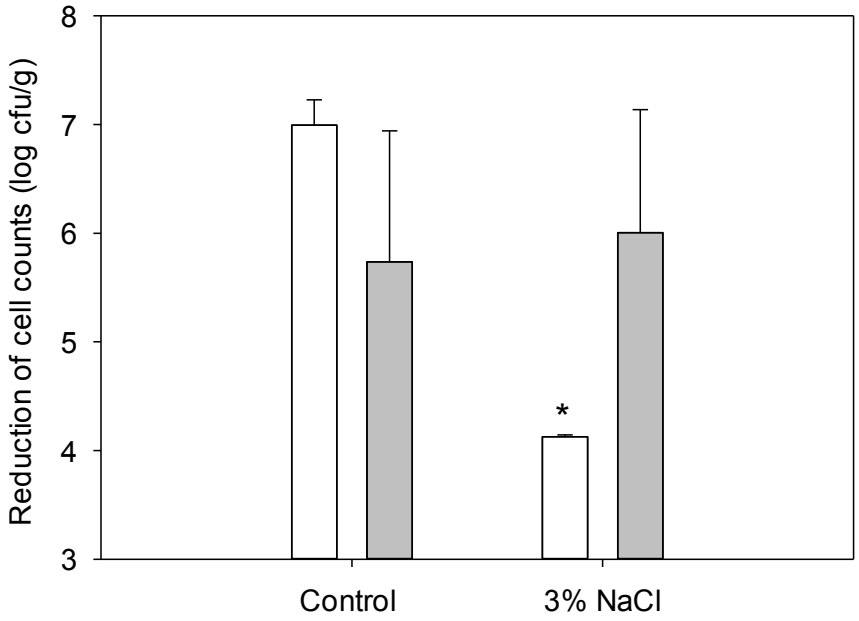

Figure 4-5 Effect of NaCl on survival of EHEC in burgers grilled to a core temperature of 71 °C. Samples were prepared and enumerated following protocol A (white bar) or protocol D (grey bar) shown in **Figure 4-1**. In each protocol, the core part of grilled burger (10 g) was sampled for enumeration. Cell reductions after grilling were determined on LB agar. Inoculated samples without NaCl addition were considered as the control group. Error bars indicate the means ± standard deviation of three independent experiments. Cell counts that are different from the cell counts of control group are indicated by an asterisk ($P<0.05$).

4.3.4 Effect of other additives on the heat resistance of *E. coli* in burger patties.

To determine whether other additives have a comparable effect as NaCl (marinade, potassium lactate) or can enhance thermal inactivation (carvacrol, chitosan), survival of *E. coli* in cooked patties was also evaluated using protocol A with *E. coli* AW1.7ΔpHR1(pLHR) and AW1.7ΔpHR1(pRK767) (**Figure 4-6A** and **B**). Chitosan enhanced thermal injury of the heat resistant strains (**Figure 4-6A**) while other additives had no effect on survival (**Figure 4-6A**). Teriyaki marinade improved survival of *E. coli*

AW1.7ΔpHR1(pRK767) in cooked patties (**Figure 4-6B**); in all other samples, the cell counts of this strain were reduced to levels below the detection limit.

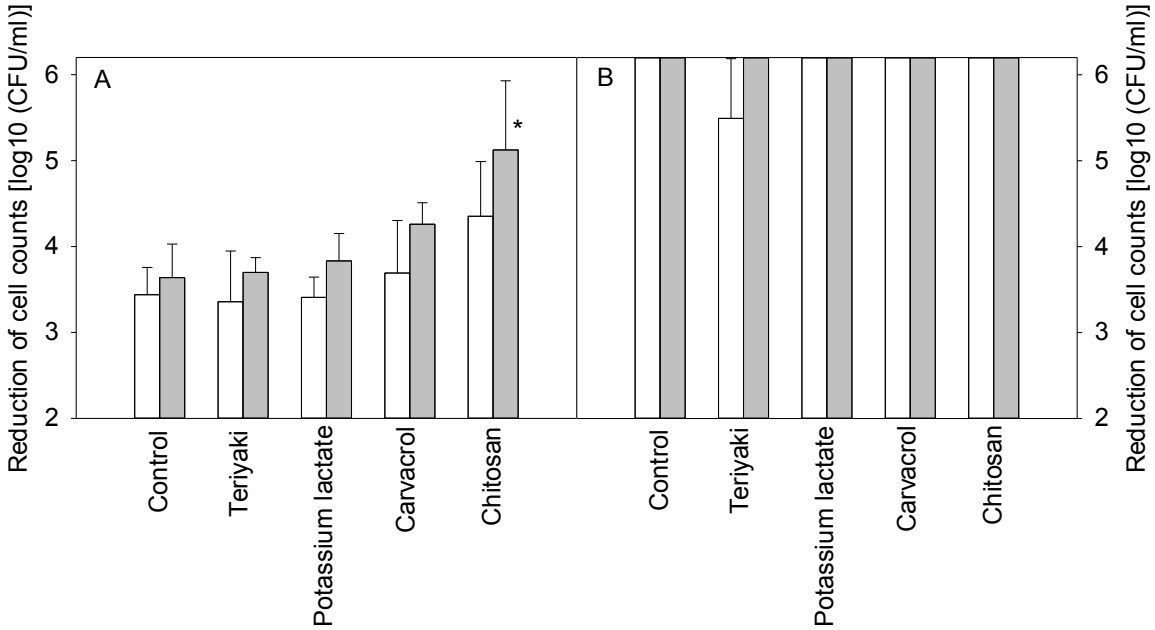

Figure 4-6 Reduction of *E. coli* AW1.7ΔpHR1(pLHR) (Panel A) and *E. coli* AW1.7ΔpHR1(pRK767) (Panel B) after grilling the burgers with different additives to 71 °C core temperature. Inoculated samples without additives were considered as control group. Core part of grilled burger (10 g) was sampled for enumeration. Cell counts were enumerated on LB agar (white bar) and VRB agar (grey bar). Error bars indicate the means ± standard deviation of three independent experiments. Differences among variables were tested for significance by one-way ANOVA with LSD test. Cell reductions that are different from cell reduction of control group are indicated by an asterisk ($P<0.05$).

4.3.5 Effect of chitosan on the heat resistance of *E. coli* in ground beef patties during 2 d cold storage.

To further validate the effect of chitosan on survival of *E. coli* in beef patties, LHR positive and negative isogenic strains of *E. coli* were inoculated into beef patties with or without chitosan, followed by refrigerated storage and cooking to 71°C (protocol E). Cell counts remained essentially stable throughout the 2 d of storage irrespective of the presence of the LHR, or the addition of chitosan. Grilling reduced cell counts of *E. coli* AW1.7ΔpHR1(pRK767) to levels below the detection limit irrespective of the presence of

chitosan (**Figure 4-7**). Cell counts of *E. coli* AW1.7ΔpHR1(pLHR) remained at about 4 log(CFU/g) after cooking to 71 °C; the addition of chitosan reduced cell counts of this strain by 0.5 to 1 log (CFU/g) (**Figure 4-7**).

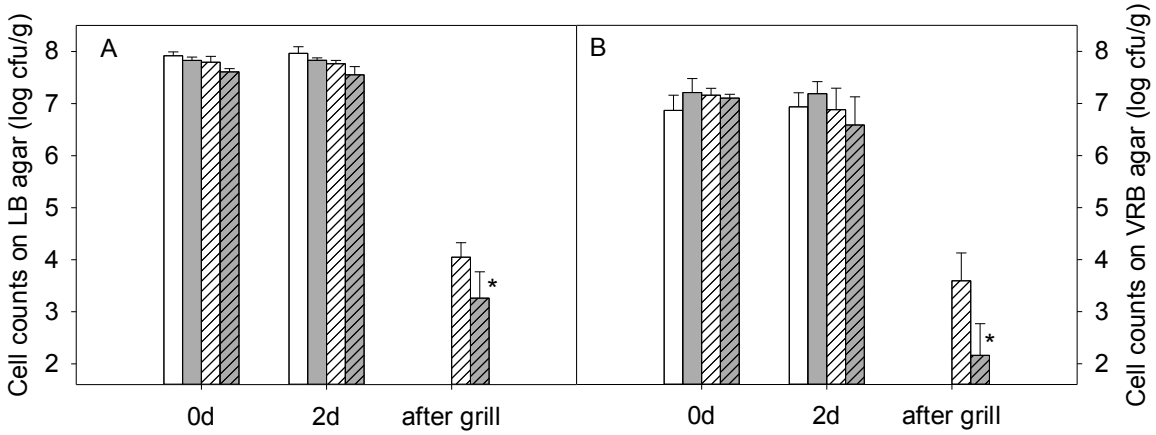

Figure 4-7 Effect of chitosan on the survival of *E. coli* AW1.7ΔpHR1(pRK767) (non-hatched bar) and *E. coli* AW1.7ΔpHR1(pLHR) (hatched bar) in burgers during 2 d of storage at 4 °C. Cell counts were enumerated on LB agar (Panel A) and VRB agar (Panel B). Core part of grilled burger (10 g) was sampled for enumeration. Cell counts of inoculated samples without chitosan and inoculated samples with chitosan were shown as white and grey bars, respectively. Error bars indicate the means ± standard deviation of three independent experiments. Differences among variables were tested for significance by two-way ANOVA with LSD test. Cell counts that are significantly lower than the cell counts of cooked samples without additives are indicated by an asterisk ($P<0.05$).

4.4 Discussion

Addition of 3% NaCl increased heat resistance in the LHR-negative isogenic strain of *E. coli* and EHEC cocktails if bacterial cells were combined with NaCl and meat simultaneously, and adapted to osmotic shock at 23-25 °C. The resulting inactivation of EHEC was less than 5 log (CFU/g) after cooking to recommended temperatures. To eliminate the risk of EHEC, a full lethality by 5 log (CFU/g) cell reduction is required by Canadian Food Inspection Agency (CFIA) (CFIA, 2015). Increased heat resistance of *E. coli* was previously reported in laboratory model systems, or in beef heated to 55.0-62.5 °C (Juneja et al., 2015; Mercer et al., 2017; Pleitner et al., 2012). This study validates the

protective effect of NaCl on survival of *E. coli* in meat cooked by the recommended cooking guidelines.

Accumulation of compatible solutes in response to hyperosmotic conditions, such as the presence of NaCl, increases resistance of bacteria to diverse environmental insults (Pleitner et al., 2012; Sleator and Hill, 2010). The capacity of compatible solutes to protect bacterial proteins and thermal ribosome against heat were demonstrated in media conditions (Herberhold et al., 2004; Pleitner et al., 2012; Ruan et al., 2003). Osmolytes that are accumulated in response to osmotic stress are preferentially excluded from the hydration shell surrounding the surface of proteins, and may increase the stability of bacterial proteins by increasing the surface tension on the exterior of the protein and decreasing the overall free energy (Parsegian et al., 2000; Timasheff, 2002). Therefore, the protective effect of NaCl against heat-induced inactivation of LHR-negative single *E. coli* strains and EHEC cocktails in ground beef may be attributed to the accumulation of compatible solutes in response to an increased osmotic stress. Remarkably, synergism of LHR and osmotic stress caused by addition of 3% NaCl in protecting cells from thermal inactivation was observed in media (Mercer et al., 2017; Pleitner et al., 2012) but not in ground beef (this study), indicating that NaCl and the accumulation of compatible solutes provide no incremental protection if LHR-positive strains are heated in a protective meat matrix.

The protective effect of NaCl was observed only if bacterial cells adapted to the osmotic shock at a temperatures above 15 °C, highlighting an appropriate response of *E. coli* to osmotic shock contributes to improved survival of *E. coli* in meat after cooking. Low temperature blocks the initiation of translation in *E. coli* (Jones and Inouye, 1994). Cold shock of cell cultures of *E. coli* at temperatures below 15°C increases the cell permeability,

and consequently decreases the heat resistance of *E. coli* (Cao-Hoang et al., 2010; Katsui et al., 1981). These findings may additionally explain why the protective effect of NaCl against heat was not observed for the cold-shocked cells in this study.

Teriyaki marinade exhibited a similar protective effect against heat as NaCl, thus extending previous results obtained with NaCl and confirming the proposed theory elucidating the role of osmotic shock in increasing heat resistance. Addition of about 1.8% (w/w) potassium lactate did not enhance the thermal inactivation of *E. coli* when ground beef was cooked to an internal temperature of 71 °C. Likewise, previous studies also demonstrated that addition of lactate at the level of 1.8%-4.5% did not affect survival of *E. coli* in meat cooked to 55-65 °C (Huang and Juneja, 2003; Mukherjee et al., 2008). Meat with a pH of 5.5, such as raw ground beef, already contains about 0.9% (wet weight) of lactic acid (Pothast and Hamm, 1976). Carvacrol at 0.5 to 1.0% (v/w) increased inactivation of *E. coli* O157:H7 in ground beef (Juneja and Friedman, 2008), while carvacrol at 0.1% (v/w) had no effect (this study). The dose-dependent bacteridical effect of essential oils needs to be balanced with their impact on food flavour (Jayasena and Jo, 2013).

Chitosan potentiated the thermal injury and inactivation of LHR-positive *E. coli* irrespective of the protocol used for application of NaCl, cells, and chitosan. Chitosan with molecular weight of 1.5 KDa also enhanced thermal inactivation of EHEC in ground beef patties stored at 4 °C for 5 days by around 2 log (CFU/g), and the combination of rutin (RT) or resveratrol (RV) with chitosan resulted in thermal destruction of EHEC by 5 log (CFU/g) (Surendran Nair et al., 2016). Chitosan is protonated when the ambient pH is below its pKa of 6.2-7.0 (Tsai and Su, 1999). Polycationic chitosan disrupts the integrity of negatively charged cell envelope of *E. coli*, including lipopolysaccharides (LPS) and the cytoplasmic

membrane, through electrostatic interactions (Helander et al. 2001, Liu et al. 2004; Mellegård et al. 2011), thus probably sensitizing *E. coli* to heat. This study validated the effect of high molecular weight chitosan on thermal destruction of LHR-positive *E. coli* in ground beef, indicating that chitosan has the potential to reduce the risk of LHR-positive *E. coli* to meat safety when used in conjunction with the recommended cooking procedures.

In conclusion, the present study demonstrated that addition of 3% NaCl increased heat resistance in LHR-negative isogenic *E. coli* strain and EHEC cocktails in ground beef if bacterial cells were well adapted to osmotic stress, resulting in cell reductions for EHEC by less than 5 log (CFU/g) after the cooking process recommended as a safe handling. These highlight that salt addition in meat may incur the generation of heat resistant EHEC, thus creating an additional risk to meat safety. Nevertheless, the protective effect of NaCl was not observed if bacterial cells were cooled to 4 °C prior to mixing with cold meat and NaCl, indicating that the response of *E. coli* to osmotic shock contributes to this effect. Therefore, chilling the meat prior to salt addition in conjunction with a subsequent chilling process after salt addition is proposed to mitigate the protective effect of NaCl against heat. Chitosan potentiated the thermal destruction of LHR-positive *E. coli* in ground beef stored at 4 °C for 2 days, indicating that the combination of chitosan with the cooking process recommended currently has the potential to reduce the potential risk of LHR-positive *E. coli* to meat safety.

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Chapter 5 Effect of locus of heat resistance (LHR) and chitosan on pressure resistance of *Escherichia coli* and *Salmonella*

5.1 Introduction

Foodborne outbreaks associated with *Escherichia coli* and *Salmonella* are continuing worldwide despite current pathogen intervention technologies (CDC, 2017; Nguyen and Sperandio, 2012; Pui et al., 2011). Pressure processing extends the shelf life of many ready to eat foods without compromising food quality and may improve food safety depending on food matrix and pathogen (Balasubramaniam et al., 2015; Buckow et al., 2013; Considine et al., 2008). *E. coli* resists pressure in meat, and considerable intraspecies variability in pressure resistance exists (Liu et al., 2015). The *rpoS*, which encodes sigma factor σ^S (or RpoS), is a central determinant in pressure resistance of *E. coli* and intraspecies variability in pressure resistance may be attributed to the sequence polymorphisms in the *rpoS* locus (Charoenwong et al., 2011, Robey et al., 2001; Vanlint et al., 2013a; Vanlint et al., 2013b). In addition, the genes involved in mitigation of oxidative stress, including *katE*, *oxyR*, *sodAB*, *soxS*, *TrxA* and *TrxB*, the *crp* encoding catabolite response protein (CRP) and the *cyaA* encoding adenylate cyclase (CyaA) also contribute to pressure resistance (Aertsen et al., 2005; Malone et al., 2006; Vanlint et al., 2013b). Nevertheless, other genetic predispositions associated with pressure resistance development may still exist (Vanlint et al., 2013b).

Locus of heat resistance (LHR), a 14-kb genomic island that was initially identified in *E. coli* AW1.7, confers an exceptional heat resistance in *E. coli* and *Salmonella* (Dlusskaya et al., 2011; Mercer et al., 2015; Mercer et al., 2017), presumably through encoding a series of stress proteins associated with protein homeostasis (Lee et al., 2016), protection against envelope stress, and mitigation of oxidative stress (Boll et al., 2017; Lee et al., 2015;

Mercer et al., 2015). Some heat resistant and LHR-positive strains of *E. coli* are also pressure resistant. Remarkably, some LHR negative *E. coli* strains that were heat sensitive were still resistant to pressure (Garcia-Hernandez et al., 2015; Liu et al., 2015), suggesting that *E. coli* may acquire pressure resistance through other routes independent of LHR. Accordingly, the role of LHR in mediating pressure resistance and the correlation between LHR and other stress-responsive genes that have already been reported to affect pressure resistance still need to be elucidated.

Limited pressure-induced inactivation of LHR positive *E. coli* strains and lack of effective hurdles that can enhance pressure lethality in ground beef (Li and Gänzle, 2016) prompt the development of other novel hurdles that can increase pressure lethality and provide additional safety assurance for consuming ground beef. Chitosan, a linear polysaccharide consisting of β-(1→4)-linked glucosamine and N acetyl-D-glucosamine that has obtained GRAS approval in the U.S.A. (FDA, 2011), exerts antimicrobial activity when its amino group is protonated below the pKA of 6.2-7.0 (Tsai and Su, 1999) and sensitizes *E. coli* to pressure in buffer and apple juice presumably through perturbation of outer membrane (Helander et al., 2001; Kumar et al., 2009; Malinowska-Pańczyk et al., 2009; Mellegard et al., 2011). However, it remains unclear that whether the LHR mediates cross-resistance to chitosan and whether chitosan and pressure processing synergistically inactivate *E. coli* or *Salmonella* in meat.

Therefore, the objectives of this study were to elucidate the effect of LHR on pressure resistance of *E. coli* and *Salmonella*, and to assess the combined effect of chitosan and pressure on inactivation of *E. coli* and *Salmonella* in buffer and ground beef.

5.2 Materials and methods

5.2.1 Bacterial strains and culture conditions

Bacterial strains and their origin are listed in **Table 5-1**. *E. coli* and *Salmonella* were cultivated at 37 °C in Luria–Bertani (LB) broth (Difco; BD, Sparks,MD, USA). Stock cultures stored at −80 °C were subcultured by streaking on LB agar (Difco; BD) for 16-18 h, followed by a second subculture and incubation in LB broth. Strains were subcultured in broth and incubated at 37 °C for 16-18 h following either of the two methods: (1) Aerobic incubation: strains were inoculated into 4 mL of LB broth in a sterile glass test tube with the length of 14.5 cm and the orifice diameter of 1.5 cm. The opening of with the tube was just covered by a sterile plastic clear cap, and the tube was fixed at a 45-degree angle from the horizontal plane. Strains were incubated with agitation at 250 rpm. (2) Microaerophilic incubation: strains were inoculated into 4 mL of LB broth in a Falcon™ 15 mL conical centrifuge tube. The screw cap was screwed tightly on the top of the tube, and the tube was fixed at a 45-degree angle from the horizontal plane. Strains were incubated with agitation at 200 rpm.

Table 5-1 Bacterial strains used in chapter 5

Strains	Description	Reference
E. coli		
AW1.7	LHR-positive wild type isolate from the carcass	Dlusskaya et al. (2011)
MG1655(pLHR)	Transgenic LHR-positive derivative of MG1655	Mercer et al. (2015)
MG1655(pRK767)	LHR-negative vector control for MG1655 carrying pLHR	Mercer et al. (2015)
MG1655Δ*cfa*(pLHR)	Transgenic LHR-positive derivative of MG1655Δ*cfa*	This study
MG1655Δ*cfa*(pRK767)	LHR-negative vector control for MG1655Δ*cfa* carrying pLHR	This study

Strains	Description	Reference
MG1655Δ*ompR*(pLHR)	Transgenic LHR-positive derivative of MG1655Δ*ompR*	This study
MG1655Δ*ompR*(pRK767)	LHR-negative vector control for MG1655Δ*ompR* carrying pLHR	This study
MG1655Δ*sodA*(pLHR)	Transgenic LHR-positive derivative of MG1655Δ*sodA*	This study
MG1655Δ*sodA*(pRK767)	LHR-negative vector control for MG1655Δ*sodA* carrying pLHR	This study
MG1655Δ*oxyR*(pLHR)	Transgenic LHR-positive derivative of MG1655Δ*oxyR*	This study
MG1655Δ*oxyR*(pRK767)	LHR-negative vector control for MG1655Δ*oxyR* carrying pLHR	This study
MG1655Δ*dps*(pLHR)	Transgenic LHR-positive derivative of MG1655Δ*dps*	This study
MG1655Δ*dps*(pRK767)	LHR-negative vector control for MG1655Δ*dps* carrying pLHR	This study
MG1655Δ*trxA*(pLHR)	Transgenic LHR-positive derivative of MG1655Δ*trxA*	This study
MG1655Δ*trxA*(pRK767)	LHR-negative vector control for MG1655Δ*trxA* carrying pLHR	This study
MG1655Δ*katE*(pLHR)	Transgenic LHR-positive derivative of MG1655Δ*katE*	This study
MG1655Δ*katE*(pRK767)	LHR-negative vector control for MG1655Δ*katE* carrying pLHR	This study
MG1655Δ*evgA*(pLHR)	Transgenic LHR-positive derivative of MG1655Δ*evgA*	This study
MG1655Δ*evgA*(pRK767)	LHR-negative vector control for MG1655Δ*evgA* carrying pLHR	This study
MG1655Δ*rpoS*(pLHR)	Transgenic LHR-positive derivative of MG1655Δ*rpoS*	This study
MG1655Δ*rpoS*(pRK767)	LHR-negative vector control for MG1655Δ*rpoS* carrying pLHR	This study
S. enterica		
ATCC43845	LHR-positive wild type strain; serovar Senftenberg.	ATCC
ATCC13311	LHR-negative wild type strain; serovar Typhimurium	ATCC
ATCC13311(pLHR)	Transgenic LHR-positive derivative of ATCC13311	Mercer et al. (2017)
ATCC13311(pRK767)	LHR-negative vector control for ATCC13311 carrying pLHR	This study

5.2.2 Determination of heat resistance in broth

Aliquots of 100 µL overnight cultures were transferred into a 200 µL PCR tube and heated in a PCR thermal cycler at 60 °C for 5 min. Serial dilutions of treated and untreated cultures in 0.1% sterile peptone (Becton Dickinson, Sparks, MD, USA) water were plated on LB agar (Difco; BD) using a spiral plater (Don Whitely Scientific, Shipely, UK). Plates were incubated at 37 °C for 16-24 h.

5.2.3 Determination of pressure resistance in broth

Pressure treatments were carried out in a high-pressure system (Micro-system, Unipress, Warsaw, Poland) as described previously (Teixeira et al., 2016). Bis (2-ethylhexyl) sebacate (Sigma-Aldrich, Germany) served as the pressure-transmitting medium. Aliquots of 123 µL overnight cultures were packed into a 3 cm Tygon tubing (Tygon S3™ E-3603 Flexible Tubings, Fisherbrand™, Pittsburgh, USA) and heat sealed after exclusion of air bubbles. Samples were held at 23-25 °C for less than 3 h until pressure treatment. After placement of the samples in the autoclave vessel filled with the pressure-transmitting medium, treatment at 400 MPa at 20 °C for 6 min were carried out. The rate of compression and decompression was 277.8 MPa/min. The temperature of the unit was maintained by submerging the autoclave vessel in a water bath (Isotemp, Fisher Scientific, USA) filled with distilled water, and the internal temperature was measured by an integrated type K thermocouple positioned inside the vessel. Immediately after pressure treatment, the tubes were opened aseptically, and then serial dilutions were made and enumerated on LB agar as described above.

5.2.4 Effect of chitosan on pressure resistance in buffer

Chitosan with high molecular weight (210 kDa) was purchased from Yuhan Ocean Biochemistry Co. Ltd. (Tauzhou, China). The deacetylation degree was 92%, which was determined by a pH titration method (Tolaimate et al., 2000). Chitosan was dissolved in 1% (w/v) acetic acid (Fisher Scientific, Canada) to the concentration of 1%, and pH of chitosan solution was adjusted to 5.4 with 10 M NaOH. This chitosan stock solution was stored at 4 ℃ for use within one week. To evaluate the effect of chitosan on pressure resistance in buffer, strains were incubated at microaerophilic conditions to the stationary phase as described above (5.2.1). Stationary cell culture (2 mL) were centrifuged with the speed of 5311 RCF at 20 °C for 20 minutes, and then the cell pellets were re-suspended with the same volume of 100 mM 2-(N-morpholino)ethane sulfonic acid (MES) buffer (pH=5.4), 0.5% acetic acid solution (pH=5.4), or 0.5% chitosan solution (pH=5.4). Aliquots of 123 μL cell suspension were packed, heat sealed, and treated at 400 MPa at 20 °C for 3 or 6 min. After pressure treatment, surviving cells were immediately enumerated on LB agar as described above.

5.2.5 Effect of chitosan on pressure resistance in ground beef

Lean ground beef (15% fat) was supplied by Cargill (Spruce Grove, Canada), divided into 5 g portions which were stored in sterile sample bags at −18 °C until use. Cell counts of uninoculated samples for each batch were determined by surface plating on LB agar and Violet Red Bile Agar (VRBA; Difco, BD). Cell counts on LB agar and Violet Red Bile Agar (VRBA; Difco, BD) were below the detection limit 2.3 log (CFU/g). *E. coli* AW1.7 or *S.* Senftenberg ATCC43845 were incubated microaerophilically to the stationary phase as described above (5.2.1). Stationary cell cultures (0.5 mL) were inoculated into meat (5

g) to achieve initial cell counts of about 8.5 log (CFU/g). The inoculated samples were manually homogenized for 2 min and held at 23-25 °C for 15 min. Afterward, inoculated meat was mixed thoroughly with 0.5 mL of 1% chitosan solution (pH=5.4) for 2 min. Aliquots of 0.3 g samples with or without chitosan were packed into a 3.5 cm Tygon tubing, heat sealed and held at 23-25 °C for 4 h until pressure treatment. Samples were treated at 400 MPa at 20 °C for 3 or 6 min, followed by immediate enumeration on LB agar and VRB agar as described above.

5.2.6 Statistical analysis

Experiments were performed in biological triplicates. All data are expressed as means ± SD. Differences among variables were tested for significance by two-way ANOVA with Least Significant Difference (LSD) test using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) for Windows 8.1. Differences at $P \leq 0.05$ were considered to be significant and n=3.

5.3 Results

5.3.1 Effect of incubation conditions, LHR and other stress-responsive genes on heat and pressure resistance of *E. coli*.

To assess the effects of incubation condition and LHR on heat and pressure resistance in *E. coli*, *E. coli* MG1655 with empty plasmid or plasmid carrying LHR were incubated aerobically or microaerophilically to stationary phase for 18 h, followed by treatment at 60 °C for 5 min or 400 MPa at 20 °C for 6 min.

After 18 h incubation, the pH of cultures that were incubated aerobically was 8.5 while the pH of cultures incubated microaerophilically was 6.5. Treatment at 60 °C for 5 min reduced cell counts of *E. coli* MG1655(pRK767) by more than 7 log (CFU/mL) unconditionally. Thermal inactivation of *E. coli* MG1655(pLHR) was less than 1.5 log

(CFU/mL) irrespective of incubation condition (**Table 5-2**). The LHR thus conferred heat resistance in *E. coli* MG1655 irrespective of incubation condition. The LHR reduced pressure-induced inactivation of *E. coli* MG1655 by 1 log (CFU/mL) when cells were incubated aerobically, while cells incubated microaerophilically resisted the pressure treatment with a reduction of cell counts by less than 1 log (CFU/mL) irrespective of the presence of LHR (**Table 5-2**).

To assess the influence of other stress-responsive genes on the protective effect of LHR, different isogenic mutant strains of *E. coli* MG1655 with or without the LHR were incubated and treated as mentioned above. Regardless of incubation condition, heat treatment reduced cell counts of all LHR negative mutants by more than 7 log (CFU/mL). Almost all LHR positive mutants resisted the heat treatment with a minimal reduction of cell counts by less than 2 log (CFU/mL), and the only exception was the *rpoS* negative mutant carrying LHR, which was heat resistant only when cells were incubated microaerophilically (**Table 5-2**). The LHR conferred pressure resistance in all mutants except for *rpoS* negative mutant only when cells were incubated aerobically. Deletion of *rpoS* eliminated the baroprotective effect of the LHR (**Table 5-2**).

Table 5-2 Cell reductions of isogenic strains of *E. coli* MG1655 after treatment at 60 °C for 5 minutes or 400 MPa at 20 °C for 6 minutes

	Cell reduction after heat [log10 (CFU/mL)]		Cell reduction after pressure [log10 (CFU/mL)]	
Strain	Aerobic	Microaerophilic	Aerobic	Microaerophilic
pRK767	> 7.0	> 7.0	5.3 ± 0.4	0.2 ± 0.2
pLHR	1.4 ± 0.5*	0.6 ± 0.2*	4.4 ± 0.2*	0.6 ± 0.2
Δ*cfa*(pRK767)	> 7.0	> 7.0	5.6 ± 0.2	0.1 ± 0.1
Δ*cfa*(pLHR)	1.6 ± 0.1*	0.8 ± 0.1*	3.9 ± 0.7*	0.6 ± 0.1
Δ*ompR*(pRK767)	> 7.0	> 7.0	4.1 ± 0.4	0.3 ± 0.2
Δ*ompR*(pLHR)	0.9 ± 0.4*	0.5 ± 0.2*	3.3 ± 0.1*	0.5 ± 0.4
Δ*sodA*(pRK767)	> 7.0	> 7.0	5.0 ± 0.9	0.3 ± 0.1
Δ*sodA*(pLHR)	1.5 ± 0.3*	0.8 ± 0.2*	4.0 ± 0.5*	0.7 ± 0.1
Δ*oxyR*(pRK767)	> 7.0	> 7.0	4.7 ± 0.7	0.7 ± 0.4
Δ*oxyR*(pLHR)	1.2 ± 0.1*	0.9 ± 0.1*	3.8 ± 0.5*	0.7 ± 0.2
Δ*dps*(pRK767)	> 7.0	> 7.0	5.7 ± 0.8	0.4 ± 0.3
Δ*dps*(pLHR)	1.9 ± 0.3*	0.7 ± 0.1*	4.5 ± 0.9*	1.0 ± 0.3
Δ*trxA*(pRK767)	> 7.0	> 7.0	4.8 ± 0.6	0.5 ± 0.1
Δ*trxA*(pLHR)	1.2 ± 0.1*	0.9 ± 0.5*	3.8 ± 0.9*	0.5 ± 0.1
Δ*katE*(pRK767)	> 7.0	> 7.0	6.1 ± 0.7	0.6 ± 0.2
Δ*katE*(pLHR)	1.5 ± 0.2*	0.5 ± 0.1*	4.8 ± 0.3*	0.9 ± 0.1
Δ*evgA*(pRK767)	> 7.0	> 7.0	5.8 ± 0.3	0.3 ± 0.2
Δ*evgA*(pLHR)	1.1 ± 0.1*	0.6 ± 0.1*	4.2 ± 0.5*	0.7 ± 0.2
Δ*rpoS*(pRK767)	> 7.0	> 7.0	> 7.0	2.0 ± 0.3
Δ*rpoS*(pLHR)	> 7.0	1.3 ± 0.5*	> 7.0	2.0 ± 0.4

Aerobic: cell reduction for stationary culture incubated aerobically.
Microaerophilic: cell reduction for stationary culture incubated microaerophilicaly.
Data are shown as means ± standard deviations of triplicate independent experiments. Asterisks indicate that the LHR positive strain was significantly more resistant than its LHR negative counterpart under the same incubation and treatment ($P < 0.05$).

5.3.2 Heat and pressure resistance of different *Salmonella* strains

To investigate the effect of the LHR on heat and pressure resistance in *Salmonella*, LHR-negative and LHR-positive *S. enterica* strains were incubated and treated at the same conditions as *E .coli*. LHR-negative *S. enterica* strains were as heat sensitive as LHR-

negative *E. coli* strains with more than 7 log (CFU/mL) cell reductions after heat irrespective of incubation condition (Table 3). Different from *E. coli* MG1655(pLHR), *S. enterica* ATCC13311(pLHR) was heat resistant only when cells were incubated microaerophilically (**Table 5-2** and **5-3**). *S. enterica* ATCC13311(pLHR) was more resistant to pressure than *S. enterica* ATCC13311(pRK767) when cultures were aerobically incubated. Remarkably, heat or pressure treatment only reduced cell counts of wild type LHR-positive *S.* Senftenberg ATCC43845 by 1 log (CFU/mL) irrespective of incubation condition (**Table 5-3**).

Table 5-3 Cell reductions of wild-type and isogenic strains of *S. enterica* after treatment at 60 °C for 5 minutes or 400 MPa at 20 °C for 6 minutes

	Cell reduction after heat [log10 (CFU/mL)]		Cell reduction after pressure [log10 (CFU/mL)]	
Strain	Aerobic	Microaerophilic	Aerobic	Microaerophilic
ATCC13311 (pRK767)	> 8.0	> 8.0	7.1 ± 0.2	1.4 ± 0.4
ATCC13311 (pLHR)	7.7 ± 0.5	0.9 ± 0.5*	5.8 ± 0.5*	3.6 ± 0.3
ATCC13311	> 8.0	7.8 ± 0.7	7.6 ± 0.6	4.8 ± 0.4
ATCC43845	1.2 ± 0.04*	0.6 ± 0.3*	1.2 ± 0.2*	1.3 ± 0.3*

Aerobic: cell reduction for stationary culture incubated aerobically.
Microaerophilic: cell reduction for stationary culture incubated microaerophilicaly.
Data are shown as means ± standard deviations of triplicate independent experiments. Asterisks indicate that the LHR positive strain was significantly more resistant than its LHR negative counterpart under the same incubation and treatment ($P < 0.05$).

5.3.3 Effect of chitosan on pressure resistance of *E. coli* and *S. enterica* in buffer.

LHR mediates pressure resistance depending on the genetic background of strains, and perhaps also mediates cross-resistance to other antimicrobial hurdles. To investigate the effect of LHR on chitosan activity, and to assess the effect of chitosan on pressure lethality in buffer, *E. coli* AW1.7, *S.* Senftenberg ATCC43845, single isogenic *E. coli* MG1655 carrying LHR and its LHR negative control were incubated microaerophilically to

stationary phase, washed by chitosan, acetic acid or MES buffer, and then subjected to treatment at 400 MPa at 20 °C for 3 or 6 min. Initial cell counts of 4 tested strains after incubation were around 8-9 log (CFU/mL) (**Figure 5-1**). Before pressure treatment, loss of viability by 1-2 log (CFU/mL) was observed only when cells were suspended in chitosan solutions for 3 h. Chitosan increased pressure-inactivation of *E. coli* strains and *S.* Senftenberg ATCC43845 by 1 and 3 log (CFU/mL), respectively, indicating that chitosan sensitized bacterial cells to pressure treatment (**Figure 5-1**).

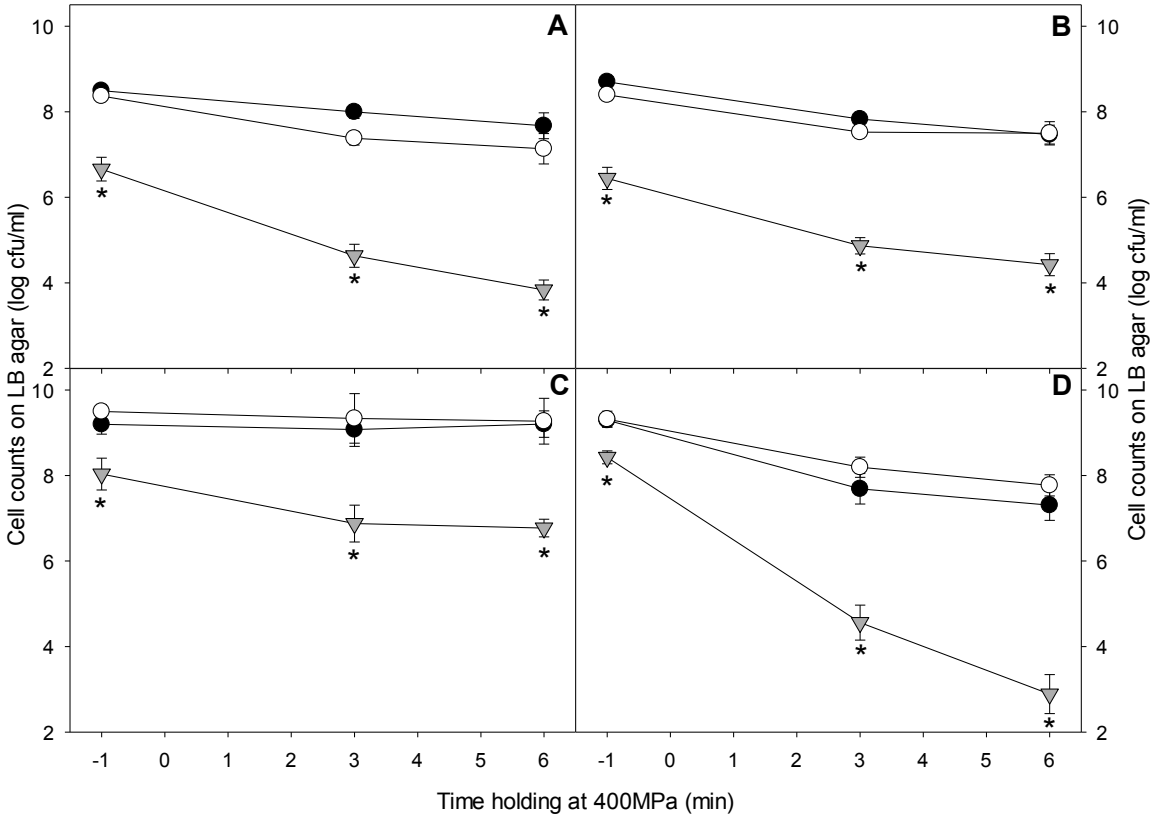

Figure 5-1 Cell counts of *E. coli* MG1655(pRK767) (Panel A), *E. coli* MG1655(pLHR) (Panel B), *E. coli* AW1.7 (Panel C) or *S.* Senftenberg ATCC 43845 (Panel D) in 100 mM MES buffer (pH=5.4, black dot), 0.5% acetic acid solution (pH=5.4, white dot), or 0.5% chitosan solution (pH=5.4, grey triangle) before and after treatment at 400 MPa at 20 °C. Samples were held at ambient condition (23-25 °C) for 3 h before pressure treatment and symbols at the holding time of −1 min represents cells counts of unpressurized samples that were held at ambient condition for 3 h. Cells counts were enumerated on LB agar. Data are shown as mean ± standard deviation of triplicate independent experiments. At each holding time point, the treatment significantly more lethal than the other two treatments is indicated by an asterisk ($P<0.05$).

5.3.4 Effect of chitosan on pressure resistance of *E. coli* and *S. enterica* in ground beef.

To assess the combined effect of chitosan and pressure treatment on survival of *E. coli* AW1.7 and *S.* Senftenberg ATCC43845 in ground beef, stationary cell cultures were inoculated into ground beef with or without the addition of chitosan, and treated at 400 MPa at 20 °C for 3 or 6 min. Before pressure treatment, bactericidal effects of chitosan by 1-1.5 log (CFU/g) were observed after holding samples at 23-25 °C for 4 h (**Figure 5-2**). Without chitosan, pressure treatment for 6 min reduced cell counts of *E. coli* and *S.* Senftenberg ATCC43845 by 1 log (CFU/g) and 4.5 log (CFU/g), respectively (**Figure 5-2A** and **C**), indicating that *S.* Senftenberg ATCC43845 was more sensitive to pressure than *E. coli* AW1.7 in ground beef. Addition of chitosan did not enhance the pressure-induced inactivation of *E. coli* or *S. enterica* (**Figure 5-2A** and **C**). Chitosan in conjunction with pressure for 6 min reduced cell counts of *S. enterica* to levels below detection limit only when samples were enumerated on VRB agar (**Figure 5-2D**).

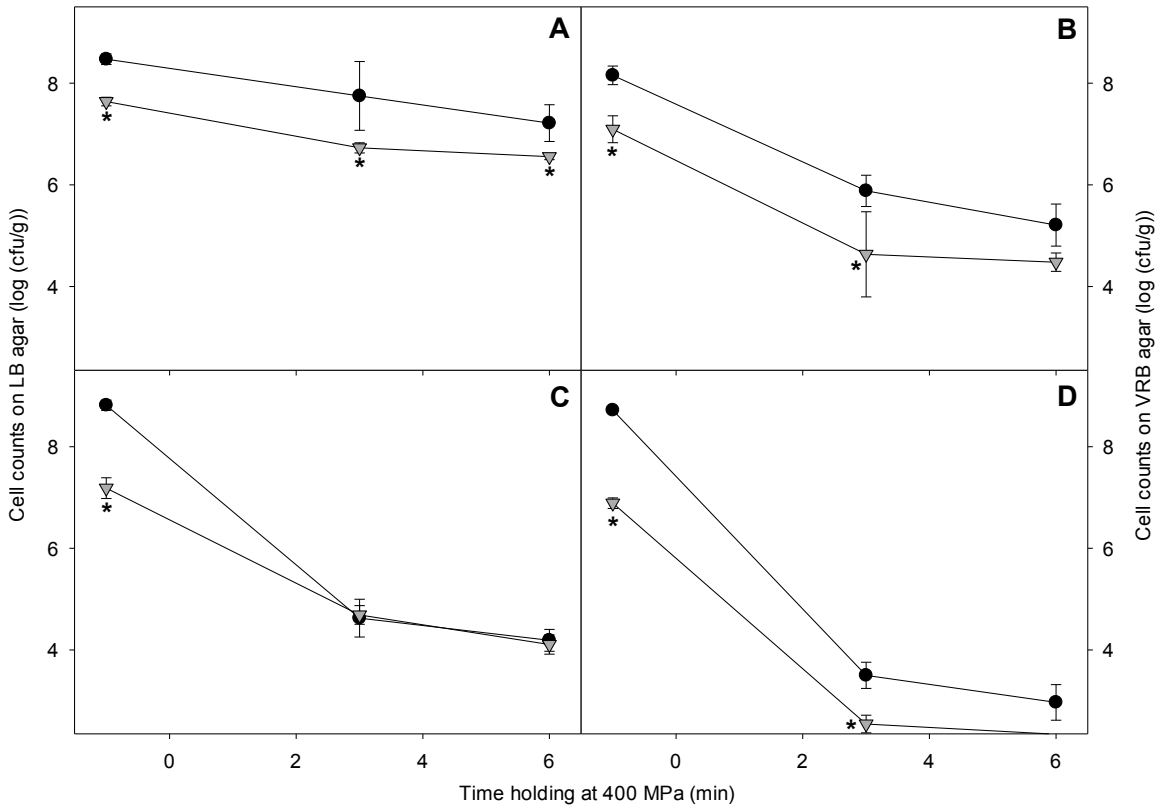

Figure 5-2 Cell counts of *E. coli* AW1.7 (Panel A and B) or *S.* Senftenberg ATCC 43845 (Panel C and D) in ground beef (black circle) or ground beef with chitosan (grey triangles) before and after treatment at 400 MPa at 20 °C. Inoculated samples with or without chitosan were held at ambient condition for 4 h before pressure treatment, and symbols at the holding time of −1 min represents cells counts of unpressurized samples that were held at ambient condition for 4 h. Cells counts were enumerated on LB agar (Panel A and C) and VRB agar (Panel B and D). Data are shown as mean ± standard deviation of triplicate independent experiments. Lines crossing the x axis indicate cell counts at or below the detection limit of 2.4 log (cfu/g). At each holding time point, cell counts that are different from the cell counts of control samples are indicated by an asterisk ($P<0.05$).

5.4 Discussion

LHR conferred pressure resistance in *E. coli* and *Salmonella* only when cells were incubated aerobically, and its contribution to pressure resistance was not as exceptional as its contribution to heat resistance. Pressure treatment inactivates *E. coli* by disrupting cell envelope, denaturing functional proteins involved in mediating cell survival, and inducing an endogenous oxidative burst (Gänzle and Liu, 2015). The LHR-encoded proteins that are

predicted to mediate heat resistance, including heat shock proteins, hypothetical proteins managing envelope stress, and hypothetical proteins mitigating oxidative stress, may also contribute to pressure resistance (Boll et al., 2017; Lee et al., 2015; Lee et al., 2016; Mercer et al., 2015). Nevertheless, the moderate baroprotective effect of LHR was highly dependent on incubation condition, highlighting that some other genetic predispositions are more critical than LHR in the development of pressure resistance. Remarkably, microaerophilic incubation resulted in cell adaption to an acidic environment and simultaneously increased pressure resistance in *E. coli* irrespective of the presence of LHR in comparison to aerobic incubation. The acid challenge increases RpoS levels (Bak et al., 2014) and an increased RpoS activity leads to the development of pressure resistance in *E. coli* (Vanlint et al., 2013a). These partially explain the acquisition of pressure resistance through microaerophilic incubation.

Among the stress-responsive genes used in the study, the protective effect of LHR was only dependent on the *rpoS*. Despite the individual contribution of *rpoS* and LHR to heat resistance (Cheville et al., 1996; Lange and Hengge-Aronis, 1991; Mercer et al., 2015; Mercer et al., 2017), acquisition of LHR in *rpoS* negative mutants was accompanied with a substantial increase in heat resistance only when cells were incubated microaerophilically, and the *rpoS* negative mutant carrying LHR was more heat sensitive than its parental strain carrying LHR. These results highlight that the *rpoS* contributes to the protective effect of LHR against heat. The RpoS regulon protects against multiple stressors including oxidative stress, osmotic stress and acid stress (Choi et al., 2000; Landini et al., 2014; Zhao et al., 2002), thus mediating cross-protection and probably acting synergistically with LHR-encoded proteins against heat. RpoS conferred pressure resistance in *E. coli*

unconditionally, while LHR did not contribute to pressure resistance in *rpoS* negative mutants, suggesting that *rpoS* is more relevant in mediating pressure resistance than LHR. RpoS promotes the pressure resistance in *E. coli* (Aertsen et al., 2005; Charoenwong et al., 2011; Malone et al., 2006; Robey et al., 2001; Vanlint et al., 2013a; Vanlint et al., 2013b) presumably through regulation of a series of proteins responsive to pressure stress, including osmoresponsive outer membrane proteins, cyclopropane fatty acid synthase, Dps, catalase, and superoxide dismutase (Landini et al., 2014). This study provides a critical appraisal of the significance of LHR and *rpoS* in mediating pressure resistance and also sheds light on the possible correlation between the baroprotective effect of LHR and the presence of *rpoS*.

Pressure lethality depends on strains, substrates and pressure intensity (de Oliveira et al., 2015; Gänzle and Liu, 2015; Hygreeva and Pandey, 2016; Rendueles et al., 2011). The present study demonstrated that *S.* Senftenberg ATCC 43845 was more resistant to pressure when suspended in MES buffer at pH 5.4 than in ground beef, extending previous results obtained with *E. coli* AW1.7 (Li and Gänzle, 2016). Pressure treatment can denature proteins containing iron-sulfur clusters, incurring the accumulation of iron in the cytoplasm. Increase in iron accelerates the formation of reactive oxygen species, thus intensifying oxidative stress to cells and leading to lethality (Gänzle and Liu, 2015). The presence of iron and iron-sulfur cluster proteins decreased the resistance of *E. coli* to pressure (Malone et al., 2006; Yan et al., 2013). Accordingly, this iron-dependent mechanism may explain why *E. coli* AW1.7 and *S.* Senftenberg ATCC 43845 were more sensitive to pressure in iron-rich ground beef than in iron-free buffer.

Different essential oils, including carvacrol, thymol, thiol-reactive allyl-isothiocyanate (AITC) and cinnamaldehyde, were not bactericidal against pressure-resistant *E. coli* in ground beef and also did not enhance pressure lethality (Li and Gänzle, 2016). In the present study, chitosan inactivated pressure-resistant strains, including *E. coli* AW1.7 and *S.* Senftenberg ATCC 43845, by 1-2 log (CFU/mL) or (CFU/g) in buffer and ground beef. Chitosan-induced inactivation is independent of the LHR and almost matches the bactericidal effects of 0.5% chitosan against *Salmonella* in chicken skin and 2% chitosan against *E. coli* in kebab (Kanatt et al., 2013; Menconi et al., 2013). Synergistic activity of chitosan with pressure in buffer observed in this study was consistent with the previous reports (Kumar et al., 2009; Malinowska-Pańczyk et al., 2009). The amino groups of chitosan are protonated at pH 5.4 and electrostatically interact with negatively charged structures of the cell envelope, including the cytoplasmic membrane and the lipopolysaccharide (LPS) in the outer membrane, thus disrupting the integrity of cell envelope, dissipating membrane potential, and causing cell leakage and death (Helander et al. 2001; Liu et al. 2004; Mellegård et al. 2011). This mode of action, which is unrelated to the mechanism of LHR-mediated defense mentioned above, may result in the bactericidal effects independent of LHR and the increased susceptibility of cells to pressure. However, chitosan did not enhance pressure lethality in ground beef. This may be attributed to the abundant proteins in ground beef, which can decrease chitosan activity by neutralizing the positive charges of chitosan (Devlieghere et al., 2004). Despite having no synergism, chitosan itself was bactericidal and the combination of chitosan and pressure caused comparable or higher lethality than the pressure treatment alone. All these indicate

that chitosan is an effective hurdle concept for reducing the risks from pressure-resistant *E. coli* and *Salmonella*.

The present study indicates that LHR confers heat resistance in *E. coli* and *S. enterica*, while deletion of *rpoS* reduces the effect of the LHR on heat resistance in *E. coli*. The LHR confers a protective effect on pressure resistance in *E. coli* that is dependent on *rpoS*, and is observed only if strains are grown under aerobic conditions. All these extend the knowledge about the role of LHR in mediating heat and pressure resistance. Chitosan and pressure synergistically inactivate *E. coli* and *S. enterica* in buffer at pH 5.4, independent of the presence of the LHR. Chitosan is bactericidal against pressure-resistant *E. coli* and *S. enterica* strains in ground beef. The combination of pressure and chitosan is more or no less lethal than pressure treatment alone in ground beef. These highlight that chitosan is an effective hurdle concept for improving meat safety.

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Chapter 6 Effect of chitosan and other antimicrobial hurdles on survival of *Listeria monocytogenes* on ham

6.1 Introduction

Listeria monocytogenes is the most significant food safety concern associated with ready-to-eat (RTE) meat due to its wide distribution in nature and ability to grow at refrigerated storage (Ferreira et al., 2014; Walker et al., 1990). With the capacity of surviving under extreme environmental conditions and forming biofilm, *L. monocytogenes* persists in food processing environment (Di Bonaventura et al., 2008; Gómez et al., 2014; Valimaa et al., 2015) and contaminates RTE meat primarily during post-cooking processing, such as slicing and packaging (Kornacki and Gurtler, 2007; Chaitiemwong et al., 2014). According to Codex Alimentarius guidelines, the amount of *Listeria* should not exceed 100 CFU/g throughout the shelf life if storage conditions do not permit growth of *Listeria*. Otherwise, the absence of *Listeria* in 25 g of the product must be guaranteed (Luber, 2011).

In addition to establishing proper hygienic systems for preventing listerial cross-contamination (Muhterem-Uyar et al., 2015), diacetate/lactate, nisin and *Carnobacterium maltaromaticum* UAL307 (also referred to as ATCC PTA-5313 or CB1), a strain producing carnocyclin A, piscicolin 126 and carnobacteriocin BM1 (Martin-Visscher et al., 2008), are commercially employed on RTE meat to control the growth of *Listeria monocytogenes*. The meat industry also processes the packaged RTE meats products at 600 MPa for 3–5 min in refrigerated processing plants to eliminate *Listeria monocytogenes* (Canadian Food Inspection Agency, 2017; Health Canada, 2017). Even though current industrial pressure processing inactivates *L. monocytogenes* on ham by 3–4 log (CFU/g) (Teixeira et al., 2016), recovery of sublethally injured *L. monocytogenes* during post-pressure refrigerated storage challenges the application of pressure for warranting the safety of RTE meat (Marcos et al.,

2008; Teixeira et al., 2018). The use of diacetate/lactate or some bacteriocins, such as nisin, enterocins, or sakacin, enhanced the immediate pressure-induced inactivation of *L. monocytogenes* or the post-pressure listericidal effect on ham (Marcos et al., 2008a; Marcos et al., 2008b; Jofré et al., 2007; Hereu et al., 2012; Teixeira et al., 2018). Nevertheless, the continuing burden of listeriosis associated with RTE meat necessitates novel antimicrobial hurdles to improve the safety of RTE meat.

Chitosan, a partially or fully deacetylated derivative of chitin, has GRAS approval in the U.S.A. (FDA, 2011). Chitosan exerts antimicrobial activity against Gram-positive bacteria by disrupting the integrity of cell surface, presumably through electrostatic interactions between its protonated amino group and negatively charged lipoteichoic acid (LTA) or wall teichoic acid (WTA) (Raafat et al., 2008). Therefore, chitosan may also enhance the efficacy of current intervention technologies. Chitosan is listericidal or listeriastatic in meat products when applied onto contaminated surface in the form of the chitosan-based film (Guo et al., 2014; Moradi et al., 2011). However, incorporation of chitosan powder into meat batter before cooking process is more feasible and practicable than the surface application of chitosan solution or chitosan-based film on cooked ham for ham production at industrial scales. It is still unclear whether incorporation of chitosan into ham exhibits antilisterial activity or acts synergistically with other antimicrobial hurdles in controlling *Listeria*. Accordingly, different types of ham formulated with chitosan or other preservatives were prepared in this study, and this study aimed to evaluate the single or combined effect of chitosan and other antimicrobial hurdles on survival and growth of *L. monocytogenes* on RTE ham.

6.2 Materials and methods

6.2.1 Bacterial strains and growth conditions.

A 5-strain cocktail of *L. monocytogenes*, including *L. monocytogenes* FSL J1-177, FSL C1-056, FSL N3-013, FSL R2-499, and FSL N1-227, which was used as "human disease cocktail" recommended for challenge studies in food (Fugett et al., 2006), was used as indicator strains in this study. *L. monocytogenes* were streaked from -80 °C stock cultures onto Tryptic Soy (TS) agar (Difco, Becton–Dickinson, Sparks, MD, USA) and incubated overnight. The single colony on the plate was inoculated into TS broth (TSB) to make an overnight subculture, followed by a second sub-culture with 1 % (v/v) inoculum and incubation for 18 h. *Listeria* was routinely incubated at 37 °C. For preparation of cocktails to be inoculated on hams, an equal volume of each individual culture was mixed to form a 5-strain cocktail of *L. monocytogenes*, followed by harvesting cell pellets of cocktails by centrifugation and resuspension of cell pellets in saline (0.85 % NaCl) to achieve a cocktail suspension with an optical density at 600 nm of 1.0.

6.2.2 Preparation of ham.

Cooked ham with or without preservatives was formulated as Table 6-1 and produced as the following: (1) Pork leg meat stored at 4 °C overnight were cut into 4 cm × 4 cm × 4 cm cubes and ground through a 0.32 cm plate (Mini-matic; HollyBerk Sales, AB, Canada). (2) Meat and other ingredients were combined as Table 6-1 depending on the type of ham, followed by tumbling (VAS-40, Glass®) under vacuum (80 kPa) at 4 °C for 10 min and holding for 1 h under the same vacuum. (3) Meat batters were stuffed into nylon casings (75 mm diameter, UniPac, AB, Canada) and stored overnight at 4 °C. (4) Raw hams were cooked in hot water at 98 °C for 10 min and held at 80 °C until the internal temperature

reaches 71 °C. (5) Hams were chilled on ice for 10 min and kept at 4 °C for further cooling. After overnight cooling, hams were sliced on a pre-sanitized slicer, vacuum packaged and stored at 0 °C prior to use. The dimension of each ham slice was 50 cm^2 surface area and 1 cm thick.

Each ham had a final sodium concentration of 0.6 % (w/w). Six types of hams were finally prepared by Danielle R. Balay and Januana S. Teixeira: control ham; ham containing sodium diacetate/sodium lactate; ham containing sodium diacetate/sodium lactate and bacteriocins; ham containing bacteriocins; ham containing chitosan and bacteriocins; ham containing chitosan (**Table 6-1**).

Table 6-1 Ingredient formulations of different hams

Ingredients (kg)	Ham 1 (control)	Ham 2	Ham 3	Ham 4	Ham 5	Ham 6
Lean meat	3 (78.94%)	3 (78.94%)	3 (78.94%)	3 (78.94%)	3 (78.94%)	3 (78.94%)
Ice	0.635 (16.71%)	0.635 (16.71%)	0.635 (16.71%)	0.635 (16.71%)	0.635 (16.71%)	0.635 (16.71%)
Sodium triphosphate (STPP)	0.019 (0.5%)	0.019 (0.5%)	0.019 (0.5%)	0.019 (0.5%)	0.019 (0.5%)	0.019 (0.5%)
Sodium chloride	0.0373 (0.98%)	0.00797 (0.21%)	0.00797 (0.21%)	0.0373 (0.98%)	0.0373 (0.98%)	0.0373 (0.98%)
Prague powder (6% sodium nitrite, 94% NaCl)	0.0126 (0.33%)	0.0126 (0.33%)	0.0126 (0.33%)	0.0126 (0.33%)	0.0126 (0.33%)	0.0126 (0.33%)
Sodium erythorbate	0.003 (0.08%)	0.003 (0.08%)	0.003 (0.08%)	0.003 (0.08%)	0.003 (0.08%)	0.003 (0.08%)
Dextrose	0.093 (2.45%)	0.093 (2.45%)	0.093 (2.45%)	0.093 (2.45%)	0.093 (2.45%)	0.093 (2.45%)
Sodium diacetate	-	0.0038 (0.1%)	0.0038 (0.1%)	-	-	-
Sodium lactate	-	0.0532 (1.4%)	0.0532 (1.4%)	-	-	-
Bacteriocins	-	-	0.0019 (0.05%)	0.0019 (0.05%)	0.0019 (0.05%)	-
Chitosan	-	-	-	-	0.019 (0.5%)	0.019 (0.5%)
Total	3.8					

* Percentage is defined as weight by weight (w/w)
* Lean meat was purchased from a federally inspected meat processing facility; Sodium chloride, sodium erythorbate, dextrose monohydrate, sodium tripolyphosphate (STPP) and Prague powder were purchased from Griffith Laboratories™, Canada; sodium lactate and sodium diacetate were purchased from Sigma-Aldrich; Chitosan was purchased from Sigma-aldrich (Catalog# 417963; CAS# 9012-76-4); Bacteriocins and ham were produced from *Carnobacterium maltaromaticum* UAL307 by Danielle R. Balay and Januana S. Teixeira. Bacteriocin activity in ham 3, 4 or 5 was 25.6 AU/g.

6.2.3 Pressure treatment and storage.

Pressure treatments were carried out in a 2.2 mL high-pressure vessel that immersed in a temperature-controlled water bath (Teixeira et al., 2016). Initial temperature in the vessel was 5 °C and the temperature increase in the pressure vessel during compression was less

than 5 °C. Ham samples were shaped, inoculated and heat-sealed as described (Teixeira et al., 2016). To assess the immediate pressure-induced inactivation, each ham column was inoculated with the 5- strain cocktail of *L. monocytogenes* to achieve cell counts of 10^7-10^8 CFU/g. To assess the effect of preservatives on the post-pressure survival of *L. monocytogenes* on ham during storage, each ham column was inoculated with the same cocktail to achieve cell counts of 10^5-10^6 CFU/g. The sealed samples were maintained at room temperature until pressure treatment. After samples were placed in the pressure vessel equilibrated at 5 °C, and then treated at 600 MPa at 5 °C for 3 min. In addition, non-pressurized and pressurized samples were stored for 4 weeks at 4 °C.

6.2.4 Detection of surviving cells.

The presence of *L. monocytogenes* was monitored immediately after pressure treatment and after storage for 4 weeks at 4 °C. Samples were opened aseptically, and the contents were transferred to a sterile 1.5 mL Eppendorf tube and homogenized for 60 s with sterile saline (0.85 % NaCl), followed by 10-fold serial dilution with the same sterile saline. Appropriate dilutions were plated on nonselective TS agar and selective PALCAM agar (Becton–Dickinson) by spiral plater to determine surviving cells and uninjured cells of *L. monocytogenes*, respectively. The plates were incubated at 37 °C for 48 h. Cell counts of *L. monocytogenes* in un-inoculated ham samples stored for 4 weeks at 4 °C were under the detection limit (2.2 log (CFU/g)).

6.2.5 Statistical analysis.

Experiments were performed in biological triplicates. All data are expressed as means ± SD. Differences between variables were tested for significance by one-way or two-way ANOVA with Least Significant Difference (LSD) test using PASW Statistics 18 (SPSS

Inc., Chicago, IL, USA) for Windows 8.1. Differences at $P \leqslant 0.05$ were considered to be significant and n=3.

6.3 Results

To assess the single and combined effect of pressure and different preservatives, ham with or without preservatives was inoculated with a 5-strain cocktail of *L. monocytogenes* at a level of 7-8 log (CFU/g) and treated at 600 MPa at 5 ºC for 3 min. None of the preservatives inactivated *L. monocytogenes*. Pressure treatment inactivated *L. monocytogenes* by 1.5-2 log (CFU/g) irrespective of preservatives and none of the preservatives enhanced the pressure-induced inactivation (**Figure 6-1A**). If cells of *L. monocytogenes* were enumerated on PALCAM agar, pressure treatment reduced cell counts on ham containing sodium diacetate/sodium lactate to levels below detection limit, whereas cell counts on other hams were still measurable after pressure treatment. This indicated that sodium diacetate/sodium lactate enhanced pressure-induced sublethal injury of *L. monocytogenes* (**Figure 6-1B**).

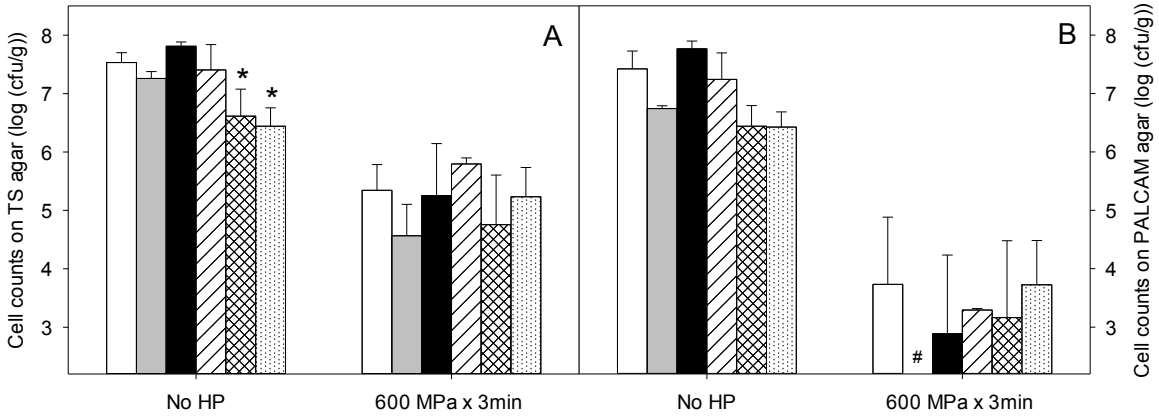

Figure 6-1 Effect of pressure on survival of *L. monocytogenes* on ham without antimicrobials (white bar) and ham containing sodium diacetate/sodium lactate (grey bar), sodium diacetate/sodium lactate and bacteriocins (black bar), bacteriocins (diagonal hatched bar), chitosan and bacteriocins (outlined diamond bar), or chitosan (dotted bar). The presence or absence of *L. monocytogenes* was monitored immediately after pressure

treatment. Cell counts were enumerated on TS agar (A) and PALCAM agar (B). Data are shown as means ± standard deviations of triplicate independent experiments. Differences among variables were tested for significance by one-way ANOVA with LSD test. Among the unpressurized hams, mean value that is significantly lower than the mean value of control ham is indicated as an asterisk (*). Among the pressurized hams, mean value that is significantly lower than the mean value of control ham is indicated as a pound sign (#) ($P < 0.05$). The detection limit is 2.2 log (CFU/g).

To investigate the effect of pressure and preservatives on survival and growth of *L. monocytogenes* on ham under refrigerated condition, hams with or without preservatives were inoculated with a 5-strain cocktail of *L. monocytogenes* at a level of 5-6 log (CFU/g) and treated at 600MPa at 5 ºC for 3 min, followed by storage at 4 ºC for 4 weeks. Initial cell counts of *L. monocytogenes* on different hams were comparable. Without pressure, control ham enabled the growth of *L. monocytogenes* by 2 log (CFU/g) after storage. Sodium diacetate/sodium lactate did not inhibit the growth of *L. monocytogenes* on ham, while chitosan or the combined use of sodium diacetate/sodium lactate and bacteriocin inhibited the growth of *L. monocytogenes* on ham after storage (**Figure 6-2**). Pressure treatment immediately inactivated *L. monocytogenes* by 2-3 log (CFU/g), and post-pressure growth of *L. monocytogenes* was not observed after storage irrespective of preservatives (**Figure 6-2**). Remarkably, cell counts of *L. monocytogenes* on pressurized ham containing sodium diacetate/sodium lactate were under detection limit after storage, indicating that the single use of sodium diacetate/sodium lactate exerts post-pressure listericidal effect (**Figure 6-2**A).

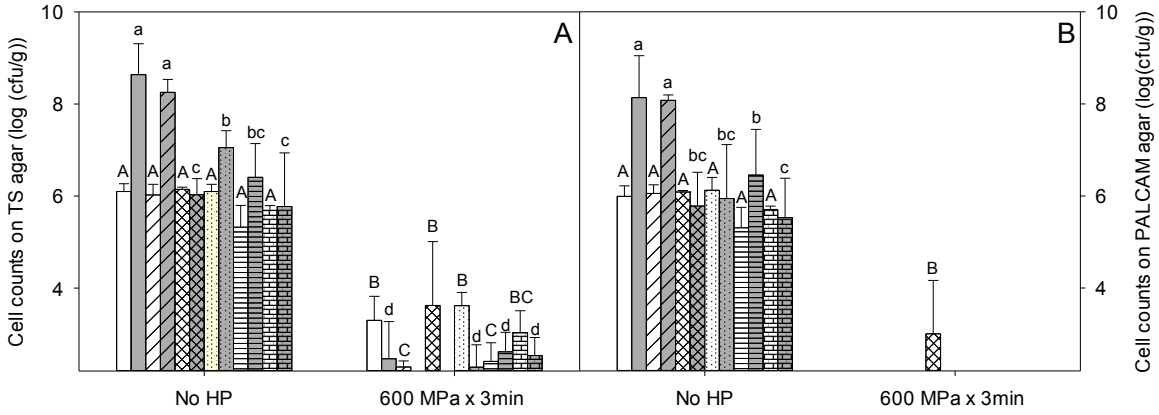

Figure 6-2 Effect of pressure on survival and post-pressure growth of *L. monocytogenes* on ham without antimicrobials (plain) and ham containing sodium diacetate/sodium lactate (diagonal hatched), sodium diacetate/sodium lactate and bacteriocins (outlined diamond), bacteriocins (dotted), chitosan and bacteriocins (horizontal hatched), or chitosan (brick). The presence or absence of *L. monocytogenes* was monitored immediately after pressure treatment (white bar) and after storage for 4 weeks at 4 ºC (grey bar). Cell counts were enumerated on TS agar (A) and PALCAM agar (B). Data are shown as means ± standard deviations of triplicate independent experiments. Differences among variables were tested for significance by two-way ANOVA with LSD test. Treatment means after storage (grey bars) within each panel with different letters (small) are significantly different ($P < 0.05$). Treatment means without storage (white bars) within each panel with different letters (capital) are significantly different ($P < 0.05$). The detection limit is 2.2 log (CFU/g).

6.4 Discussion

To control *listeria* on ham, antimicrobials or preservatives can be applied on cooked ham surface or formulated into meat batter before ham cooking. Generally, surface application is more likely to exhibit listericidal effect than internal addition. Placing chitosan-based film containing 0.389 mg chitosan/cm^2 onto the inoculated surface of ready-to-eat deli turkey meat inactivated *Listeria innocua* by 0.8 log (CFU/g) (Guo et al., 2014). Application of nisin solution on ham surface at a surface concentration of 2~19.5 μg/cm^2 reduced cell counts of *L. monocytogenes* by 1-2 log (CFU/g) (Jofré et al., 2007; Hereu et al., 2012; Teixeira et al., 2018), while observation of comparable listericidal effect requires incorporation of nisin into ham at the level of 78 μg/g (Jofré et al., 2008). Incorporation of

enterocins, potassium lactate/sodium diacetate (Marcos et al., 2008b) or the preservatives used in this study into ham also did not inactivate *L. monocytogenes*. Nevertheless, given the high cost and complexity of running surface application, incorporation of antimicrobials into meat batter before ham cooking is more suitable for production of ham at industrial scale.

Although chitosan had no bactericidal effect, its incorporation into ham inhibited the growth of *L. monocytogenes* during the four-week storage at 4 ºC. Chitosan exhibited higher bacteriostatic activity against *Listeria* than sodium diacetate/sodium lactate. The current Canadian Food Inspection Agency (CFIA) guideline states that an acceptable antimicrobial agent should allow no more than 2 log (CFU/g) increase in *L. monocytogenes* throughout the stated shelf-life of the product (Canadian Food Inspection Agency, 2017). All these indicate that chitosan is a promising RTE meat preservative and has the potential to replace diacetate/ lactate for improving RTE meat safety. The combined use of chitosan and bacteriocins produced from *C. maltaromaticum* UAL307 also allowed growth of *L. monocytogenes* by less than 1 log (CFU/g) after the 4-week storage at 4 ºC. However, chitosan and bacteriocins did not act synergistically. Considering the significant cost of bacteriocin preparations, the single use of chitosan suffices to be promising for the industrial production of ham.

Nisin, enterocin and cell culture of *C. maltaromaticum* UAL307 exhibited listericidal effects if they were applied on ham surface (Balutis, 2014; Hereu et al., 2012; Jofré et al., 2007; Marcos et al., 2008a; Teixeira et al., 2018). Incorporation of nisin into ham at the level of 78 μg/g still exhibited antilisterial activity on ham (Jofré et al., 2008), while incorporation of enterocin into ham did not affect the growth of *Listeria* (Marcos et al.,

2008b). The present study indicated that incorporation of bacteriocins produced from *C. maltaromaticum* UAL307 into ham was bacteriostatic against *Listeria*, thus extending the knowledge on antilisterial activity of bacteriocins.

Diacetate/lactate has been commercially used as RTE meat preservatives to inhibit the growth of *L. monocytogenes* (Canadian Food Inspection Agency, 2017), and is a benchmark for assessing antilisterial activity of other preservatives. Previous studies indicated that inclusion of lactate/diacetate in ham formulations inhibited the growth of *L. monocytogenes* under vacuum and refrigerated storage (Balutis, 2014; Jofré et al., 2007; Marcos et al., 2008b); in contrast, inhibitory effects of diacetate/lactate were not observed in this study. This inconsistency may be attributed to the variation in storage condition and initial inoculum level. The previous study demonstrated diacetate/lactate and pressure acted synergistically in controlling *L. monocytogenes* during refrigerated storage (Marcos et al., 2008b). The present study confirmed this finding. Remarkably, bacteriocins produced from *C. maltaromaticum* UAL307 and sodium diacetate/sodium lactate synergistically inhibited the growth of *L. monocytogenes* during refrigerated storage. The combined application of bacteriocins with sodium diacetate/sodium lactate thus provides a suitable hurdle concept for improving RTE meat safety.

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Chapter 7 General discussion and conclusion

Chitosan improves the quality and safety of various foods (Chapter 2). Nevertheless, the antimicrobial activity of chitosan against pathogens on meat products remains to be validated. It remains unclear whether chitosan is lethal to LHR positive strains on meat and whether chitosan enhances the efficacy of other antimicrobial hurdles, such as bacteriocins, heat and pressure. This research indicated that chitosan exhibited antimicrobial activity against foodborne pathogens and LHR positive strains on different meat products when used alone or in conjunction with other antimicrobial hurdles, thus providing a critical appraisal of the limitations and opportunities of the use of chitosan as a meat preservative.

7.1 The use of chitosan to improve meat safety

Numerous studies report on the application of chitosan to extend the shelf life of foods; however, only few studies validated the bactericidal effects of chitosan (Chapter 2). This research demonstrated that chitosan inactivated *E. coli* and *Salmonella*, including LHR positive strains, by 1 log (CFU/g) when 1% chitosan solutions were applied on artificially contaminated meat surfaces (Chapter 3 and 5). A bactericidal effect was not observed when a chitosan solution was mixed with ground meat prior to bacterial inoculation, when a chitosan-based film was placed between two inoculated slices of ready-to-eat sausages, or when chitosan powder was incorporated into ham (Chapter 4 and 6; Moradi et al. 2011). Variation in chitosan activity due to the application form is also observed in other food matrixes. Surface application of chitosan solutions was more lethal to *L. monocytogenes* on black radish than the chitosan-based film (Jovanović et al., 2016). Coating eggs with chitosan solutions prior to the bacterial inoculation was not lethal to *Salmonella* Enteritidis (Leleu et al. 2011), and incorporation of chitosan powder into bread also did not exhibit bactericidal effects (Lafarga et al. 2013). These findings highlight the following three

characteristics of chitosan as a food preservative. First, chitosan is bactericidal only when it is in the polycationic form and thoroughly comes into contact with the target bacteria. Second, the meat matrix strongly reduces chitosan lethality, presumably through neutralizing the positive charge of chitosan with the negatively charged proteins (Chapter 3, 4 and 6; Devlieghere et al. 2004). Due to these two characteristics, surface application of chitosan solution is preferable than chitosan-based films and internal addition of chitosan in view of attaining the bactericidal effects on meat. Third, chitosan lethality is independent of LHR (Chapter 5). LHR-encoded stress proteins are predicted to be involved in the turnover of misfolded proteins, cell envelop maintenance and mitigation of oxidative stress (Mercer et al., 2017), thus contributing to heat and pressure resistance of bacteria (Chapter 5). The antimicrobial activity of chitosan is attributed to the electrostatic interactions between polycationic structures of chitosan and negatively charged cell envelopes (Helander et al. 2001; Liu et al. 2004; Mellegård et al. 2011). This mode of action of chitosan is unrelated to the mechanism of LHR-mediated defense, resulting in lethality independent of LHR (Chapter 5). Accordingly, chitosan can be considered as an effective hurdle concept for reducing the concerns for LHR positive pathogens in meat safety.

7.2 The combined effect of chitosan and other antimicrobial hurdles.

Chitosan can disrupt the integrity of cell envelope, thus sensitizing bacterial cells to some other antimicrobial hurdles. Chitosan enhanced the thermal inactivation of pathogenic *E. coli* and LHR positive *E. coli* in ground beef (Chapter 4; Surendran Nair et al. 2016), highlighting the potential of chitosan to become part of an effective hurdle concept for

improving the safety of ground beef. However, chitosan did not enhance pressure-induced inactivation of pathogen on meat (Chapter 3 and 5).

The bactericidal effect of chitosan on meat is limited to 1.5 log (CFU/g) (Chapter 2 and 3). Combination of other biopreservatives with chitosan is considered as one solution for enhancing chitosan lethality and simultaneously meeting consumers' demand for "natural preservatives" in food products. In this research, bacteriocins and bacteriocin-producing protective culture did not enhance the antimicrobial activity of chitosan on meat (Chapter 3). Only a citrus extract acted synergistically with chitosan in reducing the contamination of *E. coli* and *Salmonella* on fresh turkey meat (Vardaka et al., 2016). However, in that report, cells of *E. coli* and *Salmonella* were enumerated on selective media. Therefore, that report only demonstrated the synergism of citrus extract and chitosan in mediating cell injury, rather than cell death. Moreover, the citrus extract is a mixture of flavonoids extracted from citrus fruits and organic acids (Tsiraki et al., 2018), and the report did not indicate the exact antimicrobials acting synergistically with chitosan (Vardaka et al., 2016). Taken together, current studies indicate that the combined use of chitosan and other biopreservatives is not more promising than the single use of chitosan for improving meat safety.

7.3 Comparison of chitosan with other biopreservatives.

Chitosan is more lethal to *E. coli* and *Salmonella* on meat than bacteriocins, organic acids, and certain essential oils, including carvacrol, thymol, thiol-reactive allyl-isothiocyanate (AITC) and cinnamaldehyde (Chapter 3, Li and Gänzle, 2016). Chitosan also enhances the efficacy of the grilling process currently recommended for eliminating the *E. coli* in ground beef. Bactericidal effects of other biopreservatives against *E. coli* and *Salmonella*,

including rutin (RT) and resveratrol (RV), were not observed or not confirmed on meat (Surendran Nair et al. 2016).

The North American beef industry applies steam pasteurization or hot water washes in combination with the application of lactic acid or peroxyacetic acid to decontaminate carcasses (Gill, 2009). However, decontaminating effects of acid solutions vary among different plants (Gill and Landers, 2003) and vanish if the solutions are applied in inadequate quantities or onto wet meat surfaces (Bosilevac et al., 2006; Gill, 2009). *E. coli* and related enteric pathogens may grow and survive in detritus that persists in processing equipment (Gill, 2009). *Salmonella* can colonize lymphoid tissues, thus resisting the surface-oriented interventions (Webb et al., 2017). Accordingly, recontamination of meat with *E. coli* and *Salmonella* may occur during carcass breaking (Gill, 2009; Mann et al., 2015). Surface application of chitosan solution inactivates *E. coli* and *Salmonella* by 1 log (CFU/g) on lean meat after steaming and is more bactericidal than acetic acid and lactic acid (Chapter 3). Taken together, these results suggest that the application of chitosan in addition to the single or combined treatment of steam and acid is particularly promising for the production of ground beef and mechanically tenderized beef and provides additional assurances for consumers.

Surface application of nisin or *Carnobacterium maltaromaticum* UAL307 exhibited bactericidal effect against *Listeria monocytogenes* after treatment and refrigeration (Balutis, 2014; Hereu et al., 2012; Jofré et al., 2007; Teixeira et al., 2018). However, incorporation of antimicrobials into ham is more practical and suitable for industrial production than the surface application. Incorporation of chitosan into ham did not inactivate *L. monocytogenes*, but it still exhibited a stronger bacteriostatic effect against *Listeria* on ham than sodium

diacetate/sodium lactate (Chapter 6) and rosemary extract (Teixeira et al., 2018). This indicates that chitosan has the potential to replace organic acids and become a novel RTE meat preservative.

Chitosan also improves meat quality independent of its antimicrobial activity by the retardation of lipid oxidation, and by improved retention of color, freshness, taste, and odor (Giatrakou et al., 2010; Bostan and Mahan 2011; Petrou et al., 2012; Vasilatos and Savvaidis 2013; Latou et al., 2014; Lekjing et al., 2016). All these may favor the application of chitosan even if the antimicrobial effect is limited.

Taken together, chitosan is a promising preservative for improving meat safety and extending the shelf life of meat products.

7.4 Future work

In this research, to assess the bacteriostatic effect of chitosan against *Listeria* on ham, ham with and without chitosan was inoculated with *Listeria* cocktails, and surviving cells of *Listeria* on ham were enumerated after inoculation and after four weeks of refrigerated storage (Chapter 6). However, monitoring cell counts every seven days up to four weeks of storage may be preferable to demonstrate the effect of chitosan.

Covering the artificially contaminated side of a ham slice with chitosan-based film inactivated the spoilage bacteria cocktail of *Brochothrix thermosphacta*, *Carnobacterium maltaromaticum*, *Leuconostoc gelidum* and *Lactobacillus sakei* by 1 log (CFU/cm^2) and delayed the growth of spoilage microbiota by one to two weeks (Zhao et al., 2018) under storage at 4 °C. Nevertheless, it remains unclear whether incorporation of chitosan into ham still exerts antimicrobial activity against spoilage microbiota. Cell culture of *C. maltaromaticum* UAL307 is listericidal on ham under vacuum and refrigerated storage

(Balutis, 2014), but the combined effect of chitosan and cell culture of *C. maltaromaticum* UAL307 on survival and post-pressure growth of *Listeria monocytogenes* on ham has not yet been explored.

The limited bactericidal effect of chitosan on meat challenges the application of chitosan for improving the meat safety, but chitosan improves meat quality in some cases by the retardation of lipid oxidation and the retention of freshness, taste, and odor (Chapter 2). This may favor chitosan applications even if the antimicrobial effect is limited. Further understanding of the application of chitosan as a meat preservative still necessitates validating the single or combined effect of chitosan and other antimicrobial hurdles on meat quality.

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