Control and Detection of Enterohaemorrhagic *Escherichia coli*

by

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Abstract

Enterohaemorrhagic *Escherichia coli* (EHEC) is a pathogen that causes severe disease in humans and has a low infectious dose. Since foodborne EHEC outbreaks continue to be a problem worldwide, improved control and detection methods for EHEC on at-risk foods, such as spinach and beef, are essential. While new methods of control and detection may prove to be effective in broth or buffer, it is important that they are also tested in a food matrix since food systems are more complex and may lead to different results. A novel intervention method was developed to control EHEC on spinach and lettuce with a volatile antimicrobial from mustard, allyl isothiocyanate (AITC). AITC released from its precursor sinigrin in mustard meal was limited by the activity of mustard's endogenous enzyme myrosinase at 4°C. While the requirement of endogenous myrosinase in mustard meal to catalyze this reaction was known, decreased activity at refrigeration temperature was not, and made this antimicrobial intervention on produce impractical. Secondly, this work explored improving EHEC detection methods. Enrichment remains a necessary but lengthy step in pathogen detection methods and a major impediment to rapid detection. Therefore, decreases in the current validated enrichment times for EHEC detection were investigated with aim to reduce pathogen detection times. Individual lag phase of heat injured *E. coli* O157:H7 cells were measured to determine necessary enrichment times. However, decreasing enrichment times increased the probability of not detecting sub-lethally injured cells since sub-lethal cell injury significantly increased lag phases and therefore overall time to detection. Lastly, a major challenge of qPCR detection is the inability to discriminate between live and dead cells within a sample and this limitation could lead to false positive result. This is especially of importance when using this method for pathogen detection in food. The current work investigated five different detection methods to determine the

concentration of viable EHEC cells on beef steaks after interventions of lactic acid, peroxyacetic acid and hot water and included 1) use of a DNA binding dye propidium monoazide (PMA) to prevent dead cell DNA amplification when used in conjunction with qPCR 2) PMA in addition to membrane emulsifying deoxycholate treatment to increase penetration of the dye and therefore increase accuracy of viable cell DNA amplification with qPCR quantification 3) mRNA and 4) rRNA qPCR quantification and 5) conventional plating. Treatment of samples with PMA and deoxycholate was reported in the literature to be successful in broth in preventing dead cell amplification in qPCR and within this research, the same treatment used within a food system confirmed these findings; however, it proved to be more complicated than in broth. While the combination treatment of PMA and deoxycholate prevented amplification of all DNA from dead cells, it also killed the sub-lethally injured cell population within samples and subsequently this rendered their DNA inaccessible for quantification in qPCR. This could lead to false negative result if the sub-lethally injured cells would have otherwise recovered and remained viable on the beef. Therefore, PMA with deoxycholate treatment requires further optimization before being considered for viable pathogen detection in qPCR. While mRNA and rRNA provided viable cell quantification in qPCR, it was more time and labor intensive and results had higher variability. Overall, conventional plating provided the most robust and reliable result for quantification of live EHEC cells on beef steaks after intervention.

Preface

This is an original work by Anna Marie Laidlaw. Supervisor Dr. Gänzle contributed to projects Chapters 2 and 3, and supervisors Dr. Yang and Dr. Gänzle contributed to projects in Chapters 4 and 5.

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List of Abbreviations

AITC	Allyl Isothiocyanate
CDC	Centers for Disease Control
cDNA	Complimentary Deoxyribonucleic Acid
CFU	Colony Forming Units
CTSMAC	Sorbitol MacConkey Agar with Cefixime Tellurite
DC	Deoxycholate
DNA	Deoxyribonucleic Acid
EFSA	European Food Safety Authority
EHEC	Enterohaemorrhagic *Escherichia coli*
FDA	United States Food and Drug Administration
Gb3	Globotriaosylceramide
GRAS	Generally Regarded As Safe
HC	Haemorrhagic Colitis
HPLC	High Performance Liquid Chromatography
HUS	Haemolytic Uraemic Syndrome
HW	Hot Water
LA	Lactic Acid
LB	Luria-Bertani
LCMS	Liquid Chromatography Mass Spectrometry
LD_{50}	Lethal Dose, 50%
LEE	Locus of Enterocyte Effacement
MIC	Minimum Inhibitory Concentration
mRNA	Messenger Ribonucleic Acid
OD	Optical Density

PA	Peroxyacetic Acid
PBS	Phosphate Buffered Saline
PCA	Plate Count Agar
PMA	Propidium Monoazide
qPCR	Quantitative Polymerase Chain Reaction
RH	Relative Humidity
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RTE	Ready-to-eat
RTqPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
STEC	Shiga Toxin-producing *E. coli*
STX	Shiga-like Toxin
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
USDA	United States Department of Agriculture
VRB	Violet Red Bile
WHO	World Health Organization

1. Introduction

Every year, over 33,000 foodborne illnesses occur in Canada due to shiga-toxin producing *Escherichia coli* (STEC; Thomas et al. 2013). Enterohaemorrhagic *Escherichia coli* (EHEC), a sub-group of STEC, is an important foodborne pathogen as it has a low infectious dose and causes severe disease in humans. Prevention of EHEC infection is difficult due to a broad range of sources that can contaminate our food supply. Novel interventions to eliminate EHEC on food, in addition to increased accuracy for viable EHEC detection on food, are essential to increase food safety and reduce the risk of infection.

1.1 Enterohaemorrhagic *Escherichia coli*

E. coli is a Gram negative, rod shaped, facultative anaerobe. Most strains of *E. coli* are commensal and exist as part of a healthy intestinal flora of humans and animals. Acquisition of virulence factors enables *E. coli* strains to cause disease and EHEC is a pathovar that contains *eae* genes for attachment and effacement and *stx* genes for shiga toxin production (Caprioli et al. 2005). Both are required to cause severe disease in humans including haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS; Bach et al. 2002; WHO 2015). EHEC's *eae* gene allows the microorganism to adhere to epithelial cells and colonize the human intestinal tract, leading to lesions and effacement of microvilli (Caprioli et al. 2005; Kaper et al. 2004). Genes responsible for lesions are found on the locus of enterocyte effacement (LEE), which is composed of 3 major regions: a type III secretion system, intimin and its receptor Tir for adhesion, and lastly, proteins for lesion formation (Delahay et al. 2001). EHEC's *stx* gene is responsible for the production of shiga-like toxins. Human receptors for this cytotoxin are on endothelial cells that express globotriaosylceramide (Gb3), which are primarily found on the renal cortex (Obrig and Karpman 2012). Their attachment to Gb3 prevents subsequent protein

synthesis causing cell death; this is the main cause of HUS and renal failure in patients (Karpman et al. 1998; Jacewicz et al. 1999).

EHEC has a very low infectious dose of only ≤ 10 cells (Willshaw et al. 1995). Initial symptoms of infection include abdominal cramps and diarrhoea, which resolves in the majority of cases within 5 to 10 d (Tarr 1995). However, infection can progress to HC characterized by severe abdominal cramps, bloody stool and colonic mucosal edema. In more severe cases, HC can develop into HUS leading to haemolytic anaemia, thrombocytopenia, acute renal failure, and death in 3-5% of cases (WHO 2015; Mele et al. 2014; Buchanan and Doyle 1997; Bach et al. 2002). Children and the elderly are most susceptible to EHEC infection, as well as the sick and immunocompromised, due to underdeveloped or weaker immune systems (Mayer et al. 2012).

There is no treatment for patients with progressed infection. Antibiotics are counter-productive and current strategies are inadequate in that they can only provide supportive care to alleviate symptoms with rehydration, blood transfusion and dialysis (Szych et al. 2014; Sockett et al. 2014). Until further notice, prevention of infection through control and detection of EHEC in our food supply is the only tool we have to reduce illness outbreaks.

1.2 Outbreaks

The primary source of EHEC is ruminants with cattle as a major reservoir, where they are able to colonize the intestinal tract without inducing disease (Naylor et al. 2003; Lim et al. 2007; Ferens and Hovde 2011; Brown et al. 1997; Doane et al. 2007; WHO 2015). In beef plants, EHEC contamination of a carcass can occur by transfer of intestinal contents during evisceration or upon removal of hide during skinning (Blagojevic et al. 2012; Ferens and Hovde 2011). Not surprisingly, raw or undercooked meat products are common sources of an EHEC outbreak

(Rangel et al. 2005; Brandt et al. 1994; Tuttle et al. 1999). In addition, EHEC is now one of the leading causes of produce outbreaks, including spinach, in the United States due to the microorganism's ability to survive outside of the host in environments such as soil and water (Ogden et al. 2001; Avery et al. 2008; Maule 2000; Olsen et al. 2002).

The consumption of contaminated food is the main route of transmission of EHEC illness outbreaks in humans (Rangel et al. 2005). It is estimated that both O157 and non O157 *E. coli* cause 33,500 cases of disease annually in Canada and an estimated 256,000 cases in the United States (Thomas et al. 2013; CDC 2015a). Furthermore, the annual cost associated with *E. coli* O157:H7 illness alone is estimated to be 26.7 and 405 million in Canada and the United States, respectively (Frenzen et al. 2005; Sockett et al. 2014). With the vast number of outbreaks and costs associated with infection, improved food safety measures are needed.

1.3 Control of EHEC on Food

Lactic acid, peroxyacetic acid, and hot water or steam application are interventions routinely used in meat packing plants to decontaminate carcasses (Gill and Landers 2003). While these interventions can reduce pathogens on food (Carlson et al. 2008), complete elimination is difficult since the food's matrix provides protection and enables survival. This creates a need for new and improved intervention methods to increase the control of pathogens on food. The use of mustard, a natural antimicrobial, could be one such method. Specifically, black and brown mustard seed contain high concentrations of the glucosinolate sinigrin (Antonious et al. 2009). When the seed's endogeneous enzyme myrosinase catalyzes the hydrolysis of sinigrin, antimicrobial allyl isothiocyanate (AITC) is released (Shofran et al. 1998; Delaquis and Mazza 1995). AITC has a broad antimicrobial spectrum with potent multi-targeted activity including membrane disruption and enzyme inhibition through interaction with amino acid residues and

thiol groups (Luciano and Holley 2009; Lin et al. 2000). Furthermore, it has the ability to kill pathogens such as *E. coli* O157:H7 on a variety of food products including beef and spinach (Nadaraja et al. 2005a; Obaidat and Frank 2009a). However, use of mustard as an intervention on food is not without challenges since effective AITC release from sinigrin is influenced by factors such as storage temperature and humidity (Dai and Lim 2014). Moreover, the antimicrobial's properties also limits its use as an intervention in food: AITC's pungent aroma and spicy flavor may decrease the sensory quality of food, and its volatility makes the compound unstable in addition to causing irritation to exposed workers (Nadaraja et al. 2005b; Sekiyama et al. 1994). Further research investigating optimal conditions for mustard AITC intervention would be beneficial, since AITC is a strong antimicrobial and its use from mustard, a natural source, would provide a clean label on food products (Khan and Abourashed 2009).

1.4 Detection of EHEC on Food

In 1994, the United States Department of Agriculture declared *E. coli* O157:H7 an adulterant in raw ground beef. Six additional non-O157 EHEC strains have recently been added to this list (O26, O45, O103, O111, O121, and O145) and along with O157, there is a zero-tolerance policy for these microorganisms in non-intact beef products in the United States (Brooks et al. 2005; USDA 2012). Similarly in Canada, raw beef is prohibited to be sold if tested positive for *E. coli* O157:H7 (Health Canada 2015a). While testing for *E. coli* O157:H7 in beef is required, it is not mandatory for produce such as spinach and lettuce (FDA 2013). Needless to say, there is a need for rapid and accurate detection methods for viable EHEC in food to retain quality and ensure safety.

Culturing methods are effective in detecting viable bacterial cells in a sample. In addition, these methods are simple and cost effective; however, they are time consuming (Sidari et al.

2011). Advances in technology have lead to improved molecular methods of detection such as real time quantitative polymerase chain reaction (qPCR). qPCR provides rapid and selective detection of microorganisms in samples, but is more complex and costly compared to conventional plating. qPCR is a commonly used detection technique for government regulatory agencies in both Canada and the United States have validated methods for EHEC detection on food that use qPCR (USDA 2015a; Health Canada 2015b; Bettelheim and Beutin 2003). Still, qPCR detection does not come without limitations. Two major barriers to successful rapid and accurate detection of EHEC by qPCR include the requirement of a lengthy enrichment step prior to analysis and the inability to distinguish between live and dead cell DNA (Wu et al. 2015). Finding solutions to these limitations would greatly increase the efficiency of qPCR pathogen detection in food.

Enrichment is a laboratory technique in which a culture or sample is added to an appropriate nutritional medium and incubated under optimal conditions (temperature, pH) that favors the growth of the culture or target microorganisms within the sample (Brehm-Stecher et al. 2009). It is an essential pre-step to pathogen detection on food: it increases low counts of target pathogen in samples, resuscitates injured or stressed cells and dilutes food inhibitors and background microflora (Dwivedi and Jaykus 2011). It is therefore often used prior to other detection methods, including conventional plating and molecular methods such as qPCR. However, its lengthy time creates a challenge with use; when coupled with rapid detection methods such as qPCR, enrichment increases overall detection time from hours to days (Brehm-Strecher et al. 2009; Ge and Meng 2009). On the other hand, enrichment can fail to detect sub-lethally injured cells if length of time is shortened, since injured cells have a longer lag phase (Li et al. 2006). Current standard enrichment time for *E. coli* O157:H7 detection in ground beef (75 g) and leafy

greens (25 g) requires 7 - 24 h to complete (Health Canada 2015b). Investigation into current enrichment time would be beneficial to determine whether these times are sufficient to detect an injured cell population, and if enrichment times could be decreased to increase speed of overall detection without risk of false negative result.

The other limitation of qPCR is that it is unable to discriminate between live and dead cells. This creates a major problem for pathogen detection on food, since it can lead to false positive result by detecting dead cell DNA within a sample (Wang et al. 2013a; Wu et al. 2015). RNA quantification in qPCR may be able to better quantify viable EHEC cells in a sample since RNA degrades rapidly upon cell death (Nocker and Camper 2009). However, RNA quantification is more complex, time consuming, and has a higher risk of sample loss due to its rapid degradation upon cell death, and therefore may not provide a practical alternative to DNA quantification. Recently, a method has been proposed to differenciate live and dead cells in qPCR by use of a DNA binding dye, propidium monoazide (PMA). PMA is a photo-inducible DNA binding dye that penetrates only into cells with compromised membranes, intercalates with DNA and upon exposure to light, forms covalent bonds with nucleic acid bases to subsequently prevent amplification (Elizaquivel et al. 2014). Unfortunately, PMA may be excluded from dead cells with intact membranes and therefore overestimation of live cells still remains an issue (Patcholewicz et al. 2013). However, PMA used in conjunction with an emulsifier, deoxycholate, can enhance PMA penetration by permeabilizing membranes of dead cells whereas viable EHEC cells have a high tolerance to bile salts due to the outer membrane (Yang et al. 2011). Investigation into this treatment may help resolve the issue of dead cell DNA amplification leading to false positive result in qPCR.

1.5 Research Hypotheses and Objectives

Effective control and detection methods for pathogens on food are critical to ensure a safe food supply and prevent human infection. Continued foodborne illness outbreaks indicate that current methods require improvement. My first hypothesis is that antimicrobial AITC released from mustard meal can be used as an intervention to control pathogens on food. My second hypothesis is that detection methods can be enhanced by a) decreasing EHEC enrichment time and b) preventing dead EHEC cell detection in qPCR.

Research aimed to meet the following objectives:

1. With respect to the first hypothesis, conduct a literature review to compare the effect of AITC intervention on food in relation to food sensory quality and health (Chapter 2).
2. With respect to the first hypothesis, determine the efficacy of a novel AITC intervention by investigating its use as a volatile gas released from mustard meal to control EHEC on spinach and lettuce at refrigerated temperature (Chapter 3).
3. With respect to the second hypothesis, investigate necessary enrichment times through analysis of lag phases of heat injured individual EHEC cells with spectrophotometer measurement, and growth of heat injured EHEC cells over 24 h with qPCR quantification (Chapter 4).
4. With respect to the second hypothesis, evaluate five different detection methods to determine the most accurate method to quantify viable EHEC cells on beef steaks after intervention, including PMA treatment or PMA with deoxycholate treatment prior to qPCR DNA quantification, mRNA and rRNA RTqPCR quantification, and conventional plate count method (Chapter 5).

2. A Review of Allyl Isothiocyanate Encompassing Food and Health: from Antimicrobial Activity against Foodborne Pathogens and Sensory Effect on Food to Anti-cancer Properties

2.1. Introduction

Consumers prefer a clean, all-natural label on food to foods labeled with chemical preservatives or intervention technologies such as irradiation, and industry is happy to comply with the wishes of consumers (Sloan 2014; Tiwari et al. 2009; Heath 1981; Yeung and Morris 2001). Biopreservatives such as phages and bacteriocins provide food with clean label; however, a disadvantage to their use is that they are specific to one target microorganism (USDA 2015b; Cleveland et al. 2001). Natural antimicrobials such as organic acids and essential oils also provide a clean label and have the benefit of being non-specific which enables them to target a wide range of microorganisms; however, they also interact with food matrix and can change food properties such as pH, flavor and color (Perricone et al. 2015; Lucera et al. 2012). In this case, a balance needs to be achieved between antimicrobial efficacy and sensory quality of these preservatives on food if they are to be used as a practical intervention by industry. Allyl isothiocyanate (AITC) is a compound naturally found in cruciferous vegetables and is the main compound in mustard essential oil (Shofran et al. 1998, FDA 2015a). It has antimicrobial properties and has been approved for use as a preservative in the United States and Japan (Sekiyama et al. 1994; FDA 2014a). Moreover, when AITC is used from a natural source, it does not require listing as a synthetic preservative (Khan and Abourashed 2009). However, its use comes with challenges related to volatility as well as antimicrobial efficacy and sensory quality in food. In addition, AITC is responsible for the pungent odor and spicy flavor characteristic of wasabi, horseradish and mustard which may affect sensory quality of food.

Concentrations of AITC effective in killing pathogens on some food products have also shown to have acceptable sensory quality (Table 2.2, 2.3 & 2.5). Furthermore, AITC is versatile in that it can be applied to food in various forms such as a vapor, liquid or as a natural ingredient such as mustard powder (Figure 2.1, Table 2.2 & 2.3). In addition, new forms of AITC have been developed to control volatility and increase manageability for practical use as an intervention, including packaging films, coatings, and microencapsulation (Figure 2.1, Table 2.3). Interestingly, AITC is also linked to cancer prevention, a devastating disease that kills many people (Table 2.7). The objective of this review is to discuss AITC's antimicrobial efficacy and sensory effect on food as well as AITC's anti-cancer effect.

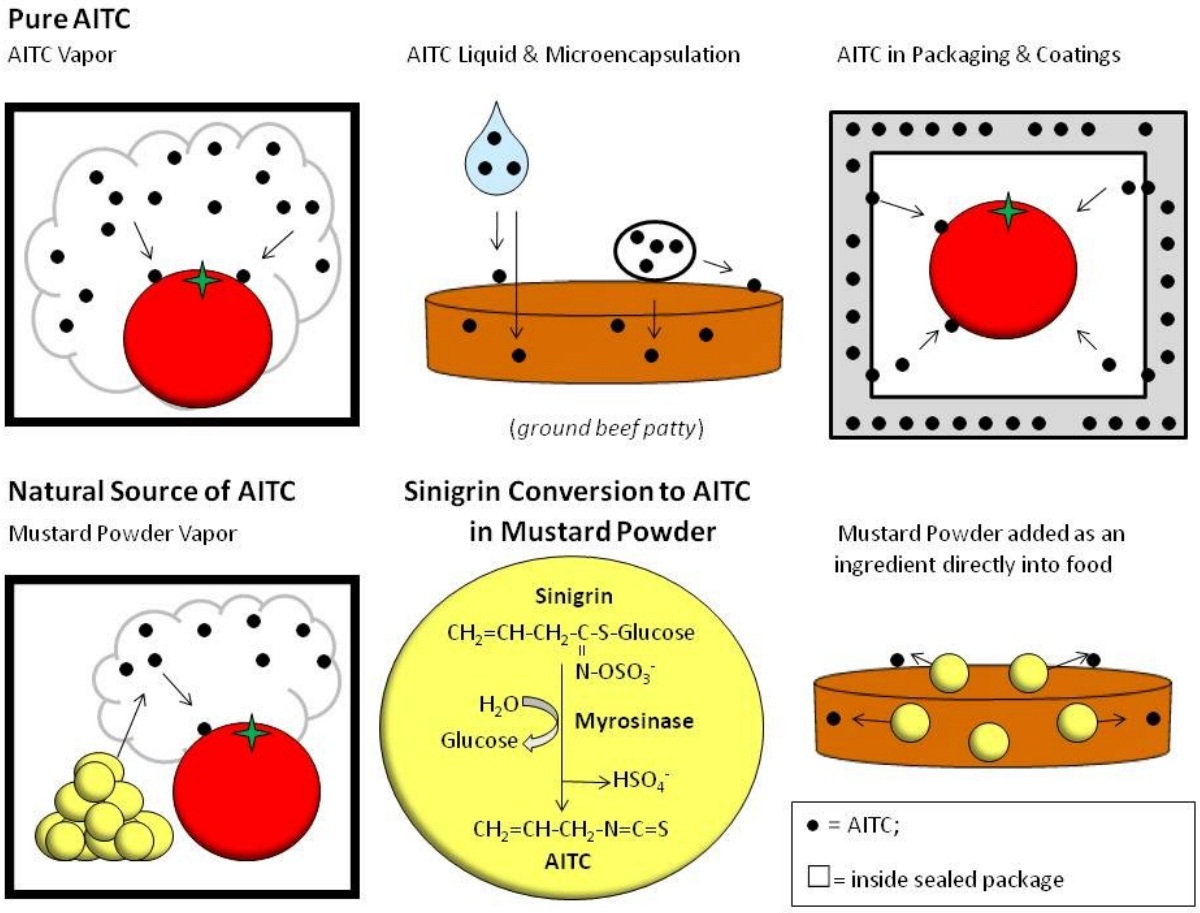

Figure 2.1. Different forms of AITC intervention on food

2.2. Regulatory Aspects of AITC use in Food

AITC and mustard essential oil (>93% AITC) are generally regarded as safe (GRAS; FDA 2015a). However, AITC exposure can cause irritation to skin, eyes and respiratory tract, and is therefore prohibited for use in topical cosmetic products in Canada (Health Canada 2014; CDC 2015b). Furthermore, consumption of AITC (40 µL, 0.125% AITC) can cause irritation to oral cavity (Simons et al. 2003) and may also cause gastrointestinal irritation (CDC 2015b). Although no toxicity studies are currently available for humans, the level of toxicity in mice (LD_{50}) is approximately 310 mg AITC/kg body weight (WHO 2006; EFSA 2010) and based on this data levels of AITC used in food research (Tables 2.2-2.4) are likely safe for human consumption without harmful effect. In addition, some natural plant sources of AITC such as mustard have recently been listed as an allergen (Health Canada 2012a). However, AITC and mustard essential oil likely are not allergenic as the protein that causes allergic reaction is removed during processing and only trace amounts remain (Lerbaek et al. 2004, EFSA 2007).

The addition of AITC to food is regulated in many countries as a preservative or flavor enhancer. AITC has been approved for use as a preservative in food systems in the United States and Japan, and approval is currently being sought after in Europe (FDA 2014a; Sekiyama et al. 1994; EFSA 2010). In the US, the amount of AITC allowed as a preservative on food is 30-50 ppm AITC or 200 µg AITC per L air in vapor phase (FDA 2005; FDA 2014a). Though not approved as a preservative in Canada, it is used as a flavor enhancer (Health Canada 2015c). While there is no maximum amount listed when used for this purpose, use is usually limited by its pungency. The FDA states that when used as a food additive, AITC should be used in the minimal quantity required to produce the intended effect (FDA 2015b). Furthermore, AITC is already present in condiments such as mustard, wasabi, horseradish, and foods to which these

condiments are an ingredient and these levels are not regulated as AITC has not been added but rather is present naturally from the hydrolysis of its precursor compound sinigrin (Delaquis and Sholberg 1997; Shofran et al. 1998). AITC released from sinigrin in plant sources used as a preservative agent would provide a clean, all-natural label on food products. On the other hand, use of AITC in food systems is regulated in any form; pure AITC and volatile mustard oil are similarly regulated due to the essential oil's high concentration of the compound; however, they are listed differently on a food label as the source, either synthetic or natural, needs to be indicated (Khan and Abourashed 2009). Consumers are becoming increasingly discriminative in their food choices leading to a demand for all-natural food products. Therefore, the use of AITC from natural plant sources as a food preservative would satisfy this request (Sloan 2014; Tiwari et al. 2009; Heath 1981).

2.3. Minimum Inhibitory Concentration

AITC's minimum inhibitory concentration (MIC) against several foodborne pathogens has been investigated (Table 2.1). There is no indication that resistance differs between microorganisms as AITC has a broad antimicrobial spectrum and kills both Gram negative and Gram positive bacteria. MICs of AITC against foodborne pathogens are influenced by temperature. While MICs on Table 2.1 were carried out between 30-37°C, a reduction in temperature to 21°C reduced the MIC of *Listeria monocytogenes* from 100 to 40 mg/L and for *Salmonella* Typhimurium from 80 to 10 mg/L (Olaimat and Holley 2013). Moreover, pH also affects AITC inhibitory concentrations on pathogens. MICs on Table 2.1 were carried out at neutral pH; however, an increase in pH leads to an increase in MIC. For *Escherichia coli* O157:H7, MICs increased from 25 mg/L at pH 5.5 to 500 mg/L at pH 8.5, respectively, and for

L. monocytogenes, MIC increased from 27 mg/L at pH 5 to 100 mg/L at pH 7 (Luciano and Holley 2009; Miyague et al. 2015).

Table 2.1. MICs of AITC on Foodborne Pathogens

Species	MIC range (mg/L)	Reference
Escherichia coli	30-300	Luciano et al. 2009; Borges et al. 2015; Dussault et al. 2014; Kyung and Fleming 1997; Palaniappan and Holley 2010
Salmonella spp.	30-300	Olaimat and Holley 2013; Dussault et al. 2014; Palaniappan and Holley 2010; Kyung and Fleming 1997
Bacillus cereus	300	Dussault et al. 2014
Listeria monocytogenes	75-300	Olaimat and Holley 2013; Lara-Lledo et al. 2012; Borges et al. 2015; Dussault et al. 2014; Kyung and Fleming 1997; Miyague et al. 2015
Pseudomonas spp.	100-300	Borges et al. 2015; Dussault et al. 2014
Staphylococcus aureus	15-300	Borges et al. 2015; Dussault et al. 2014; Palaniappan and Holley 2010; Kyung and Fleming 1997
Campylobacter jejuni	5-200	Borges et al. 2015; Dufour et al. 2012

2.4. Mechanism of Action

Isothiocyanates are reactive molecules and react readily with glutathione, amino acids and proteins including sulfhydryl, alcohol and thiol groups and water; upon reaction, AITC degrades into other non-antimicrobial products (Shofran et al. 1998). Due to its high reactivity with many compounds, AITC is a non-specific antimicrobial that has multi-targeted activity with several mechanisms of action. One mode of action is enzyme inhibition due to the isothiocyanate group's interaction with amino acids and thiol groups (Lin et al. 2000a; Turgis et al. 2009;

Cejpek et al. 2000; Luciano et al. 2008). Moreover, AITC inhibits carriers in the electron transport chain, leading to loss of cell viability due to inhibition of cell respiration (Kojima and Ogawa 1971). AITC also causes cell membrane damage, due to both protein interaction as well as its oxidative activity leading to disintegration of disulfide bonds, key regulators of the homeostasis of the cell (Luciano and Holley 2009). All damage that is inflicted to a cell has yet to be fully explored; however, AITC likely interferes with various essential proteins for cell viability.

Due to AITC's non specific binding to proteins, its use in a food system can present a challenge if foods are abundant with these compounds. Rather than exerting effect on microorganisms present, AITC may bind to alternative reaction partners in the food matrix and be neutralized. This is also true for other non-specific antimicrobials such as sodium hypochlorite, a strong oxidizing agent in buffer or on hard surfaces, but when used in the presence of organic matter is inactivated (McDonnell and Russell 2009; CDC 2009).

2.5. AITC as an Intervention on Food

AITC can control pathogens on at-risk foods including meat and produce (Tables 2.2 and 2.3). However, application of the antimicrobial differs within a food system; concentrations of AITC required for antimicrobial effect vary with food composition and moreover, food matrix can protect cells and enable survival (Schirmer and Langsrud 2010). While AITC's antimicrobial action involves binding to the amino acids and proteins in microorganisms, foods that are abundant in these compounds gives AITC an alternative binding site and neutralizes its antimicrobial effect (Ohta et al. 1995). Within food systems AITC is adaptable as an intervention which allows it to be tailored to food product, with different forms including liquid, vapor, coatings and packaging films and natural plant sources (Figure 2.1). For food surfaces easily

bruised such as the tender skin or leaves of produce, AITC vapor may be the best method of application. However, in ground beef patties or sausages, mustard powder could be the best method of application since it is already used as a flavoring agent in these products and can be directly mixed into meat batter to provide antimicrobial activity throughout the product.

2.5.1. AITC Vapor

AITC is volatile and therefore can be applied in vapor form as an intervention on food. AITC is vaporized into headspace by placing a source of AITC with a food product in a closed environment. Different sources from which AITC can be volatilized include liquid solutions, beta cyclo-dextrin inclusion complexes, calcium alginate beads, desorption from deoiled brown algae or brown seaweed matrices or vapor generating films and labels (Lin et al. 2000; Piercey et al. 2012; Seo et al. 2012; Chen et al. 2013; Winther and Nielsen 2006; Siahaan et al. 2013; Siahaan et al. 2014). AITC volatilization from these sources can be hard to control and factors that influence rate of volatility include vapor pressure, temperature and humidity (Sekiyama et al. 1994).

There is a wide range of AITC vapor concentrations effective in killing pathogens on food (Table 2.2). AITC gas is more effective on produce than on meat. On spinach, concentrations as low as 4 μL AITC vapor per L headspace reduced *E. coli* O157:H7 by 4 log CFU but higher concentrations of at least 450 mg AITC gas per L headspace were required on meat for similar antimicrobial activity (Obaidat et al. 2009a; Muthukumarasamy et al. 2003). This relates to mode of action as AITC readily reacts with sulfhydryl groups of which meat has many, and produce does not. This causes AITC to become neutralized and therefore requires higher concentrations of the antimicrobial for observable effect on meat. In addition, AITC's antimicrobial effect is more pronounced on pathogens inoculated onto intact produce surfaces as

opposed to cut or damaged produce surfaces, as the latter enables the food's matrix to provide increased protection from the intervention and a better attachment site for the microorganism (Obaidat and Frank 2009a, 2009b). Overall, AITC vapor activity is temperature dependant and is more lethal at higher temperatures (Seo et al. 2012, Chen and Brody 2013, Obaidat and Frank 2009a, 2009b).

2.5.2. Liquid or Microencapsulated AITC

AITC can also be applied as liquid intervention (Table 2.3). However, there are few studies on the efficacy of liquid intervention on food and of these studies, a wide range of AITC concentrations are used (2 mg/L – 9.5 mg/L) which are not always effective. Since AITC in liquid form, similar to vapor form, is highly unstable it creates challenge for practical use as an intervention. Microencapsulated AITC has been developed to overcome this limitation and has successfully reduced *E. coli* O157:H7 on dry cured hams and in beef and sausages (Table 2.3).

2.5.3. AITC in Food Packaging and Coatings

An alternative method of AITC intervention is incorporation into packaging and coatings of food (Table 2.4). Two main types of coatings for direct application to food have been developed: non edible coatings (polylactic acid-based) beneficial for non consumed surfaces of food (rinds, egg shells), and edible coatings (chitosan-based) beneficial for use on surfaces of foods that are consumed (vegetables, cooked Ready-To-Eat (RTE) shrimp). In a study comparing both types of AITC incorporated coatings, polylactic acid based coatings were slightly less effective than chitosan based coatings for reduction of pathogens on food; however, this is not surprising since chitosan also has antimicrobial activity which creates an additive effect (Jin et al. 2013). Food composition also plays a role in antimicrobial efficacy of coatings; application of 9 $\mu L/cm^2$ AITC was less effective for *Salmonella* spp. reduction on egg shell than on the surface

Table 2.2. Use of AITC Vapor to Control Pathogens on Food

Food	AITC Concentration	Pathogen	Initial Inoculation	Antimicrobial Activity	Reference
Lettuce	100 µL/L	*S.* Montevideo	8 log CFU/g	8 log CFU/g reduction in 2 d at 4°C	Lin et al. 2000b
	50 µL/L		8 log CFU/g	1 log CFU/g reduction in 2 d at 4°C	
	88 µL/L	*E. coli* O157:H7	7 log CFU/g	7 log CFU/g reduction in 2 d at 4°C (undetected)	
	25 µL/L		7 log CFU/g	1 log CFU/g reduction in 2 d at 4°C	
Lettuce	4 µL/L	*E. coli* O157:H7	5.8 log CFU/leaf	4.2 log CFU/leaf reduction in 4 d 4°C	Obaidat and Frank 2009a
Spinach	4 µL/L	*E. coli* O157:H7	5 log CFU/leaf	4 log CFU reduction 4 d at 4°C	
Spinach	68 mg/L	*E. coli* O157:H7	5.6 log CFU/leaf	2.6 log CFU/leaf reduction at 4°C by 5 d (85% RH)	Seo et al. 2012
Spinach	50g/L	*E. coli* O157:H7	6 log CFU/g	>5 log CFU/g reduction after 2 days at 4°C	Huang et al. 2012
Tomato (whole)	8.3 µL/L	*E. coli* O157:H7	7.8 log CFU/ site	5 log CFU/site reduction in 4 d at 4°C	Obaidat and Frank 2009b
	33.3 µL/L			>4 log CFU/site reduction in 10 d at 10°C	
				>3.5 log CFU/site reduction in 10 d at 25°C	
	8.3 µL/L	*S.* Enteriditis	7 log CFU/ site	>4 log CFU/site reduction in 4 d at 4°C	
	33.3 µL/L			>5 log CFU/site reduction in 7 d at 10°C	
				>2 log CFU/site reduction in 7 d at 25°C	
Tomato (whole)	125 µL/L	*S.* Montevideo	8.6 log CFU/g	8 log CFU/g reduction in 2 d at 13°C	Lin et al. 2000b
Tomato (whole)	40 µL/L	*S.* Typhimurium	N/A	>5 log CFU/g reduction in 18 h at 22°C (undetected)	Yun et al. 2013

Onion (cut)	200 µL/L	*L. monocytogenes*	3.5 log CFU/g	2.5 log CFU/g reduction after 10 d at 5°C	Piercey et al. 2012
Alfalfa Sprouts	200 -500 mg/L	*S.* Typhimurium	7.5 log CFU/g	7.5 log CFU/g reduction 4 d at 10°C (undetected)	Weissinger et al. 2001
Raw ground beef	450 mg/L	*E. coli* O157:H7	6 log CFU/g	>3 log CFU/g reduction after 21 d at 4°C	Nadaraja et al 2005a
Raw ground beef	1300 mg/L	*E. coli* O157:H7		5 log CFU reduction after 25 d at 4°C	Muthukumarasamy et al. 2003
Raw chicken breast	600 ug*/container	*L. monocytogenes*	4 log CFU/g	< 1 log CFU/g reduction at 4°C after 21 d (1.2 µg/h release rate)	Shin et al. 2010
	600 ug*/container	*S.* Typhimurium	4 log CFU/g	1 log CFU/g reduction at 4°C for 12 d (1.2 µg/h release rate)	
Raw catfish fillets	0.04 mg/L	*P. aeroginosa*	3.5 log CFU/g	0.8 log CFU/g reduction after 1d at 8°C	Pang et al. 2013
Cooked ham (sliced)	0.4 mg/L	*L. monocytogenes*	4 log CFU/g	Did not inhibit growth, no reduction at 4°C by 28 d	Chen and Brody 2013

*container size unknown

Table 2.3. Use of Liquid or Microencapsulated AITC for Control of Pathogens on Food

Food	AITC Concentration	Pathogen	Initial Inoculation	AITC Antimicrobial Activity	Reference
			Liquid		
Raw tuna (fatty)	50 mg/L	*Vibrio para-haemolyticus*	2 log CFU/mL	Growth inhibited at 37°C in 8 h	Hasegawa et al. 1999
Raw tuna (lean)				No inhibition; grew 4.5 log CFU/mL at 37°C in 8 h	
Raw egg	9.5g/L	*Yersinia pestis*	1 log CFU/mL	1 log CFU/mL reduction by 11 d at 4°C	Gurtler et al. 2010
Fermented cabbage	2g/L	*E. coli* O157:H7	6 log CFU/g	Inhibited growth, no reduction in 4 d at 10°C	Inatsu et al. 2005
		S. Enteriditis	6 log CFU/g	Inhibited growth, no reduction in 4 d at 10°C	
		S. aureus	6 log CFU/g	Inhibited growth, no reduction in 4 d at 10°C	
		L. monocytogenes	6 log CFU/g	Inhibited growth, no reduction in 4 d at 10°C	
			Microencapsulation		
Dry cured ham	0.2 mg/kg	*E. coli* O157:H7	7 log CFU/g	3.2 log CFU/g reduction in 45 d ripening	Graumann and Holley 2009
	0.3 mg/kg			5.7 log CFU/g reduction in 45d ripening	
Fermented sausage	500 mg/kg	*E. coli* O157:H7	6.5 log CFU/g	4.75 log CFU/g reduction in 28 d ripening	Chacon et al. 2006a
	750 mg/kg			6.5 log CFU/g reduction in 21 d ripening	
	1000 mg/kg			6.5 log CFU/g reduction in 16 d ripening	
Raw beef (chopped)	4980 mg/kg	*E. coli* O157:H7	8 log CFU/g	8 log CFU/g reduction,18 d,4°C	Chacon et al. 2006b
	28 28 mg/kg			3 log CFU/g reduction,18 d, 4°C	
	333 mg/kg			No reduction in 18 d at 4°C	

Table 2.4. Use of AITC in Coatings or Packaging Films to Control Pathogens on Food

Food	Concentration	Pathogen Targeted	Initial Inoculation Level	Antimicrobial activity	Reference
Coating (applied directly to food)					
Cantaloupe (whole)	9 µL/cm^2	*Salmonella* spp.	6.8 log CFU/cm^2	5.6 log CFU/cm^2 reduction after 24 h at 10°C	Chen et al. 2012
Raw shrimp (whole)	~3 µL/g	*L. innocua*	8.2 log CFU/g	4.48 log CFU/g reduction after 2 h at room temperature	Guo et al. 2013a
Cooked shrimp (whole)	~3 µL/g	*L. innocua*	7.75 CFU/g	>5.5 log CFU/g reduction after 1 d at 4°C	Guo et al. 2013b
Egg shell	3 µL/ cm^2	*S. enterica*	6.8 log CFU/cm^2	1.1 log CFU/cm^2 reduction after 1 d at 22°C	Jin et al. 2013
	9 µL/ cm^2		6.8 log CFU/cm^2	1.7 log CFU/cm^2 reduction after 1 d at 22°C	
Raw chicken breast	25 µL/g†	*C. jejuni*	6.18 log CFU/g	3.74 log CFU/g reduction in 5 d at 4°C	Olaimat et al. 2014
	50 µL/g†		6.18 log CFU/g	>6 log CFU/g reduction in 5 d at 4°C (not detected)	
Packaging film or container					
Liquid egg albumin	0.5% AITC (coated container)	*Salmonella* spp.	7 log CFU/mL	7 log CFU/mL reduction after 21 d at 10°C (not detected)	Jin and Gurtler 2011
Cooked chicken (shredded)	28% AITC (film)	*S.* Choleraesuis	5.7 log CFU/g	2.8 log CFU/g reduction in 10 d at 4°C	Dias et al. 2013
Cooked turkey (sliced)	5% AITC* (film)	*L. monocytogenes*	5 log CFU/g	>4 log CFU/g reduction after 35d at 10°C	Guo et al. 2015

*coating or film contains 1.5-2% chitosan and 2% lactic, acetic and levulinic acid

† coating or film contains 0.2% K-carrageenan and 2% chitosan

of whole cantaloupe (Chen et al. 2012; Jin et al. 2013). AITC can also be incorporated into packaging films or containers (Table 2.4). Similar to coatings, packaging can be chitosan or polylactic acid based and have successfully reduced pathogens on food with incorporation of 0.5-28% AITC (Table 2.4) and AITC plays the main role in their antimicrobial activity (Guo et al. 2015). As AITC antimicrobial packaging and coatings are relatively new, further optimization on concentrations effective against target pathogens on various food matrices and under different storage conditions are warranted.

2.5.4. Mustard and Other Natural Sources of Sinigrin as a Precursor for AITC

Natural plant sources can also be used as a vector for AITC release in food interventions. Cruciferous vegetables of the plant family *Brassicaceae* including cauliflower, garlic, Brussels sprouts, collard greens and mustard naturally contain AITC in the form of a stable precursor compound, the glucosinolate sinigrin. Black, brown and Oriental mustard seed contain the highest levels of sinigrin as compared to other cruciferous vegetables, and therefore focus has been given to these species for AITC intervention in food safety systems (Antonious et al. 2009).

Within the mustard seed, volatile AITC is stabilized in the form of sinigrin. AITC production from sinigrin can occur in two ways upon disruption of the seeds tissues. When no heat treatment is applied to mustard meal (also called “hot” or “non-deheated” mustard), endogenous enzyme myrosinase within the seed catalyzes the hydrolysis of sinigrin to release the AITC. By this method, AITC is the major compound produced at neutral pH, and the only product of this reaction to have antimicrobial activity (Shofran et al. 1998; Brabban and Edwards 1995; Antonious et al. 2009). If mustard meal is subjected to heat treatment (“deheated” mustard), the plant’s myrosinase enzyme is inactivated and instead, some microorganisms (including pathogens such as *E. coli* O157:H7) that contain enzymes with myrosinase-like

activity can catalyze the hydrolysis of glucosinolates to isothiocyanates. In this manner, it is hypothesized that these microorganisms unintentionally release AITC while trying to extract a glucose molecule, and in doing so, are killed (Luciano et al. 2011). Both deheated and non-deheated mustard meal have been investigated as interventions in food, and interestingly, a combination of the two may be the most lethal (Cordiero et al. 2014).

Mustard seed meal is the most investigated natural source of AITC in food safety research. Similar to pure AITC, it can be applied as intervention in different forms including AITC released from sinigrin in mustard meal as vapor, mustard meal directly added as an ingredient in food products, or mustard meal incorporation into food packaging films. Since mustard is already used in the meat industry as a binder, filler and flavoring agent, it could also be used as an antimicrobial in these same products without the need to add new ingredients as preservative agents. Direct application of mustard meal (2% -20%) as an ingredient in ground beef and dry fermented sausages has successfully reduced *E. coli* O157:H7 by up to 5-7 log CFU/g (Cordiero et al. 2014; Nadaraja et al. 2005a). Furthermore, mustard incorporated into packaging film successfully reduced *E. coli* O157:H7 and *L. monocytogenes* on fresh beef and RTE bologna sausages with 1300 ppm mustard sinigrin and 5% mustard, respectively, by > 4 log CFU/g (Herzallah and Holley 2015; Lara-Lledo et al. 2012). Efficacy of mustard intervention is influenced by concentration, storage time and temperature, target species and food matrix. There is some research on other natural sources of AITC for intervention on food which has included cauliflower juice, horseradish vapour and fresh garlic, all of which have shown lethal activity against foodborne pathogens (Brandi et al. 2006; Delquis and Mazza 1995; Ward et al. 1998; Al-Delaimy et al. 1971).

2.5.5. Comparison of AITC Intervention Forms on Food

AITC vapor intervention has been extensively researched and has proven efficacy in controlling pathogens on food (Table 2.2). Intervention studies with liquid AITC are few and available research suggests it does not have strong effect on pathogens in food. Liquid AITC was not able to reduce pathogens in a low pH food (kimchi), which was surprising since AITC had stronger inhibitory effect at lower pH in broth (Luciano and Holley 2009; Inatsu et al. 2005). Microencapsulated AITC is a recent development but shows promise as an effective intervention. The concentration of AITC required for effective control of pathogens on meat is dependent on whether AITC is applied to surface only or within a meat batter. This is because the latter leads to increased surface area of meat that comes into contact and possible react with the antimicrobial (such as protein amine and thiol groups), thus reducing its efficacy. AITC vapor is effective in killing surface pathogens on fresh ground beef at 450 mg/L. Microencapsulated AITC can either be added onto meat product surfaces or mixed as an ingredient into meat batter; when added onto the surface of dry cured hams, 0.3 mg/kg was effective in reducing pathogens but when added into fresh ground beef, a concentration of 333 mg/kg had no effect on pathogen reduction (Graumann and Holley 2009; Nadaraja 2005b; Chacon et al. 2006a). This suggests AITC intervention is best suited for surface decontamination of meat.

There are many influences on efficacy of AITC. Temperature plays a major role in AITC efficacy but differs with AITC form and target pathogen (Olaimat and Holley 2014). Other factors include increased pathogen survival after AITC exposure when in a nutrient rich matrix or medium, at neutral to acidic pH (Luciano and Holley 2009; Miyague et al. 2015), dry (Park et al. 2000), high fat content (Hasegawa et al. 1999), and on cut or damaged produce rather than

intact surface (Obaidat and Frank 2009a, 2009b). AITC form and concentration, food matrix, storage time and temperature, and species targeted needs to be considered for effective intervention application.

2.5.6. Multi-Hurdle Intervention Approach

AITC is also useful in a multi-hurdle intervention approach. AITC has been used in conjunction with modified active packaging (MAP) with atmospheres of 100% N_2 to 70-50% N_2 and 50-30% CO_2 to increase intervention lethality on food (Nadaraja et al. 2005a; Shin et al. 2010; Pang et al. 2013). The efficacy of AITC can also be increased when used in combination with other antimicrobials. Use of chitosan with AITC had synergistic effect against foodborne pathogens (Inatsu et al. 2005; Jin et al. 2013). Furthermore, its application in a step wise intervention successfully increased antimicrobial effect when used with chitosan coating combined with cryogenic freezing (-75°C, 2 min) and an ozone wash (1.9 ppm ozone) to reduce *L. innocua* on shrimp (Guo et al. 2013a). AITC (2 mg/L) has also increased lethality of intervention when used in combination with high hydrostatic pressure (600 MPa; Raouche et al. 2011).

2.6. Sensory Evaluation of AITC on Food

Sensory quality of food can be influenced by the application of AITC as an intervention. Though pure AITC is colorless, its oxidative properties may cause undesirable color changes to food (CDC 2015b). Furthermore, AITC has a spicy flavor and pungent aroma which can be transferred to food (Sekiyama et al. 1994). AITC has a flavor threshold of 375 ppm and if present in higher amounts than this in food, its odor and flavor can be sensed (Belitz et al. 2004). Different food products are affected differently by AITC exposure; while some foods remain unaffected, others have unacceptable changes in odor, flavor, smell, texture and color. The

changes in sensory quality of food are dependent on food composition, storage temperature, duration of exposure and AITC concentration and form (Tables 2.5 and 2.6).

2.6.1. Effect of AITC on Food Flavor and Odor

Analysis of flavor and odor changes to food with application of AITC was conducted by panelists using hedonic scales for assessment (Table 2.5). Grain products were only acceptable with minimal AITC exposure (<1 mg/L), as unacceptable changes to flavor and aroma occurred in rice and bread at higher levels (1 mg/L-2 mg/L) after only 1-2 d storage (Nielsen and Rios 2000; Kim et al. 2002). On the other hand, raw cabbage had acceptable flavor and odor with up to 50 mg/L AITC vapor after almost two weeks of storage (Banerjee et al. 2015). For mustard powder, 5-10% was acceptable in ground beef patties whereas at 20%, patties were deemed too spicy and brittle (Nadarajah et al. 2005). Liquid or microencapsulated AITC added to fermented sausages at 0.5 g/kg was slightly spicy but acceptable in flavor while levels above this (0.75-1 g/kg) were deemed unacceptable (Chacon et al. 2006b). Similarly in kimchi, acceptable flavor and odor was observed with <1 g/kg AITC; levels above this were unacceptable (Inatsu et al. 2005). Moreover, length of AITC exposure influences food sensory quality. In lightly fermented cabbage, 2 g/kg was acceptable but in kimchi (a prepared fermented cabbage dish), 2 g/kg was found to be unacceptable. While both were stored at 10°C, in the former study storage time was only 1 d and the latter, 2 weeks, and therefore time of exposure to AITC could explain the discrepancy between the two studies (Ko et al. 2012; Inatsu et al. 2005). Interestingly, other foods had improved sensory quality with increased storage time after initial AITC application because food lost pungency over time; semi-hard cheese applied with 93 mg/L AITC vapour had a strong mustard flavor and was unacceptable after 1-3 weeks, but at 12 weeks, mustard flavor had dramatically decreased and flavor was acceptable (Winther and Nielsen 2006).

Table 2.5. Sensory Evaluation of AITC in Food

Food	Form of AITC	Concentration	Sensory Quality	References
			Trained Panelists	
Cabbage (shredded)	Vapor	5-50 mg/L	Acceptable taste, texture and aroma after 12 d at 10°C	Banerjee et al. 2015
		100 mg/L	Acceptable taste and texture, unacceptable aroma after 12 d at 10°C	
Lightly fermented cabbage	Liquid	2 g/kg	Acceptable color, taste, texture and odor after 1 d at 10°C	Inatsu et al. 2005
Kimchi	Microen-capsulated	≤1 g/kg	Acceptable taste and odor after 15 d at 4 or 10°C	Ko et al. 2012
		1.5-2 g/kg	Unacceptable odor and taste after 15 d at 4 or 10°C	
Rice	Vapor	2 mg/L	Unacceptable odor after 12 h and taste after 2 d at 10°C	Kim et al. 2002
			Untrained Panelists	
Cooked ground beef	Mustard Flour	5-10%	Acceptable level of spiciness	Nadarajah et al. 2005b
		20%	Unacceptable level of spiciness and texture (too brittle)	
Fermented sausage	Microen-capsulated	0.5g/kg	Acceptable level of spiciness	Chacon et al. 2006a
		0.75-1 g/kg	Unacceptable level of spiciness	
Rye bread	Vapor	<1 mg/L	Acceptable aroma and taste after 1 d at room temperature	Nielsen and Rios 2000
		2.4 mg/L	Unacceptable flavor and aroma after 1 d at room temperature	

Hot dog bread	<1 mg/L	Acceptable aroma and taste after 1 d at room temperature
	1.8-3.5 mg/L	Unacceptable flavor and aroma after 1 d at room temperature

2.6.2. Effect of AITC on Food Color

Color changes after AITC application to food has been assessed by use of a colorimeter or chromameter (Table 2.6). No observable color changes occurred on rice with 1-2 mg/L AITC vapor. On dark meat such as beef, mustard powder has little influence on color change but on a light colored meat such as pork, slight yellowing is observed with 2% mustard and was still acceptable (Saleemi et al. 1993). At 300 µg/container, pure AITC vapor did not affect the color of raw chicken breast but at 600 µg AITC, the surface became discolored (Shin et al. 2010). Color changes were also observed on produce where 30 mg/L to 25 g/L (2.5%) AITC vapor caused spinach to turn brown within 4-8 d, and browning was accelerated with increased concentrations of AITC and temperatures (Obaidat and Frank 2009a; Huang et al. 2012). However, on raw cabbage the opposite trend was observed. Higher concentrations of AITC (50-100 mg/L) prevented blackening of raw cabbage edges compared to low concentrations (5-10 mg/L) or untreated controls (Banerjee et al. 2015). Furthermore, AITC application can also cause loss of color to produce, as 40 mg/L AITC was observed to reduce redness of tomatoes (Yun et al. 2013). AITC effect on color differs greatly depending on food composition, AITC concentration and storage time and temperature.

Table 2.6. Instrumental Analysis (Colorimeter or Chromameter) of AITC's Effect on Food Color

Food	Form of AITC	Concentration	Effect on Food Color	Reference
Cabbage (shredded)	Liquid	1 mL/L	No color change after 5 d at 10°C	Hamanaka and Izumi 2008
Cucumber (sliced)			No color change after 5 d at 10°C	
Cabbage (shredded)	Vapor	5-10 µL/L	Discoloration (blackening of edges) by 12 d at 10°C	Banerjee et al. 2015
	Vapor	50-100 µL/L	No color change by 12 d 10°C	
Tomatoes (whole)	Vapor	40 mg/L	Discoloration (reduced redness) after 21 d at 10°C	Yun et al. 2013
Raw chicken breast	Vapor	302 µg/container*	No color change after 21 d at 4°C	Shin et al. 2010
		604 µg/container*	Discoloration (yellowish green) after 18 d at 4°C	
Cured ground pork	Mustard Powder	2%	No adverse effect on color after 20 d at 4°C (slight yellowing)	Saleemi et al. 1993
Cooked ground pork			No adverse effect on color after 20 d at 4°C (slight yellowing)	

*container size unknown

2.6.3. Other Sensory Effect of AITC on Food

In addition to flavor, odor and color changes to food, other effects to food after AITC application have been observed. AITC concentrations of 50-500 mg/L greatly reduced quality of alfalfa seeds by decreasing seed viability and preventing germination (Park et al. 2000; Weissinger et al. 2001). AITC application can also cause loss of nutrients and phytochemicals in produce, such as vitamin C and lycopene in tomatoes (Yun et al. 2013). In addition, AITC

intervention can influence texture; it caused undesired softening of tomatoes (AITC vapour; Yun et al. 2013) and brittleness in ground beef patties (mustard powder; Nadaraja et al. 2005a) while on the other hand, improved the texture of kimchi (microencapsulated AITC; Ko et al. 2012).

2.6.4. Range of AITC Concentrations on Various Food Products with Acceptable or Unacceptable Sensory Quality Changes

Up to five percent mustard powder or 500 mg/kg pure AITC added to meat has no adverse effect on sensory quality (Saleemi et al. 1993, Nadaraja et al. 2005b, Chacon et al. 2006a). Produce has a range of acceptable limits depending on the plant. AITC vapor of 50 mg/L is acceptable on cabbage; levels above this cause adverse effect on flavor and aroma, whereas levels below this cause adverse effect on color (Banerjee et al. 2015). In kimchi, <1 g/kg liquid AITC is acceptable without adverse sensory effect (Ko et al. 2012). On tomatoes and spinach, under 30-40 mg/L AITC vapor should be used to prevent adverse color changes during storage (Yun et al. 2013). Lastly, AITC should not be used on grain products such as bread and rice since even though there was no significant difference in color after storage, they had unacceptable flavor and odor with only 1-2 mg/L AITC (Nielsen and Rios 2000; Kim et al. 2002).

2.7. Stability of AITC during Storage

The stability of AITC has been investigated to determine influences such as storage time and temperature, humidity and effect of medium or food matrix. Pure AITC (99.9%) is stable when stored alone. After 1 year of storage, almost 96% of the compound remained at refrigerated temperature, and surprisingly 88% remained at an elevated temperature of 60°C (Sekiyama et al. 1994). These same authors observed an increased stability when headspace was replaced with nitrogen and the sample was kept out of direct sunlight. In contrast, the half life of AITC in

aqueous solution was shortened to 31 d at room temperature, and was further decreased to 22 d when the pH of the aqueous solution was increased to 9 (Tsao et al. 2000). Others have observed even faster decomposition in water with an 87% decrease after 22 d at room temperature (Li et al. 2015). In broth, degradation was ten times faster with the same degradations after only 2 d (Olaimat and Holley 2013). This is because in aqueous solution, AITC can decompose due to the nucleophillic attack of water molecules and hydroxide ions on its isothiocyanate group (Cejpek et al. 2000). In comparison, AITC can be stabilized under the same storage conditions and time (room temperature, 21 d) with nanoparticles composed of zein and caseinate, where only a 20% decrease in AITC from original concentration was observed (Li et al. 2015). This prevented AITC degradation 67% when compared to storage in water alone. AITC is also miscible in ethanol and when stored together for 2 weeks at just above room temperature (28°C), it decreased by 10% from original concentration and therefore provided more stability than water. AITC is also fat soluble as it is a component of the essential oil of mustard. The stability of AITC for long-term storage was analyzed in two different oil-in-water emulsions, a saturated fat (medium chain triglyceride) and an unsaturated fat (soybean oil; Liu and Yang 2010). Increasing concentrations of oil (10-30%) further stabilized AITC and in addition, was more stable in saturated fat than unsaturated fat. This is because the latter is susceptible to oxidation, producing hydroperoxides and free radicals that can accelerate the rate of AITC decomposition (Liu and Yang 2010). Understanding what factors influence AITC degradation are important to prevent decomposition during intervention application and storage. Overall, AITC is therefore more stable in saturated fat emulsions than aqueous or ethanol solutions and in lower pH mediums; however, is most stable when stored alone at low temperature in nitrogen replaced headspace and out of direct sunlight.

Not surprisingly, AITC stability becomes more complicated in a food system. Since fat stabilizes AITC, it is not surprising that creamy wasabi (mayonnaise or tartar) or creamy horseradish sauces had significantly less AITC decomposition throughout storage than non-creamy, tomato-based sauce. A 2 and 11% loss was observed at 2 months for mayonnaise or tartar sauce, respectively, compared to 90% loss at 1 month for the tomato-based sauce (Sultana et al. 2009). Temperature also influences compound stability in creamy foods with 62% further degradation of AITC when stored at 18˚C as compared to 2˚C (Kosson et al. 2009). AITC stability in food during processes such as cooking has yet to be investigated. However, when pure AITC was subjected to temperatures of 160, 170 and 180ºC, the compound decreased to 97.4%, 93.9% and 71.2%, respectively, after 16 h (Sekiyama et al. 1994). Though this was not conducted in food, it suggests that high temperatures possibly used in cooking or processing do not immediately or completely decompose AITC. Further research on the stability of AITC in different foods, and residual amounts remaining after processing or cooking is warranted.

2.8. Chemopreventive Properties and Health Benefits of AITC

Intake of cruciferous vegetables has been linked to multiple health benefits, with AITC identified as a compound that contributes to these benefits. One of the most researched health benefits of AITC is cancer prevention. There are meta-analyses available for anti-cancer effects of cruciferous vegetable intake, and *in vivo* animal model studies indicate the amount of AITC needed for this effect (Table 2.7). Meta- analyses suggest 4 or more servings of raw cruciferous vegetables per week can significantly reduce the risk of bladder (Liu et al. 2013a) and kidney cancer in humans (Liu et al. 2013b); however, the amount of AITC related to this intake for anti-cancer effect was not calculated and could be beneficial as a comparison to *in vivo* animal studies available on the anti-cancer effects of AITC. As 1 serving of vegetables is approximately ½ cup

or 75 g (Health Canada 2007), this information can be used to calculate the range of AITC provided by raw cruciferous vegetables in 4 servings (300 g) per week from the meta-analyses. It is important to note that the concentration of AITC's precursor compound, sinigrin, varies in different vegetables and moreover, that the subsequent release of AITC from sinigrin can also fluctuate and this makes it difficult to determine the actual amount of AITC available to the body from cruciferous vegetable consumption. AITC production depends on efficacy of myrosinase's ability to catalyze the hydrolysis of sinigrin upon mastication of the plant, as this is where the majority of AITC is released (pH of mouth 6.2-7.2). Furthermore, sinigrin not yet converted in the mouth can be catalyzed by gut by enzymes produced by the microbiota to produce AITC (Krul et al. 2002). AITC released from these methods has high bioavailability (90%) and is absorbed in the small intestine. From calculated sinigrin levels, the potential amount of AITC released from a variety of raw vegetables is as follows: Brussels sprouts 0.87 mg/g, cauliflower 0.92 mg/g, cabbage 0.77 mg/g, kale 1.03 mg/g, wasabi 2.9 mg/g, and in black and brown mustard up to 2.6 - 3.5 mg/g (Kushad et al. 1999; Olivier et al. 1999; Sultana et al. 2002). Therefore, a rough estimate of AITC through the consumption of 1 serving or 75 g of the aforementioned vegetables ranges from 56-263 mg AITC per g of vegetable; if consumed four times per week, an estimated 224 -1052 mg AITC could be provided from 4 servings of raw cruciferous vegetables. Therefore, this amount of AITC corresponds to a reduction in cancer risk from the meta-analyses. In addition, it also satisfies AITC levels administered in *in vivo* animal studies for anti-cancer effects (1 mg/kg/day). In comparison, 4 servings per week leads to an average range of 32-150 mg AITC/day and since CDC estimates that the average weight of a man and a woman in the US to be 89 kg and 75 kg (CDC 2012), respectively, this amount of AITC translates to an average range of 0.35-1.68 and 0.43-2 mg/kg/day for men and women,

respectively. This concentration of AITC also remains below amounts shown to have adverse effect in mice (EFSA 2010).

In vitro studies show a common mechanism of action of AITC against different types of cancer cells including brain, prostate, colorectal, liver, breast and bladder: cells arrest in G_2M phase and inducing apoptosis (Tsai et al. 2012; Bhattacharya et al. 2010;

Table 2.7. Studies Documenting Anti-Cancer Effect of AITC

Cancer	Concentration	Anti-cancer Effect	Reference
		Meta-analysis (human)	
Kidney	~ 4+ servings cruciferous vegetables/week	Significant decrease in renal cell carcinoma risk	Liu et al. 2013a
Bladder	~ 4+ servings cruciferous vegetables/week	Significant decrease in bladder cancer risk	Liu et al. 2013b
		***In vivo* study (animal)**	
Lung	1.1 mg/kg/day/10 d	Inhibited tumor formation, no effect on developed tumors	Manesh and Kuttan 2003
Colorectal	1 mg/L, 3x per week/26 d	Reduced tumor volume and weight	Lau et al. 2010
Prostate	1 mg/L, 3x per week/28 d	Inhibited tumor growth	Srivastava et al. 2003
Bladder	1 mg/kg/day/21 d	Inhibited tumor formation and muscle invasion	Bhattacharya et al. 2010

Hwang and Lee 2006; Lau et al. 2010; Xiao et al. 2003; Chen et al. 2010). The range of AITC concentrations at which this has been observed is 0.1-10 mg/L AITC for 24 h. Interestingly, viability of healthy human prostate cells are minimally affected by levels of AITC that are highly

toxic to cancerous human prostate cells (10-40 μM AITC), which makes AITC a promising anti-cancer agent (Xiao et al. 2003). In addition to anti-cancer effects (Manesh et al. 2003), other health benefits of AITC have been proposed including a significant reduction in angiogenesis in acute colitis (Davaatseren et al. 2014), inflammation (Wagner et al. 2012), hyperglycemia (Mori et al. 2013), improvement in immune system and neurological disorders (Xiang et al. 2012), and inhibition of platelet aggregation (Kumagai et al. 1994; Lee et al. 2014) as well as improvement of insulin resistance (Ahn et al. 2014).

2.9. Balancing Preservation, Sensory and Regulation of AITC on Food

AITC is a dose-dependent intervention; not surprisingly, higher AITC concentrations prove to have stronger antimicrobial activity against pathogens on food (Obaidat and Frank 2009a, 2009b). However, higher concentrations of AITC may also cause adverse effects to food's sensory quality. Therefore, a balance needs to be achieved between effective control of pathogens on food and acceptable sensory quality of food for the development of practical use of AITC as an intervention method.

In relation to MICs of foodborne pathogens, that ranges from 5 to upwards of 300 ppm, flavor threshold of AITC is 375 ppm. Therefore, it is possible to find a range of AITC concentrations that do not influence food flavor and are effective for pathogen control (Table 2.1; Belitz et al. 2004). However, as MICs are performed in buffer, some foods may require higher concentrations above the flavor threshold. When this is the case, it does not become a question of whether you can sense AITC in food, but rather if it is acceptable on the specified food product. AITC's flavor has been found acceptable in sausages and beef patties which is not surprising as mustard containing AITC is already used as a flavoring agent in these products (Chacon et al. 2006a; Nadaraja et al. 2005 b), in prepared cabbage dishes such as kimchi where

this compound is already present in the cruciferous vegetable (Ko et al. 2012; Inatsu et al. 2005) but has been found not acceptable in products that do not normally use this compound as a flavoring agent and is not already present naturally, which includes grain products such as bread and rice (Kim et al. 2002; Nielsen and Rios 2000). On produce, concentrations of AITC required to reduce pathogens are lower than the flavor threshold and therefore may be used without adverse flavors (Table 2.2).

AITC intervention is effective against pathogens on produce at concentrations allowed on food for use as a preservative in the United States. However, in comminuted meat products, 10-100 times more AITC is required for effective control of pathogens although this concentration of AITC was found to have acceptable sensory quality, it is not in the range of AITC levels allowed for use as a preservative on food (FDA 2005, Chacon et al. 2006a; Nadaraja et al. 2005 b). Concentrations of AITC within FDA allowable levels have been successfully applied to the surface of intact or whole muscle meat products (Graumann and Holley 2009). Alternatively, mustard powder could be used as a natural source of AITC for preserving food. It would be a practical intervention in meat as it is already used as a flavoring agent in meat products. Moreover, mustard is not regulated as a preservative since AITC is in the form of its precursor sinigrin, which would render it in compliance with the regulatory authority. Overall, this makes mustard a more suitable candidate for intervention on meat than pure AITC. At 5% mustard powder, ground beef patties are acceptable in spiciness, color and texture while having effective lethal activity against pathogens (Nadaraja et al. 2005a).

On produce, concentrations of pure AITC in compliance with allowable levels are also able to control pathogens, as well as retain acceptable sensory quality. On cabbage, 50 ppm can be used for intervention while still remaining acceptable in sensory quality and also prevents

blackening of leaves. On spinach and tomatoes, <30 mg/L AITC is an effective intervention concentration that avoids adverse color change during storage which may occur on these foods with higher concentrations of AITC (Banjeree et al. 2015; Yun et al. 2013; Obaidat and Frank 2009b). AITC may not be suitable for grain products as only minimal levels (<1 mg/L) can be applied without adversely affecting sensory quality, but this level does not prevent fungal (3.5 mg/L) or pathogen growth (Table 2.2; Nielsen and Rios 2000).

AITC is a powerful antimicrobial that can control both Gram negative and Gram positive pathogens on a variety of food products at concentrations that do not affect sensory quality. However, AITC needs to be tailored to the food product as efficacy depends on storage time and temperature, microbial species targeted, AITC form and concentration, as well as food matrix, water activity and pH. More research is needed for AITC intervention on food at levels regulated by governing authorities. However, AITC used in combination with other intervention hurdles may increase antimicrobial effect while decreasing the amount of AITC applied, to be in compliance with allowed levels. Natural sources of AITC intervention are also an attractive alternative to pure AITC intervention, since they provide a clean, all natural label on food as opposed to a chemical preservative. However, if AITC produced from sinigrin hydrolysis from a natural plant source is to be used as intervention, one needs to master the art of releasing AITC from its precursor which may present another challenge in itself. In sum, AITC intervention has the potential to reduce the risk of foodborne illness and furthermore, AITC consumed in the diet from raw cruciferous vegetables can reduce the risk of one of the world's most devastating diseases, cancer.

3. Investigation Into A Novel Antimicrobial Intervention For The Control Of EHEC And *Listeria* On Spinach And Lettuce Using Volatile AITC From Mustard

3.1. Introduction

Plants of the family *Brassicaceae*, including mustard, cabbage, cauliflower and Brussels sprouts, contain the secondary plant metabolites glucosinolates that are as precursors to antimicrobial isothiocyanates (Shofran et al. 1998; Nielsen and Rios 2000). In brown mustard seed (*Brassica juncea*) the dominant glucosinolate is sinigrin, a stable precursor to volatile allyl isothiocyanate (AITC) (Zrybko et al. 1997; Zasada and Ferris 2004; Cools and Terry 2012, Brabban and Edwards 1994; Antonious et al. 2009; Delaquis and Mazza 1995). Sinigrin hydrolysis is catalyzed by the plant enzyme myrosinase. Disruption of the plant's tissues provides access of myrosinase to the substrate, resulting in sinigrin hydrolysis and release of AITC (Delaquis and Mazza 1995; Shofran et al. 1998). This reaction acts as the plant's defence system against invading insects and microorganisms, and may serve as natural antimicrobial in food protection. AITC has a broad antimicrobial spectrum and studies have shown that AITC can kill a variety of foodborne pathogens including *E. coli* O157:H7 and *L. monocytogenes* (Delaquis and Sholberg 1997; Rhee et al. 2003; Luciano and Holley 2009). AITC's antimicrobial action is due to the isothiocyanate group (-N=C=S), which binds to amino acids and proteins, leading to enzyme inhibition, cell membrane damage, and leakage of cell contents (Luciano and Holley 2009; Lin et al. 2000a; Turgis et al. 2009).

Pure volatilized AITC successfully inhibits the growth of pathogens on food (Park et al. 2000; Obaidat and Frank 2009a, 2009b; Piercey et al. 2012). However, AITC is unstable, toxic, and highly volatile, making it difficult to manage. Various forms of AITC may increase its manageability for use as an antimicrobial in food preservation, one such way is by use of

mustard meal. Within the meal, the antimicrobial remains in the form of its stable precursor sinigrin until water is present and it is released as a vapor to the surrounding headspace. Another added benefit of using sinigrin as a precursor to AITC release in a mustard meal intervention is that it allows for a clean label on food rather than listing it as a chemical preservative, as required with pure synthetic AITC (Khan and Abourashed 2009). Mustard meal is often used as a flavoring agent, binder or filler in meat products and has been successfully applied as an antimicrobial agent to reduce *E. coli* O157:H7 when added as an ingredient in dry fermented sausage batter and ground beef patties (Cordiero et al. 2014; Nadaraja et al. 2005a). To my knowledge, little has been researched on a system wherein AITC first remains in the form of sinigrin in mustard meal to be later released as a gaseous antimicrobial in headspace for the control of pathogens on food.

Consumption of contaminated fresh produce, including spinach and lettuce, is one of the leading causes of EHEC outbreaks and is also a cause of listeriosis outbreaks in recent years (CDC 2016). EHEC is an important pathogen as it has the ability to cause severe disease in humans at only 10 cells or less (Kiranmayi et al. 2010; Willshaw et al. 1994). Symptoms of EHEC infection include bloody diarrhoea, abdominal cramping, and in severe cases can lead to haemolytic uraemic syndrome (HUS) and in 0.5% of cases, death (Rangel et al. 2005). In comparison, *Listeria* is a foodborne pathogen with a higher infectious dose of 10^6 cells, however it also has a much higher mortality rate of up to 21% of cases (PHAC 2012; Garner and Kathariou 2016). Symptoms of listeriosis include fever, muscle aches, and nausea which may be followed by meningitis encephalitis and/or septicaemia, either of which can cause death (CDC 2015c). New methods of intervention are essential to increase the safety of at-risk food products and protect consumers from potentially fatal outcomes due to infection from these pathogens.

Therefore, the objective of this study is to investigate a novel method of antimicrobial intervention to eliminate EHEC and *Listeria* on the surfaces of spinach and lettuce leaves by use of volatile AITC from mustard meal.

3.2. Material and Methods

3.2.1. Bacterial Strains and Inoculum

A 5 strain cocktail of shiga toxin negative *E. coli* O157:H7 (02-0628, 02-1840, 02-0304, 02-0627, 00-3581; Luciano et al. 2011) was prepared by growing individual strains overnight in Luria-Bertani broth (LB; Oxoid, Missisauga, Ontario, Canada) at 37ºC and 250 rpm, and combining them the next day. *L. innocua* (ATCC 33090) was used as a model organism for the foodborne pathogen *L. monocytogenes* and prepared by growing the strain overnight in Tryptic Soy Broth (TSB; Oxoid) at 37°C. One hundred microlitres of each culture was spot inoculated onto separate surfaces of filter paper, spinach or lettuce leaves and dried for 30 min to allow for attachment of bacteria to surfaces.

3.2.2. Preparation of Filter Paper, Spinach and Lettuce

Bagged spinach leaves were purchased from a local grocery store in Edmonton, AB and stored at 4°C until use, within 24 h of purchase. Individual leaves were removed from the bag and cut into 2.0 ± 0.5 cm x 2.0 ± 0.5 cm squares using a sterile scalpel. Likewise, iceburg lettuce was purchased from a local grocery store in Edmonton, AB. The outer plastic was removed from the head of lettuce and outer leaves were removed and discarded. The interior leaves were cut into 2.0 ± 0.5 cm x 2.0 ± 0.5 cm squares using a sterile surgical knife. Leaf samples were used in 4°C temperature trials only since wilting was observed in elevated temperature trials and therefore was impractical to use. Filter paper (Whatman; Sigma-Aldrich, Mississauga, Ontario,

Canada) was used in place of leaves in trials at elevated temperatures. Filter paper was autoclaved (120 min for 121°C) and cut to the same dimensions as leaves with a sterile scalpel.

3.2.3. Mustard Preparation

Brown mustard seed (*B. juncea*; Viterra, Lethbridge, Alberta, Canada) used for active AITC treatment was crushed by mortar and pestle into a meal. The mustard meal was weighed to 0.5 g meal per 50 mL of air of petri dish volume on an analytical scale, corresponding to10 g meal per litre of air (10g/L). Either canola meal or autoclaved brown mustard meal served as negative control; canola seed does not contain sinigrin as the dominant glucosinolate (Szmigielska et al. 2000) and the high temperature of the autoclave inactivated myrosinase, preventing the production of AITC from sinigrin. Canola meal (10 g/L) and autoclaved mustard meal (10 g/L) was prepared in the same manner as described above; however, before weighing autoclaved mustard, the dry meal was spread in a thin layer between two sheets of aluminum foil and autoclaved for 120 min at 121°C to inactivate myrosinase.

3.2.4. Sinigrin Extraction from Mustard and Quantification in HPLC

Sinigrin was extracted from mustard meal used in challenge trials (n=6 for 4°C trials; n=2 each for 15 & 30°C trials) with a simple extraction technique adapted from methods previously described (Cataldi et al. 2007). Briefly, mustard meal from challenge trials were each added to separate 15 mL centrifuge tubes, sealed, and placed in a water bath at 80°C for 10 min to inactivate myrosinase. Five mL of boiling water:methanol (90:10) was added to the tube and held for 20 min at 80°C, followed by centrifugation. A lower temperature was used to inactivate the enzyme than in section 3.2.3 (for the entire batch of autoclaved mustard meal) as the mustard meal was divided into smaller portions in tubes (0.5 g) from the challenge trials and wet heat was applied allowing for more efficient inactivation. Supernatant was kept and extraction was

repeated again with the pellet, and supernatants were combined and freeze dried. Glucotropaeolin (LGC, Manchester, New Hampshire, USA) was used as an internal standard to determine extraction efficiency (87%). Sinigrin was separated from crude extract, identified and quantified on a 1200 series high performance liquid chromatography unit (HPLC; Agilent Technologies, Palo Alto, California, USA) coupled to a 4000 QTRAP LC-MS/MS System (MDS SCIEX-Applied Biosystems, Streetsville, Ontario, Canada) adapted from methods previously described (Cataldi et al. 2007, Skutlarek et al. 2004). Sinigrin was separated on a Luna C18 RP-HPLC column (5 µm, 250 x 4.6 mm, Phenomenex, Torrance, California, USA) and eluted at 0.5 mL per min with mobile phases water (Eluent A) and methanol (Eluent B) both containing 15 mM ammonium formate. Mobile phases were run on a gradient of 1 min, 95% A; 12 min, 70% A; 18 min, 95% A. Sinigrin was identified and quantified with multiple reaction monitoring scan mode (MRM). LC-MS/MS was operated in negative ESI mode using manual optimization of parameters as follows: 358 mass-to-charge ratio (*m/z*; precursor ion) → 97 & 195 *m/z* (product ions); Ion spray voltage, -4,500 V; Temperature, 550; Declustering potential DP, -70 V; Collision energy CE, -28 V; Collision cell exit potential CXP, -15 V, retention time, 6 min. A sinigrin standard (99% pure; Sigma-Aldrich) was used as a reference and to create a calibration curve within range of samples, and sinigrin in samples was quantified using Agilent Quantification Wizard software (Agilent Technologies). Sinigrin concentrations in mustard meal were quantified to determine the maximum amount of AITC released into the headspace of the petri dish during challenge trials, since sinigrin is a stable compound easily quantified in HPLC. Furthermore, AITC is the main compound produced at neutral pH from the hydrolysis of sinigrin and has antimicrobial activity, therefore lethal effect would be attributed to the release of this compound (Shofran et al. 1998).

3.2.5. Use of Mustard Meal to Control Microorganisms on Spinach, Lettuce and Filter Paper

A sterile 50 mL plastic petri dish was divided into 3 areas, wherein an inoculated paper or leaf was placed in one area, mustard treatment in a sterile weigh boat in a second, and a sterile cotton ball (moistened with 1 mL sterile Milli-Q water) in a third. All 3 sections were kept separated to avoid contact. Mustard treatments included 10 g/L mustard meal, 0 g/L mustard meal negative control and 10 g/L autoclaved mustard meal or canola meal negative control, with a sterile cheese cloth placed on top of meal in the weigh boat to prevent meal particles from escaping into the other sections of the petri dish. Petri dishes were sealed with parafilm to create a closed system. Samples were stored at 4, 15 and 30°C for up to 4 d. For spinach and lettuce trials, all samples were also plated on Plate Count Agar (PCA; Oxoid) for enumeration of total mesophilic microorganisms to determine natural microbial population on leafy greens before inoculation and to determine whether these microorganisms were also sensitive to mustard treatment. During 4 d storage, water from the cotton ball created humidity within the system as witnessed by condensation inside of the sealed petri dish.

3.2.6. Sampling and Microbiological Analysis of Filter Paper, Spinach and Lettuce

Filter paper and leaves were sampled and analyzed on 0, 2 or 4 d for all temperatures. Upon sampling, parafilm was removed from the petri dish surround, paper or leaf removed with a sterile tweezer, and placed in 2 mL 0.1 % peptone water (Difco, Becton-Dickinson, Sparks, Maryland, USA) in a 15 mL centrifuge tube. Samples were vortexed intermittently for 2 min, diluted and then plated using an automatic spiral plater (Don Whitley Scientific Ltd., West Yorkshire, UK). For spinach and lettuce EHEC challenge trials, Violet Red Bile agar (VRB; Oxoid) was used for enumeration of total coliforms and sorbitol maconkey agar with cefixime

tellurite supplement (CTSMAC; Oxoid) for differentiation of *E. coli* O157:H7. In addition, plate count agar (PCA; Oxoid) was used for total aerobic mesophilic count. For EHEC challenge trials on sterile filter paper, LB agar was used for enumeration of *E. coli* O157:H7; for *Listeria* enumeration, tryptic soy agar (TSA; Oxoid) was used. All agars were incubated at 37ºC for 18 hr followed by enumeration of colonies. After preliminary experimentation, 3 trials were performed on spinach and lettuce, respectively, and each trial done in technical repeat (2 repeats per sample). For filter paper trials, 2 trials were performed per experiment with technical repeat, with the exception of *Listeria* at 30°C wherein 4 trials were performed.

3.2.7. Data Analysis

Data was evaluated by two way analysis of variance followed by evaluation of pair-wise comparison of means from different treatment groups, by use of Statistical Analysis Systems (SAS Institute, Inc.). A probability (p) of <0.05 was considered statistically significant, with P between 0.05 and 0.01 was considered to be approaching statistical significance.

3.3. Results

3.3.1. Sinigrin Quantification in Mustard Meal from Challenge Trials over 4 d Storage at 4, 15 or 30°C

Concentration of sinigrin in mustard meal was measured using a sophisticated quantification technique as quantification of substrate was preferred over volatile product. A decrease of the gluconsinolate in enzyme active meal was observed throughout storage, though this was temperature dependent (Figure 3.1). Concentration of sinigrin in mustard meal did not decrease throughout 4 d storage at 4°C. However, at 15 and 30°C sinigrin was reduced by ~30 µmol/g in 4 d ($p<0.05$). This decrease in sinigrin corresponds to a maximum release of ~30 mg/L air of AITC in petri dish headspace from 10 g/L mustard meal. No significant reduction of

sinigrin was observed in the autoclaved mustard meal control throughout 4 d storage at 4, 15 or 30°C (p>0.05; data not shown).

3.3.2. *E. coli* O157:H7 Recovered on LB and *L. innocua* Recovered on TSA From Filter Paper Exposed to Mustard Meal at 4, 15 or 30°C Over 4 d Storage

To show that sinigrin was converted to antimicrobial AITC at 15 and 30°C and not refrigerated temperature, mustard meal was added as a treatment against *E. coli* O157:H7 and *L. innocua* on filter paper to see if it would correlate to bacterial inactivation. In addition to no

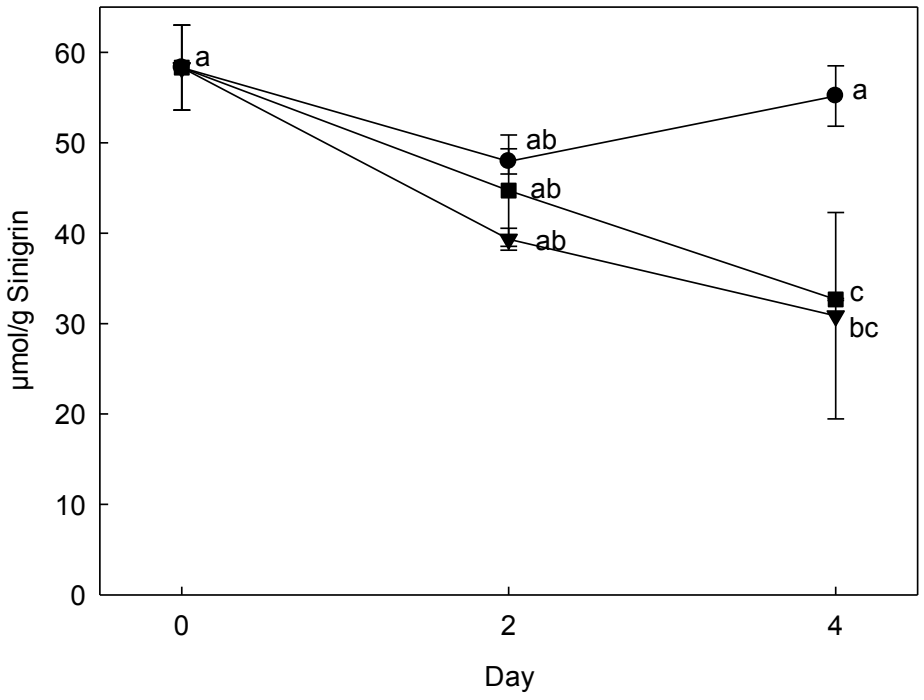

Figure 3.1. Sinigrin quantified in 10 g/L mustard meal samples from challenge trials over 4 d storage at 4, 15 or 30°C. Values are means of µmol/g sinigrin ± standard deviation (SD), n=6 (4°C) or n=2 (15 & 30°C). Circle, 4°C; triangle, 15°C; square, 30°C. Values that do not share a common superscript differ significantly ($p < 0.05$).

mustard as negative control (0 g/L; data not shown), autoclaved mustard meal (10 g/L) was used as a second control since the myrosinase enzyme was inactivated by the autoclave's high temperature and pressure, thereby preventing AITC production from sinigrin. *E. coli* O157:H7 on filter paper was not reduced by 10 g/L mustard meal at any storage temperature over 4 d

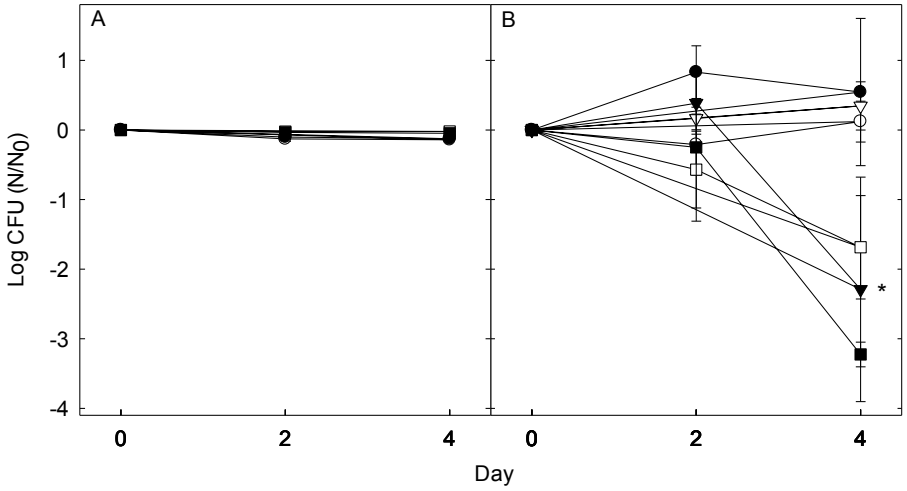

Figure 3.2. (A) *E. coli* O157:H7 recovered on LB agar from filter paper exposed to 10 g/L mustard meal or 10 g/L autoclaved mustard meal over 4d at 4 or 30°C. Values are means of log CFU (N/N_0) ± SD, n=2. (B) *L. innocua* recovered on TSA from filter paper exposed to mustard over 4 d at 4, 15 or 30°C. Values are means of log CFU (N/N_0) ± SD, n=2 (4 & 15°C) or n=4 (30°C). Circle, 4°C; triangle, 15°C; square, 30°C; black, 10 g/L mustard meal; white, 10 g/L autoclaved mustard meal. A control without mustard meal (0 g/L) was also used during trials and results were similar to 10 g/L autoclaved mustard meal at 4, 15 and 30°C (data not included). *Significant reduction from control (15°C; $p<0.05$).

storage (Figure 3.2 A). Similarly in *L. innocua* challenge trials, 10g/L mustard meal did not reduce the microorganism on filter paper at 4°C over 4 d storage and was not different from 10 g/L autoclaved mustard control (Figure 3.2 B). However, at elevated storage temperatures of 15 and 30°C, *L. innocua* was sensitive to 10 g/L mustard meal and a 2.3 and 3.2 log CFU (N/N_0) reduction was observed by 4 d, respectively. No sensitivity of *L. innocua* was observed at 15°C to the enzyme inactive autoclaved mustard meal; both 10g/L autoclaved mustard control and 0 g/L negative control remained at initial inoculation counts throughout storage (0.3 and 0.2 log CFU (N/N_0), respectively). Reduction of *L. innocua* by 2 log CFU (N/N_0) from controls at 15°C with 10 g/L mustard meal was significant ($p<0.05$). However, at 30°C, *L. innocua* was reduced in all treatments over time; and a trend observed in both 10 g/L autoclaved mustard control and 0 g/L negative control with a reduction of ~1.5 log CFU (N/N_0) by 4 d, indicating that the microorganism does not survive as well at this higher temperature on filter paper. However, from the controls, *L. innocua* was further reduced by 1.6 log CFU (N/N_0) at 30°C with 10 g/L mustard meal, which was not significant but rather was approaching significance ($p<0.1$).

3.3.3. Total Coliforms and *E. coli* O157:H7 Recovered from Spinach and Lettuce After Exposure to Mustard Meal over 4 d at 4°C

To investigate inactivation of *E. coli* O157:H7 with high and low background microbiota, 1 or 10 g/L mustard meal was exposed to inoculated spinach and lettuce at refrigerated temperature over 4 d with negative controls of 0 g/L mustard meal and 10 g/L canola meal (Figure 3.3). Trials were also performed at 15 and 30°C, but rapid wilting prevented sampling. Furthermore, different bags of spinach leaves were used each trial (3 bags total), which explains high error bars. To detect effect of mustard meal on spoilage microorganisms, spinach was used directly from bags and not further washed. The natural microbial load on spinach was > 5 log CFU/cm^2

indicating that leaves were more highly contaminated than interior leaves of lettuce, which were below detection limit, but reasonably clean with early shelf life since natural contamination from aerobic mesophiles on spinach can reach over 8 log CFU/g (Valentin-Bon et al. 2008).

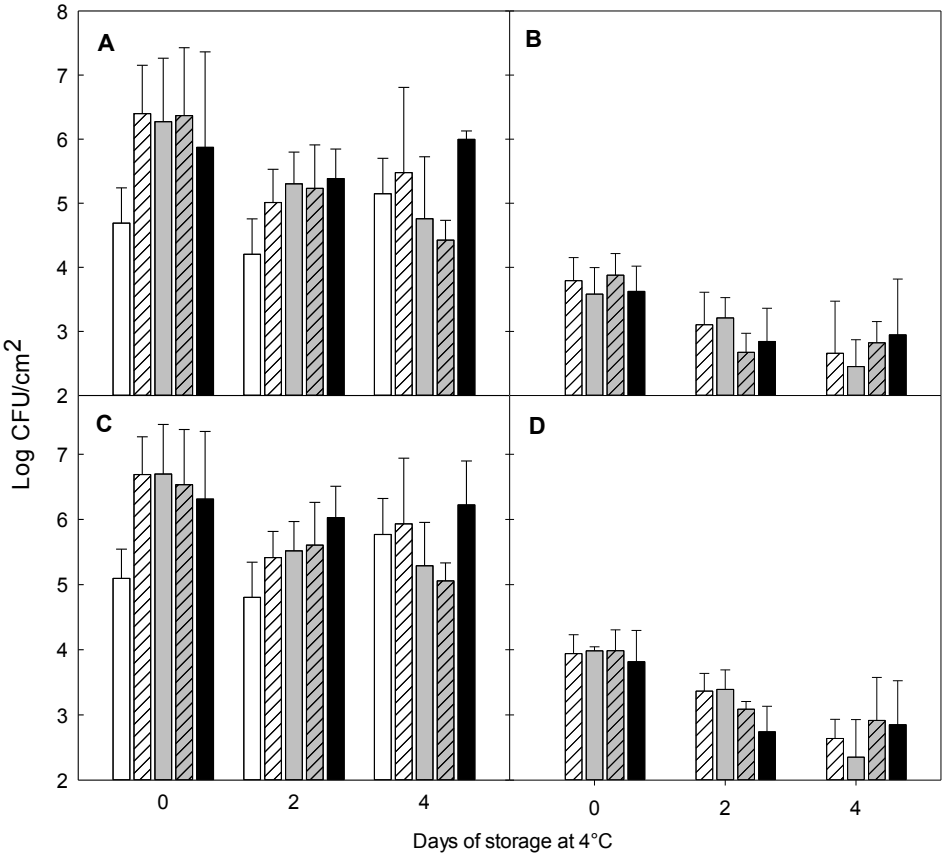

Figure 3.3. Total coliforms recovered on VRB agar (A) and total mesophilic microorganisms recovered on PCA (C) from spinach, and *E. coli* O157:H7 recovered on CTSMAC agar (B) and total mesophilic microorganisms recovered on PCA (D) from lettuce, exposed to mustard treatment over 4 d at 4°C. Values are means of log CFU/cm^2 ± SD, n=3. White bars, uninoculated control; white hatched bars, inoculated control; grey bars, 1 g/L mustard meal; grey hatched bars, 10 g/L mustard meal; black bars,10 g/L canola meal.

Background microbiota on spinach was reflected by uninoculated control counts recovered on VRB agar (A; total coliforms) and PCA (C; total aerobic mesophiles). After inoculation with a 5 strain *E. coli* O157:H7 cocktail and its subsequent attachment to spinach leaves, a ~2 log CFU/cm^2 increase was observed from initial uninoculated counts. No significant reduction (p>0.05) was observed for either total coliforms or total aerobic mesophiles on spinach over 4 d exposure to 1 or 10 g /L mustard meal. Mustard meal of 100 g/L was used as a treatment for EHEC on spinach at 4°C over 4 d storage, but met with similar results (data not shown). However, it was also noted that this volume of mustard meal was impractical and therefore not used further.

On the interior leaves of lettuce (B, D), uninoculated controls were very clean with no spoilage microorganisms recovered. This is not surprising as the interior leaves were protected by the outer leaves of the lettuce head from environmental microbial contamination. At day 0, *E. coli* O157:H7 inoculated samples were initially ~3.5 log CFU/cm^2, but by 4 d, the pathogen did not show sensitivity to the mustard treatment. No significant difference (p>0.05) was observed for reduction of *E. coli* O157:H7 recovered on CTSMAC agar (B) or total aerobic mesophiles on PCA (D) on lettuce with 10 g/L mustard at 4°C over 4 d storage. For PCA counts on lettuce, which mainly recovered the *E. coli* O157:H7 cocktail, counts decreased slowly over 4 d, though this was not significant (p>0.05).

3.4. Discussion

This study demonstrates that AITC gas from mustard can be used to control microorganisms but not at 4°C. This is most likely due to slow activity of mustard's endogenous myrosinase enzyme at this temperature, since the optimal temperature of mustard myrosinase is 60°C at neutral pH (Van Eylen et al. 2008). Previous research has confirmed that AITC release

from mustard meal is significantly lower at 5°C as compared to a higher temperature of 35°C (2 mg/g vs 17 mg/g; Dai and Lim 2014). With a decreased of sinigrin by 30 μ mol/g from 10 g/L mustard meal at 15 and 30°C, the concentration of AITC in headspace is never higher than 30 mg/L due to its reactive nature. Mustard meal of 10 g/L was selected for use in challenge trials as it was found to be an acceptable amount of mustard to apply in this intervention model; mustard meal at higher volumes than this (ie 100 g/L) were not practical. Mustard meal at 10 g/L had amounts of sinigrin which correlated to concentrations of volatile AITC (~60 mg/L) that were within ranges previously demonstrated to be lethal towards pathogens on food (Obaidat and Frank 2009a, 2009b). However, complete degradation of sinigrin was not observed throughout 4 d experimentation during our study. At 4°C storage, no degradation of sinigrin was observed whereas at 15 and 30°C storage, sinigrin decreased in mustard by ~ 50% of the total. AITC released throughout 4 d with 10 g/L mustard meal; however, was not enough to kill EHEC in challenge trials, though *L. innocua* did show reductions form controls that were either significant (2 log CFU (N/N_0) at 15°C) or approaching significance (1.6 log CFU (N/N_0) at 30°C).

Pure volatilized AITC is lethal against *E. coli* O157: H7, *L. monocytogenes* and spoilage microorganisms on produce at refrigerated temperature (Lin et al. 2000b, Obaidat and Frank 2009a, Piercey et al. 2012). A > 2.0 and > 3.0 log CFU decrease of *E. coli* O157:H7 on sliced tomatoes at 4 and 10°C, respectively, was observed with 17 mg/L pure volatilized AITC after 10 d (Obaidat and Frank 2009a). In addition, >4 log CFU reduction in *E. coli* O157:H7 on intact lettuce surfaces was observed with 4 mg/L pure volatilized AITC at 4°C in 4 days and at10°C, this reduction was observed in 2 days (Obaidat and Frank 2009b). Furthermore, a 7 log CFU decrease in *E. coli* O157:H7 was observed on iceburg lettuce with 88 μL/L pure volatilized AITC at 4°C in 2 d (Lin et al. 2000b). In another investigation, 200 ppm of volatilized AITC

reduced *L. monocytogenes* by over 1.5 log CFU/g from controls on fresh cut onions at 5°C for 8 d (Piercey et al. 2012). AITC vapour is also effective against spoilage microorganisms; concentrations of 50-100 mg/L AITC inhibited the growth of total mesophilic microorganisms on minimally processed cabbage during storage at 10°C over 12 d (Banerjee et al. 2015), and inhibited both mesophile and coliform bacterial growth on lightly fermented cabbage during storage at 10°C for 4 d with 2 g/L AITC (Inatsu et al. 2005). These investigations show that pure AITC has antimicrobial activity, and can reduce or inhibit pathogen and spoilage microorganisms on produce, at low temperatures.

Since AITC is active at refrigerated temperature, absence of antimicrobial activity at 4°C in our study relates to inactivity of myrosinase in mustard meal since the endogenous enzyme is essential for AITC release from sinigrin. Moreover, the lack of antimicrobial activity at 4°C is supported by our finding that sinigrin was stable in mustard meal throughout 4 d storage at 4°C. This also corresponds to research by other authors (Dai and Lim 2014), who investigated the release rate of AITC from mustard: the myrosinase-glucosinolate reaction in mustard occurs at a faster rate, thereby producing a higher volume of AITC, at higher temperatures (35°C) versus at lower temperatures (5°C). Therefore, the intervention developed in the present study using mustard meal as a vector for AITC release to eliminate EHEC on leafy greens was ineffective, as this product requires refrigerated temperature during storage.

However, there are many benefits to using mustard meal for AITC as an intervention on food. The high volatility and instability of pure AITC has presented challenges for use; some authors have even noted this has prevented further experimentation with the compound in food or in packaging (Dussault et al. 2014; Martinez-Abad et al. 2013). Using mustard meal increases manageability since volatile AITC remains in the form of its stable precursor sinigrin until

antimicrobial release directly into the system is facilitated by myrosinase and water. The use of mustard meal as an antimicrobial has been successfully applied in meat interventions; research has shown that adding mustard meal directly into dry fermented sausages and ground beef patties can kill foodborne pathogens such as *E. coli* O157:H7 (Cordiero et al. 2014; Nadaraja et al. 2005b). On the other hand, the application of AITC as a gas or fumigation agent is more ideal for non meat products that do not use mustard as a flavoring agent, and foods that are easily bruised or damaged. For example, Nielsen and Rios (2000) found that volatile AITC killed spoilage fungi in bread (*Penicillium commune*, *P. roqueforti*, *Aspergillus flavus*, *Endomyces fibuliger*) and increased product shelf life. Overall, mustard meal as an antimicrobial is a novel approach to food safety and the present research adds further insight into controlling pathogens on food by its use in a way that has not been done before.

To reduce the risk of foodborne illness, the development of new and innovative intervention methods are a necessity. The use of mustard meal is one such novel intervention. As AITC is highly unstable, mustard meal allows for controlled release of the antimicrobial by keeping it in the form of sinigrin until myrosinase catalyzes hydrolysis to produce AITC. However, our study showed that the effectiveness of this antimicrobial application is dependent on storage temperature. At refrigerated temperatures, no sinigrin decrease in mustard meal was observed over 4 d and no antimicrobial effect was observed for either *Listeria* or EHEC on spinach and lettuce or filter paper. However, at higher temperatures of 15 and 30°C, sinigrin decreased in mustard meal and a significant reduction of *Listeria* was observed but no such reduction was observed for EHEC. Therefore, this method could be useful for controlling some microorganisms on food stored at room temperatures.

4. Effect of sub-lethal heat treatment on growth and detection of *E. coli* O157:H7 single cells

4.1. Introduction

Improved detection techniques are necessary to increase the safety of our food supply. Enterohaemorrhagic *Escherichia coli* (EHEC) is a pathogenic subgroup of *E. coli* with shiga-like toxins (stx) that can cause severe disease in humans (Lim et al. 2010). EHEC serotype O157:H7 causes an estimated 73,000 illnesses in the United States and 13,000 illnesses in Canada per year, with outbreaks linked to raw or undercooked meat and fresh produce (Rangel et al. 2005; Thomas et al. 2013). At-risk foods such as these require more accurate detection methods to decrease the prevalence of infection.

Conventional plating methods to detect pathogens on food are simple but time consuming and labor intensive. In comparison, molecular detection techniques such as quantitative polymerase chain reaction (qPCR) are more rapid and selective. However, qPCR comes with limitations; it is not able to detect low cell numbers in a sample or differentiate between live and dead cell DNA (Bae and Wuertz 2009). To overcome the former limitation, enrichment is required prior to qPCR detection to increase target cell number to a concentration at which it can be detected, in addition to resuscitating injured target cells and diluting food inhibitors, background flora and dead cell DNA (Ge and Meng 2009). Because of the necessity of a lengthy enrichment step, detection times in qPCR are increased from hours to days. However, enrichment is important for EHEC detection in food, as this pathogen can cause disease with only ten cells or fewer (Willshaw et al. 1994).

E. coli O157 is a prevalent EHEC serotype that can cause severe disease in humans and is frequently implicated in foodborne outbreaks (Thomas et al. 2013). While *E. coli* O157:H7 is

regulated in both Canada and the US, it remains difficult to control and detect on food. Common detection methods validated by Health Canada for shiga toxin-producing *E. coli* in ground beef and leafy greens are: VIDAS® UP method for selective capture of *E. coli* O157 with a solid phase receptacle coated with recombinant phage tail fiber protein and subsequent fluorescence detection, and qPCR methods including Assurance GDS™ method with immunomagnetic bead capture of *E. coli* with antibody specific coatings and BAX® method with a combination of primer and fluorescent dye, all of which require enrichment times prior to detection of between 7-24 h (Health Canada 2005, 2011, 2012b). Though these enrichment times are validated by Health Canada, there is a lack of information on necessary enrichment times for detection of injured *E. coli* cells, since extended lag phases increase time to detection. If current enrichment time provides more than sufficient time for this population of cells to be detected, minimizing enrichment time would be beneficial for faster detection of EHEC on food. On the other hand, it is possible that current enrichment times may be inadequate for injured cell detection, and thus further decreasing of enrichment time would result in false negative result.

Heat intervention causes disruption of cell membrane and irreversible protein denaturation and is an effective method for inactivation of microorganisms on food (Lee and Kaletunc 2002). Hot water or steam interventions are often used for carcass decontamination in meat packing plants; while a temperature of 85°C is used for pasteurization, the carcasses themselves are below 30°C and therefore microorganisms present are exposed to a range of temperatures up to 85°C once the hot water or steam hits the carcass (Gill and Bryant 2000; Yang et al. 2014). In addition, mild heat treatments can be applied to leafy greens such as spinach to increase the efficacy of organic acid intervention against *E. coli* O157:H7 (Huang and Chen 2011). Heat interventions can kill microorganisms present on food surfaces, but if they are subjected to a

lower, non-lethal temperature, they may be sub-lethal injured instead. Sub-lethally injured cells recover, but have extended lag phases before regaining ability to replicate. Heat stressed individual *Sacchomyces cerevisia* cells and heat injured single *Lactobacillus plantarum* cells, in addition to foodborne pathogens *Salmonella* Typhimurium, *Listeria monocytogenes* and *E. coli* O157:H7 cells, have previously been investigated for effect of injury on lag phase and show extended lag phase and detection with injury (Stephen et al. 1997; Tibayrenc et al. 2011; Guillier et al. 2005; Li et al. 2006).

Single cell detection is a suitable method to detect length of a cell's lag phase, as lag phases can be deduced from times for individual cells to reach an optical density threshold (Baranyi and Pin 1999; Smelt et al. 2002; Guillier and Augustin 2006). Stress applied to cells can cause variability of individual lag times, and furthermore, heat application is a good way to measure extended lag phases since heat treatment influences lag phases of individual cells more than other stresses such as acid (HCl, lactic acid), alkali (NaOH), chlorine, starvation and osmotic stress (Guillier et al. 2005). There is little information on the lag phases of sub-lethally injured *E. coli* lag phase and necessary time to detection. While Li et al. (2006) explored the lag phase of *E. coli* after a 50°C treatment, there is no research on the lag phase of *E. coli* after a lethal heat treatment has been applied to cells. Furthermore, while the previous authors measured lag phases at an incubation temperature of 37°C, injured cells are better resuscitated at 25°C (by up to 28%) which may have prevented detection of a percentage of the sample's injured cell population (Hara-Kudo et al. 2000). Lastly, there is a lack of information on the lag phase of sub-lethally injured *E. coli* extending over 24 h; this research would be invaluable in determining if current enrichment times are sufficient to detect injured cells (7-24 h) and to explore if these times can be further decreased for more rapid detection of *E. coli* in food.

4.2. Materials and Methods

4.2.1. Bacterial Strains and Media

One strain of *E. coli* O157:H7 (02:3581; Luciano et al. 2011) without stx was grown for 16 h in Luria-Bertani (LB; Oxoid, Missisauga, Ontario, Canada) broth at 37°C and 250 rpm and was used in further experimentation for both spectrophotometer and qPCR measurement. A second microorganism, stx-less *E. coli* O157:H7 (02:0627; Luciano et al. 2011), was grown in the same manner and used as a positive control (no treatment applied) in spectrophotometer measurement to confirm that under the conditions of the spectrophotometer, the microorganism would grow; these conditions included LB broth at 25°C, a temperature chosen for better resuscitation of injured cells (Hara-Kudo et al. 2000). For qPCR measurement, high and low cell density samples were grown at optimal temperature of 37°C in LB broth. For plating conditions, all samples were plated on LB agar and incubated at 30°C for 24 h.

4.2.2. Inflicting Sub-lethal Injury to *E. coli* O157:H7 Cells By Application of Heat Treatments

Four different treatment groups included untreated control, a cold treatment of 4°C for 24 h, and heat treatments of 66°C for 1 min 20 s or 58°C for 5 min 15 s. Heat treatments were chosen since they provided a 2 log reduction in cell numbers as determined from plating on LB and VRB agars (described in section 4.2.3; Figure 4.1). The original overnight culture was pipetted (500 µL) into 2 mL Eppendorf tubes for each treatment in duplicate. For heat treatments, tubes were placed in a water bath for the specific temperature and holding time then removed and immediately placed on ice. For the cold treatment, tubes were placed in a refrigerator (4°C) for 24 h; this treatment was used to represent the requirement of samples to be held at 4°C before use, if not sampled immediately (Health Canada 2013, USDA 2015c).

4.2.3. Determination of Growth Curves in Spectrophotometer

For all treatment groups, ten-fold serial dilutions from original treated samples were prepared in LB broth, up to the point of cell exhaustion (10^{-10} log CFU/mL). Dilutions estimated to grow 30-300 colonies per plate were manually plated (100 µL) on LB and Violet Red Bile (VRB; Oxoid) agar, to confirm lethal reduction in viable cells as well as injury to viable cells (Figure 4.1). Plates were incubated at 30°C for 24 h and colonies were enumerated next day. Plating of samples was performed immediately prior to each measurement of optical density of each treatment. From the ten-fold serial dilutions prepared from original treated samples, the dilution containing an estimated 1-10 cells/mL was used as the first dilution on the microtiter plate and pipetted into the third column of a 96-well microtiter plate. From this column, subsequent two-fold dilutions with LB broth were performed across the microtiter plate to the point of sterility. In between the original dilution to cell exhaustion in LB broth, estimated single cell growth was detected on the microtiter plate. Columns one and two were reserved for controls; column one was positive control and column two was negative control (LB broth only). The microtiter plate was sealed with a clear optical adhesive film (MicroAmp, Applied Biosystems, Thermo Fischer Scientific, Burlington, Ontario, Canada) to prevent evaporation, loss of culture or contamination. The microtiter plate was then placed in a spectrophotometer (Multiskan Ascent 96/384 Plate Reader, Thermo Fischer Scientific) which monitored the turbidity of *E. coli* O157:H7 (A=600 nm) at 30 min intervals for up to 5 d at 25°C with Multiskan Ascent Software (Version 2.6, Thermo Fischer Scientific). This temperature was chosen since injured cells are better resuscitated at 25°C (Hara-Kudo et al. 2000). Detection times for the growth of *E. coli* O157:H7 were calculated as time elapsed until estimated single cells increased in optical density (OD) by 0.02 from baseline, the lowest OD measurement to

accurately detect growth in the spectrophotometer used. Authors Baranyi and Pin (1999) estimated the initial number of cells per well (x_0) on a microtiter plate by mathematical equation: $x_0 = rX_{det}$, where r = the sample's dilution ratio and X_{det} = the detection limit of the spectrophotometer (10^7 cells). Similarly in the present study, wells selected as single cells were those where growth was detected beside a column of sterile wells on the microtiter plate and where estimated single cells could be calculated from dilution used across the plate. The experiment was performed to obtain growth information from at least 50 estimated single cells per treatment. The number of estimated single cells per treatment group was: control, n=103; 4°C, n=70; 58°C, n=65; 66°C, n=58 (Figure 4.2).

4.2.4. Determination of Cell Density in Spectrophotometer

The lowest amount of growth, or increase in turbidity of a sample, that could accurately be detected in our spectrophotometer was an increase in OD by 0.02. When OD increased by this value from the baseline measurement, the sample's cell density is 10^6. This was confirmed by growing *E. coli* O157:H7 overnight in LB at 37°C and 250 rpm, and preparing ten-fold serial dilutions (up to the point of cell extinction) in LB broth the next day. All dilutions were measured in spectrophotometer to provide an OD for each sample. Each dilution was also manually plated (100 µL) on LB agar and incubated at 30°C for 24 h. Plates that had between 30-300 colonies on the plate were enumerated and compared to OD measurement. The lowest detected increase in OD in samples was from 0.089 ± 0.001 to 0.107 ± 0.010 (therefore, an increase in OD by 0.02), corresponding to enumeration data on LB agar from 5.38 ± 0.35 to 6.20 ± 0.35 log CFU/mL. The blank (LB broth without culture) or baseline measurement in spectrophotometer was OD 0.088 ± 0.001, in addition to all other dilutions that corresponded to

enumeration data of ≤ 5 log CFU/mL. The experiment was performed 3 times to measure experimental variability (n=3).

4.2.5. Sampling of Heat or Cold Treated *E. coli* O157:H7 over 24 h for qPCR Analysis

E. coli O157:H7 strain 02:3581 was grown overnight in 20 mL LB broth at 37°C and 250 rpm. The selected treatment groups (heat treatment, 66°C for 1 min 30 s; cold treatment, 4°C for 24 h; untreated control) were prepared as described previously. These treated cultures were then serially diluted ten-fold until sterility (up to 10^{-10} CFU/mL) in LB broth and a range of these dilutions were manually plated (100 µL) on LB agar that were estimated to provide 1 single colony on the plate. This range of dilutions was incubated at 37°C and 250 rpm and sampled at 12 and 24 h, and this temperature was used at it is optimal growth temperature for *E. coli* O157:H7 often used in enrichment technique for EHEC detection methods on food (Health Canada 2015b). The corresponding plates were incubated at 30°C for 24 h. Plates were enumerated next day and the dilutions that corresponded to 1 single colony on the plate were kept as 10-100 cell/mL estimated initial count. The dilution under this was kept as 1-10 cells/mL estimated initial count with no colony growth on the corresponding plate and if the broth remained clear with no growth for the subsequent dilution (ie, diluted to cell extinction). All other dilutions were discarded. For sampling at 12 and 24 h, 1 mL was removed per treatment group, transferred to a 2 mL Eppendorf tube, centrifuged at 8000 *x g* for 3 min and the supernatant was discarded while the pellet was stored at -20°C until DNA extraction step. The experiment was performed in 3 trials for each treatment with technical repeat (n=3).

4.2.6. DNA Extraction and Quantification in qPCR

DNA was extracted from pelleted samples using DNeasy blood and tissue kit (Qiagen, Mississauga, Ontario, Canada). Instructions were followed for Gram negative bacteria with the following exceptions: cells suspended in buffer ATL (supplemented with proteinase K) were incubated for 2 h instead of 1-3 h, and DNA was eluted from mini columns twice with 100 µL of butter AE, instead of once with 200 µL buffer AE. Primers were selected to target the *E. coli*'s *uidA* gene (β-glucuronidase), with primers URL-301 (5'-TGT TAC GTC CTG TAG AAA GCC C-3') and URR-432 (5'-AAA ACT GCC TGG CAC AGC AAT T-3' (Sigma-Aldrich, Mississauga, Ontario, Canada; Bej et al. 1991). A stock solution of primers was prepared in sterile nuclease free water. qPCR master mix was prepared as follows (for 100 reactions): 1250 µL of SYBR green (Qiagen), 250 µL each of 10 µM forward and reverse primers, and 250 µL water; 20 µL of the master mix was used per reaction with 5 µL template DNA. A standard curve was created to quantify DNA in samples, in which the PCR product of *E. coli* O157:H7 DNA was purified (QIAquick PCR Purification Kit, Qiagen) and serial ten-fold dilutions were prepared from the purified product at a concentration range of 0.001 pg/µL to 100 pg/µL. A 7500 Fast Real-Time PCR system (Applied Biosystems) quantified samples by one initial denaturation of template DNA and activation of Taq DNA polymerase at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 1 min and extension at 72°C for 1 min. After each qPCR run was finished, data was acquired from the 7500 Software (version 2.0.5, Applied Biosystems) and analyzed. Melt curves of final products were used to verify that non-specific products or primer dimers did not form. All DNA samples from three trials (n=3) with technical repeat were quantified in qPCR in duplicate reaction per plate.

4.3. Statistical Analysis

Statistical Analysis Systems (SAS Institute, Inc.) was used to analyze the data. Two way analysis of variance (ANOVA) and pair-wise comparison of means analyzed significant differences between treatment means, with a probability (p) of <0.05 considered statistically significant.

4.4. Results and Discussion

To achieve two lethal heat treatments that differed in intensity and reduced *E. coli* O157:H7 by 2 log CFU/mL, a heat treatment with a higher temperature and shorter holding time of 66°C for 1 min 30 s and a heat treatment with a lower temperature and a longer holding time of 58°C for 5 min 15 s were chosen (Figure 4.1). When *E. coli* O157:H7 was held at 4°C for 24 h plate counts were similar to untreated cells, indicating that holding samples at refrigerated temperature does not reduce or cause injury to cells.

Detection time includes lag phase followed by growth of cells to a concentration at which they can be detected. While cell growth rate remains constant, it is the lag phase that causes variation in detection times due to recovery of injured individual cells before growth. By collecting a population (>50 cells) of times for detection of single cells on spectrophotometer per treatment group, a population distribution of healthy and sub-lethally injured single *E. coli* O157:H7 cells was created. Next, this data was converted to percent cumulative frequency (# of cells detected per treatment group/total cells in treatment group*100%) over time at which cells were detected, to show the difference in detection time of heat treated cells in comparison to untreated (control) or cold treated cells (Figure 4.2).

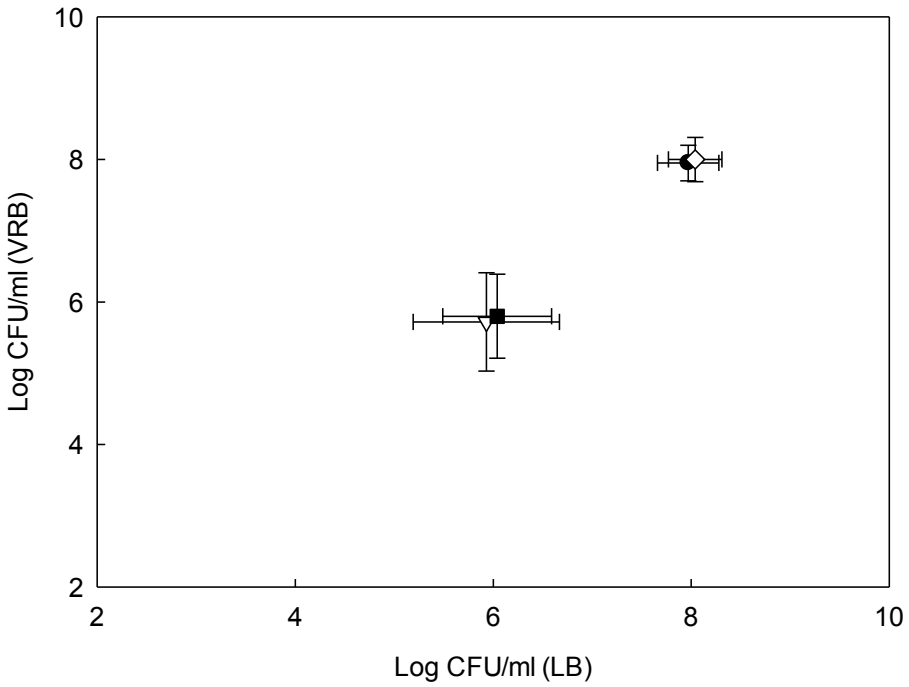

Figure 4.1. Cell counts of heat treated *E. coli* O157:H7 prior to estimated single cell detection by OD readings in spectrophotometer, plated on LB and VRB. Values are means ± SD. ●, control (n=27); ◇, 4°C (n=6); △, 58°C (n=8); ■, 66°C (n=8).

Non-injured single cells in untreated and cells held at 4°C had detection times between 10 – 16 h without an extended lag phase. Similarly, others found that cold stress, at an increased intensity of -25°C for 48 h, did not have a large influence on lag time of *L. monocytogenes* individual cells (Guillier et al. 2005). Heat injured single cells had a broader range of detection times due to extended lag phases, with detection times ranging from 11 to 118 h. The longest times to detection observed for each heat treatment was 67 h for 58°C and 118h for 66°C.

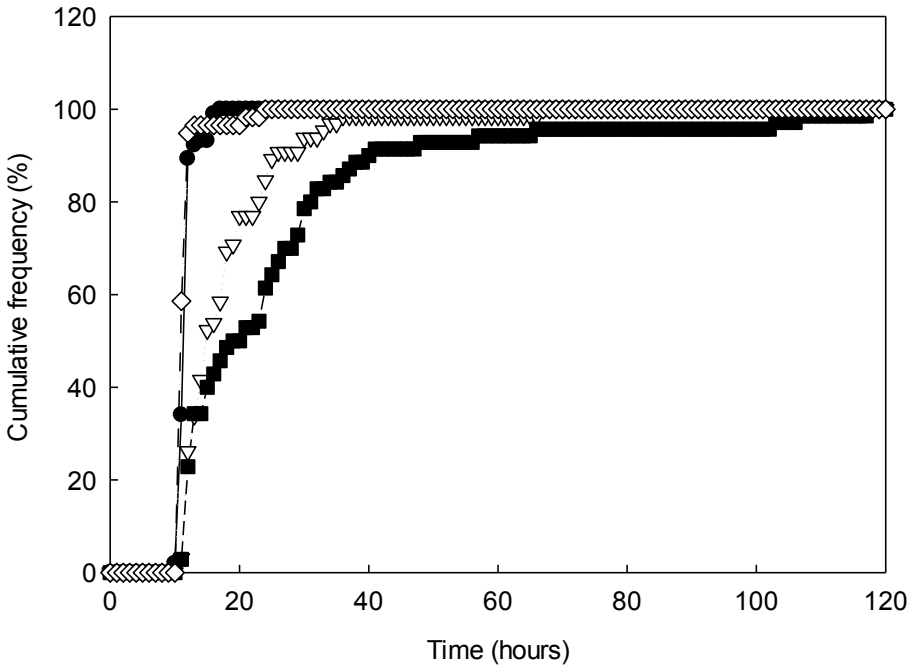

Figure 4.2. Percent cumulative frequency of estimated single *E. coli* O157:H7 cell detection in spectrophotometer every 30 min for 120 h at 25°C. Values are estimated single cells grown to detection of OD 0.02. ●, control (n=103); ◇, 4°C (n=70); △, 58°C (n=65); ■, 66°C (n=58).

The detection limit of the spectrophotometer is high (10^6 cells or 20 generations) in comparison to qPCR's detection limit of 10^3 cells or 10 generations. Generation time from single cell detection data in a spectrophotometer was 30 min at 25°C, which assumes no lag phase for the first time of detection (10 h). Therefore, subsequent times to detection were attributed to lag time. Since the first detection time of 10 h in the spectrophotometer has a cell density of 10^6 (section 4.2.5), to correspond this to the lower detection limit of qPCR (10^3 cells), half the spectrophotometer detection time would be required (a 5 h incubation time). While adjusting for the 5 h detection time difference, at 7 h qPCR or 12 h spectrophotometer detection, 80-90% of uninjured cells are detected whereas only 30% of injured cells are detected; at 24 h qPCR or 29 h

spectrophotometer detection, at least 80% of all cells will grow to qPCR detection limit. Hence, by 24 h incubation the majority of all cells are detected but when this time is reduced, the risk of not detecting injured single cells is increased.

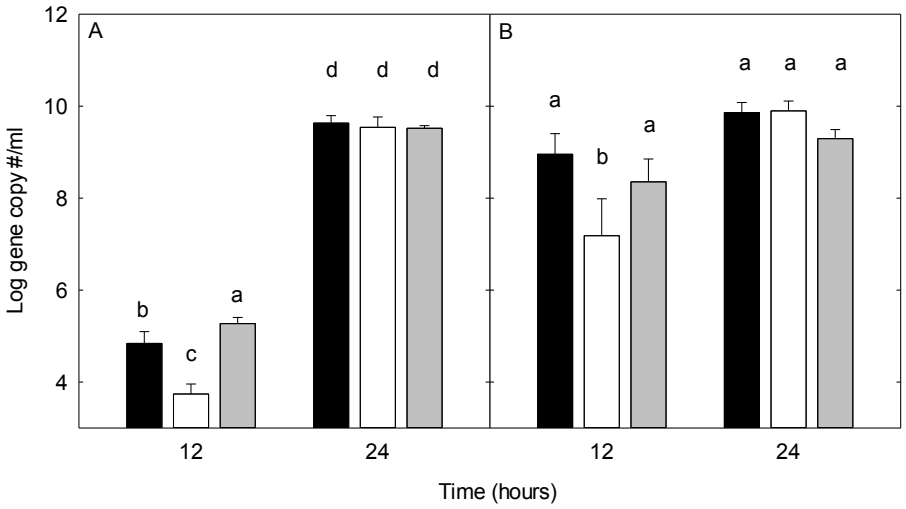

Figure 4.3. (A) Growth of 1-10 or (B) Growth of 10-100 *E. coli* O157:H7 cells/mL in LB broth after heat or cold treatment for 12 or 24 h and quantified by qPCR. Values are means ± SD, n=3. Black bars, control; white bars, 66°C; grey bars, 4°C. Values that do not share a common superscript differ significantly within a group and between groups ($p<0.05$).

Figures 4.3 A and 4.3 B show qPCR quantification of sub-lethally injured cells compared to non injured cells with low initial cell counts of 1-10 cells/mL or 10-100 cells/mL, respectively, after 12 or 24 h incubation at 37°C in LB broth. While the gene copy numbers of the cells treated at 4°C and untreated cells were not different from each other with 10-100 initial cell count/mL samples, heat injured cells grew to significantly lower cell counts ($p<0.05$) at 12 h

measured by qPCR (corresponding to 17 h OD measurement in spectrophotometer). By 24 h, there was no significant difference across treatments with qPCR quantification (corresponding to 29 h OD measurement). In comparison, samples with lower initial cell counts (1-10 cells/mL) had significantly lower gene copy numbers of heat injured cells by 12 h incubation coupled with qPCR quantification, than both untreated cells and cells treated at 4°C ($p<0.001$). In addition, untreated controls were also significantly lower than cells treated at 4°C at 12 h coupled with qPCR quantification, and although the difference was not as large as the former ($p<0.01$), it indicates that in samples with a low initial cell count samples there is greater variability in detection time among treatments. This is also confirmed by previous research determining that there is greater variation in detection time with a decrease in inoculum size due to variation in individual cell lag phase (Baranyi and Pin 1999). By 24 h incubation followed by qPCR measurement, there was no significant difference between treatment groups of 1-10 initial cell count /mL samples. Therefore, with a higher initial cell density within a sample (10-100 cells/mL), the majority of cells in both uninjured and injured cell populations were detected by 12 h in qPCR: 90, 89 and 72% of untreated, cold treated and heat treated groups, respectively, as compared to 24 h recovery. However, with a lower initial cell count (1-10 cells/mL), only 38% of sub-lethally heat treated cells were detected at this time versus 49 and 53% of untreated and 4°C treated groups at 12 h, when compared to 24 h recovery. Therefore, with 1-10 cells/mL in initial samples coupled with 12 h incubation at 37°C and use qPCR detection method, all cells are detected but sub-lethally injured cells approach detection limit but further reduction of incubation time or extension of the cell lag phase will lead to false negative result. However, an increase in initial cell count in a sample reduces this distinction. By 24 h incubation coupled with

qPCR quantification, all cells in different treatment groups are detected in 1-10 or 10-100 initial cell count/mL samples without risk of being undetected.

A population distribution of individual lag phases of *E. coli* O157:H7 cells after 24 h enrichment at 37°C showed that while most untreated cells were detected at 3 h, the majority of lag phases for heat stressed (50°C for 2 h) cells increased to 6 h (Li et al. 2006). In comparison, the present study applied higher heat treatments (over 50°C) and included a much longer incubation time (5 d). This is important since heat stress of ≤50°C extended the lag phase of *E. coli* no more than 10 h (Li et al. 2006), while current work shows that lag phase of sub-lethally injured cells were extended to over 100 h. Furthermore, the present study also gives a more accurate description of lag phase distribution, with over ten times more individual *E. coli* cell measurements. Other researchers have also investigated the lag phase of individual cells treated with mild heat. Over a 48 h period, a broad distribution of lag phases and detection times were discovered for single cells of heat-injured *Salmonella* (53.5°C for 15 min), with the majority of injured cells detected with 20 h enrichment at 37°C (Stephens et al. 1997). Moreover, lag times of individual *L. plantarum* cells increased after heat treatment (51°C for 30 s); while all untreated cells grew by 112 h enrichment, the lag phase of heat-injured cells were up to 196 h at 15°C (Smelt et al. 2002). Likewise, heat treatment of 55°C for 5 min extended the lag phase and increased detection time of individual *L. monocytogenes* from 18.5 to 34 h (Guillier et al. 2005). Therefore, variation in lag time after injury is not only true for *E. coli* but for all other microorganisms as well.

Interventions that aim to eliminate pathogens on food products can sub-lethally injure cells. Heat injured single cells of *E. coli* O157:H7 have a longer lag phase and therefore take a longer time to grow to a level of detection. The range of current validated enrichment times (7-

24 h) may not be sufficient to detect all injured single cells in a sample; with 7 h sample enrichment coupled with qPCR (or 12 h OD) detection, there is a probability of not detecting 10 and 70 % of uninjured and heat injured cells, respectively, whereas at 24 h detection in qPCR (29 h OD), all uninjured cells are detected and there is only a 10 – 20 % chance that injured cells go undetected. Moreover, detection of EHEC in food samples is more complex than in media; this can also affect enrichment and time to detection. Overall, short enrichment times increase the probability of not detecting heat injured cells and as such, increase the risk of false negative detection while longer enrichment times increase the probability of detecting all cells within a sample, including the sub-lethally injured population.

5. Comparative Assessment of qPCR Methods to Distinguish between Live and Dead Enterohaemorrhagic *Escherichia coli* on Meat

5.1. Introduction

Molecular detection methods have become a mainstay in assessment of microbiological composition and activity within microbial ecosystems, including food safety, as they are faster and more selective than labor-intensive and time consuming conventional plating methods (Ge and Meng 2009). Quantitative polymerase chain reaction (qPCR) is one of the most used methods of gene quantification and is a validated method for pathogen detection on food (Wong and Medrano 2005; Health Canada 2015b). However, qPCR is limited in that it does not discriminate between DNA from live and dead cells in an enrichment of a food sample, which may lead to false positive result (Bae et al. 2009).

Monoazide DNA binding dyes can enter dead cells with compromised membranes, intercalate with DNA bases and form covalent bonds upon exposure to light (Taskin et al. 2011; Nocker and Camper 2006a). This results in DNA insolubility causing loss during extraction, as well as preventing its amplification in qPCR. Ethidium monoazide (EMA) has previously been investigated for this purpose, but was found to cause significant DNA loss from viable cells after extraction (Nocker et al. 2006; Flekna et al. 2007). A better alternative to EMA is propidium monoazide (PMA), as it can penetrate into cells with compromised membranes without affecting viable cells with intact membranes (Pan and Breidt 2007). However, PMA may be excluded from dead cells with intact membranes and therefore the overestimation of live cells in qPCR remains an issue (Løvdal et al. 2011; Pachelowicz et al. 2013; Nocker et al. 2007). To better select for live cells in qPCR, a sodium deoxycholate treatment prior to PMA application can enhance its penetration due to the emulsifier's ability to permeabilize intact membranes of dead

cells (Yang et al. 2011). While *E. coli* is highly tolerant to bile salts, the effect of deoxycholate on injured cells is unknown. Another solution is to quantify RNA in qPCR as an indicator of cell viability as it degrades upon cell death as compared to DNA, with mRNA degradation occurring more rapidly than rRNA degradation (Keer and Birch 2003). However, RNA quantification is more time consuming and due to its instability has a higher risk of sample loss as compared to DNA quantification, which makes DNA quantification more practical.

With cattle as a major reservoir of *E. coli* O157:H7, foodborne illness has been linked to the consumption of contaminated beef (Rangel et al. 2005; Blagojevic et al. 2012). Interventions implemented in beef packing plants to control EHEC on carcasses include lactic acid and peroxyacetic acid washes as well as hot water application (Bacon et al. 2000). *E. coli* O157 is regulated in Canada and the US and current methods of EHEC detection on food include enrichment in conjunction with qPCR. However, qPCR does not differentiate between live cells and cells killed by intervention. Improved methods for detection of live EHEC on beef are therefore necessary to reduce illness outbreaks. The objective of the current investigation is to compare five different detection methods to determine which method is best able to accurately quantify live EHEC without detecting dead cells on beef steaks in qPCR after lactic acid, peroxyacetic acid and hot water intervention and includes conventional plating, mRNA and rRNA quantification in qPCR in addition to PMA or PMA and deoxycholate treatment prior to DNA quantification in qPCR.

5.2. Materials and Methods

5.2.1. Preparation of Inoculum

Five shiga toxin (stx) negative *E. coli* O157:H7 (02-0628, 02-1840, 02-0304, 02-0627, 00-3581; Luciano et al. 2011) were grown individually overnight in Luria-Bertani broth (LB; Oxoid,

Missisauga, Ontario, Canada) at 37ºC and 250 rpm. Each strain was centrifuged and resuspended pellet was washed in 0.1% (w/v) peptone water (Difco, Becton-Dickinson, Sparks, Maryland, USA) next day and combined to create a 5 strain cocktail of *E. coli* O157:H7.

5.2.2. Primers

For quantification of DNA in qPCR, primers URL-301 (5'-TGT TAC GTC CTG TAG AAA GCC C-3') and URR-432 (5'-AAA ACT GCC TGG CAC AGC AAT T-3') that target β-glucuronidase activity of *E. coli* were selected as they have previously been confirmed to be able to target *E. coli* O157:H7 (Bej et al. 1991; Sigma-Aldrich). For quantification of rRNA in reverse transcription qPCR (RT-qPCR) primers targeting partial 16S rRNA, HDAf (5'-ACT CCT ACG GGA GGC AGC AGT-3') and HDAr (5'-GTA TTA CCG CGG CTG CTG GCA C-3') were used (Walter et al. 2000, Wang et al. 2013b; Sigma-Aldrich). For quantification of mRNA in RT-qPCR, primers targeting glyceraldehyde-3-phosphate dehydrogenase were selected: gapAf (5'-GTT GAC CTG ACC GTT CGT CT-3') and gapAr (5'-ACG TCA TCT TCG GTG TAG CC-3'; Sigma-Aldrich). While the latter two primers were not species specific, they were chosen because they were able to amplify *E. coli* O157:H7 and since the steaks were clean, quantification was attributed to the microorganism.

5.2.3. Preparation of Beef Steaks for Intervention and Control Samples

For each trial, fresh beef (eye of round) was purchased from a local grocery store. It was then boiled in water for 2 min to decontaminate surface, the cooked outer layer (2 mm) was removed with a sterile knife, and the beef cut was sliced into 2 cm thick steaks with surface area of 88 cm^2. This provided clean steaks for experimentation. One mL of the 5 strain cocktail of *E. coli* O157:H7 was inoculated onto beef steak surfaces in a biosafety cabinet and dried for 30 min to allow for attachment. Three trials were performed per intervention and for each trial, eight steaks

were inoculated with the 5 strain *E. coli* O157:H7 cocktail: four for application of interventions (2 x 0 min and 2 x 30 min sampling) and four for inoculated control (no application of intervention; 2 x 0 min and 2 x 30 min sampling). One steak was left uninoculated as a control sample per trial to confirm steak was clean, where no intervention was applied but rather it was immediately placed in 99 mL PBS buffer in a stomacher bag, manually massaged for 2 min and suspension plated on LB and VRB (100 µL per plate; incubated at 30°C for 24 h; enumeration yielded below detection limit (BDL)) and also 3 x 1 mL samples were kept for DNA qPCR analysis (section 5.2.9); similar to plating, quantification yielded negative result denoted by either amplification at or above 35 Ct with species specific primers (*uidA*) for DNA qPCR or 10 Ct above last sample's Ct value for non specific primers (HDA, gapA) used in both RNA RTqPCR experiments.

5.2.4. Lactic Acid or Peroxyacetic Acid Intervention

Prior to application of interventions, inoculated and control steaks were placed in separate aluminum trays (23 x 15 x 1.5 cm). A 5% (w/v) lactic acid (Sigma-Aldrich, Mississauga, Ontario, Canada) or 200 mg/l peroxyacetic acid (Inspexx, Ecolab Inc., St. Paul, Minnesota, USA) solution was prepared and connected to equipment; the equipment used for application of antimicrobials has been previously described in detail (Youssef et al. 2012). The aluminum trays containing steaks were placed on the stainless steel conveyor belt that carried trays under a spray nozzle, exposing steaks to a 5% lactic acid spray or 200 mg/l peroxyacetic acid spray at a volume of 0.4 mL of fluid per cm^2 steak surface. The steaks were sampled immediately (0 min) or after 30 min of holding at room temperature.

5.2.5. Hot Water Intervention

A 4 L beaker was filled with 2.5 L of sterile water and brought to 80°C on a hot plate. Once the target temperature was reached, steaks were submerged individually for 10 s using sterile stainless steel tongs and immediately removed and sampled at 0 or 30 min.

5.2.6. Sampling of Steaks after Intervention

Upon sampling, each steak was placed in a sterile stomacher bag (VWR International, Radnor, Pennsylvania, United States) with 100 mL buffer. Either 100 mM PBS (includes buffering agents monopotassium phosphate and disodium phosphate) was used to neutralize pH for lactic acid or hot water intervention samples, or neutralizing buffer (includes buffering agent monopotassium phosphate and reducing agent sodium thiosulfate) was used for peroxyacetic acid intervention samples to neutralize pH as well as quench oxidation. Steaks in buffer were then massaged manually in stomacher bags for 2 min. From sample suspension, 15 x 1 mL aliquots were transferred to separate 2 mL transparent Eppendorf tubes. Tubes were centrifuged at 10,000 *x g* for 5 min, supernatant discarded, cells washed in 1 mL peptone water, centrifuged and resuspended in 300 µL peptone water. Each tube was designated to one of 5 different sampling groups for subsequent treatment application, in triplicate: DNA extraction (control; no PMA and deoxycholate), PMA treatment and DNA extraction, PMA with deoxycholate treatment and DNA extraction, RNA extraction, plating (Figure 5.1). Samples for RNA extraction were further washed with RNAprotect Bacteria Reagent (Qiagen) according to the manual to stabilize and protect RNA from degradation, and the pellet was kept frozen (-20°C) until use.

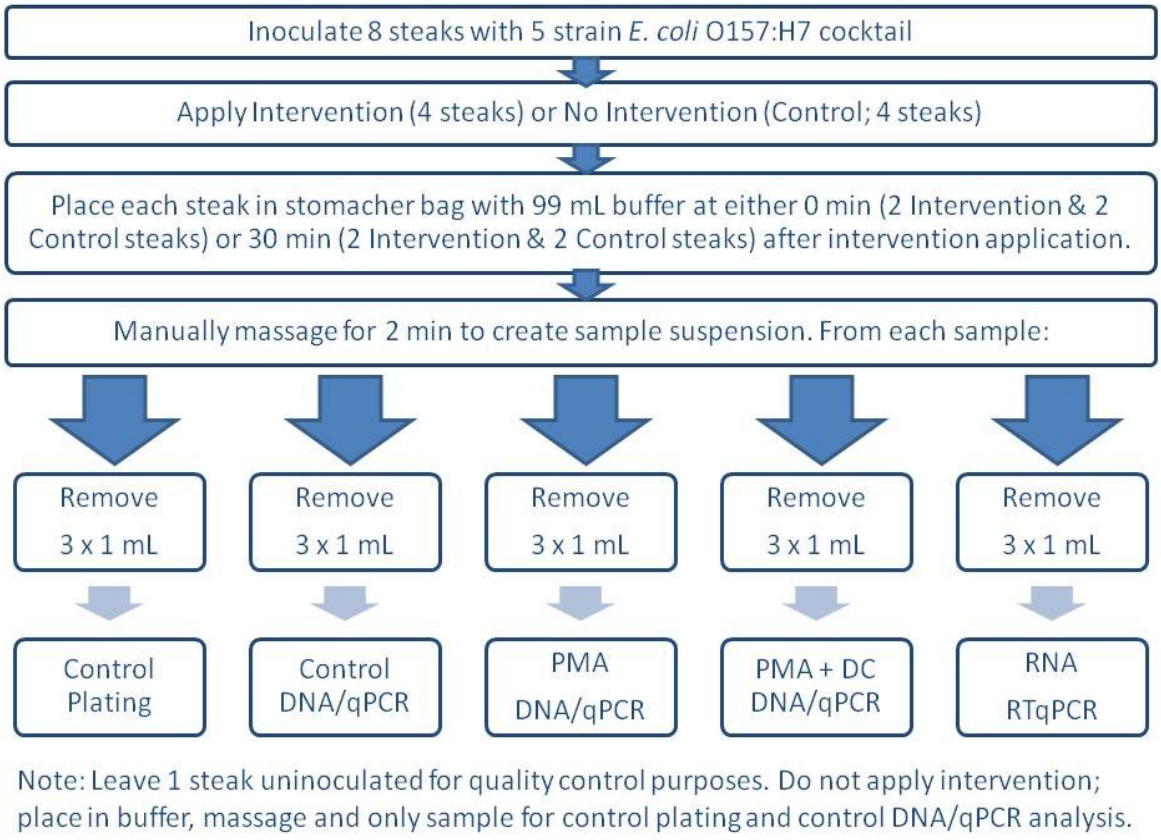

Figure 5.1. Experimental Flow Chart For Each Trial

5.2.7. PMA and Deoxycholate Treatment

For PMA treatment of samples, a 20 mM stock solution of PMA (Biotium, Inc. Hayward, California, USA) was prepared and stored at 4°C until use. For each sample suspension (300 µL) in 2 mL Eppendorf tubes, 1.5 µL of 20 mM PMA was added and vortexed thoroughly. Tubes were placed on ice horizontally in the dark for 5 min followed by exposure to a 650-W halogen lamp for 2 min, held 20 cm above the tubes, while slowly rocking samples back and forth to ensure uniform exposure. Samples were then removed from light, centrifuged at 8,000 *x g* for 3 min, the supernatant was discarded, and the cells were washed with peptone water. The cell pellet was stored at -20°C until further analysis.

For samples treated with both PMA and deoxycholate, deoxycholate was applied to samples before PMA treatment. A 1% (w/v) solution of deoxycholate (Sigma-Aldrich) previously optimized (Yang et al. 2011) was prepared and stored at -20°C until use. Sample suspensions (300 µL) in 2 mL Eppendorf tubes were centrifuged at 8,000 *x g* for 3 min, and the supernatant was discarded. The pellet was resuspended with 300 µL 1% deoxycholate and incubated in a rocking water bath at 37°C for 30 min. After incubation, the suspension was treated with PMA as described above.

5.2.8. Enumeration of *E. coli* O157:H7 on Beef Steaks

On the same day as experimentation, samples were plated. Ten fold dilutions were prepared in sterile 0.1% peptone water from original sample suspensions of beef steaks in stomacher bags with buffer, and 100 µL of dilutions estimated to provide 30-300 colonies per plate was used to manually plate onto LB and Violet Red Bile (VRB; Oxoid) agar. Plates were incubated at 30°C for 24 h and enumerated next day. The number of uninjured cells were enumerated on VRB agar and the numbers of uninjured and injured but viable cells were enumerated on LB agar.

5.2.9. DNA Extraction and Quantification in qPCR

DNA was extracted from samples (DNeasy blood and tissue kit; Qiagen, Mississauga, Ontario, Canada) following instructions for Gram negative bacteria with exceptions. Cells suspended in buffer ATL (supplemented with proteinase K) were incubated for 2 hr, and DNA was eluted from mini columns twice with 100 µL of butter AE. For quantification in qPCR, primers URL-301 and URR-432 were used to target *uidA* gene (Sigma-Aldrich). qPCR master mix was prepared as follows per reaction: 12.5 µL of SYBR green (Qiagen), 2.5 µL each forward and reverse primer, and 2.5 µL nuclease free water, with 5 µL template DNA added to 20 µL of master mix for a total volume of 25 µL per reaction. A standard curve was prepared

from purified PCR product of *E. coli* O157:H7 (00-3581) DNA (QIAquick PCR Purification Kit; Qiagen), which was diluted to 100 mg/l and then further diluted in serial ten fold dilutions to 10^{10}. From here, six dilutions were used as points on the standard curve in range of DNA concentration within samples, 10^3 to 10^8. Nuclease free water served as negative control to ensure no contamination within master mix and uninoculated steak served as negative control for any native *E. coli* that may be already present on steak, as described previously (section 5.2.3). A 7500 Fast Real-Time PCR system (Applied Biosystems) was used for absolute quantification of DNA by one initial denaturation of template DNA and activation of Taq DNA polymerase at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 1 min, extension at 72°C for 1 min. Quantification data was acquired from 7500 Software (version 2.0.5, Applied Biosystems) and analyzed. Melt curves of final products verified no formation of non-specific product or primer dimers. Each sample was quantified in duplicate per qPCR plate.

5.2.10. RNA Extraction and Quantification in RTqPCR

RNA was extracted from samples according to protocol (RNeasy Mini Kit; Qiagen), reverse transcribed to cDNA following the kit's manual (QuantiTect Reverse Transcription Kit; Qiagen). Two sets of primers were selected for RNA analysis, one targeting partial 16S rRNA (rRNA quantification; HDAf and HDAr; Sigma-Aldrich) and the second targeting the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (mRNA quantification; gapAf and gapAr; Sigma-Aldrich). Stocks were prepared of each primer set with sterile nuclease free water. Master mix recipe per reaction was prepared as follows: 12.5 µL SYBR green (Qiagen), 0.5 µL each forward and reverse primers, 10.5 µL nuclease free water, and 1 µL sample cDNA. A standard curve was developed by extracting genomic DNA from an overnight culture of *E. coli* O157:H7 (00-3581)

using Wizard Genomic DNA purification kit (Promega) as specified. Extracted genomic DNA was run in qPCR with respective primers (HDA or *gapA*) and the PCR product was purified according to purification kit instructions (QIAquick PCR Purification Kit, Qiagen) and diluted to 100 mg/l. For each primer set, this concentration was further diluted ten fold (up to 10^{-8}) with dilutions chosen for final standard curve of 10^{-3} to 10^{-8}. Standard curves analyzed by qPCR with samples for absolute quantification. Negative controls included nuclease free water and samples after genomic DNA Wipeout step in reverse transcription protocol (Qiagen) without supplementation of reverse transcriptase, to ensure no contamination of gDNA. The Reverse Transcription qPCR (RTqPCR) method was one initial denaturation of template DNA and activation of Taq DNA polymerase at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, extension at 72°C for 30 s. Data was acquired from 7500 software (version 2.0.5, Applied Biosystems) and analyzed. Melt curves of final products were used to verify that non-specific products or primer dimers did not form. Samples were run in triplicate reaction per plate.

5.2.11. Statistical Analysis

Statistical Analysis Systems (SAS Institute, Inc.) was used to evaluate data by two way analysis of variance (ANOVA) followed by pair-wise comparison of means from different treatment groups with Bonferroni adjustment. A probability (p) of <0.05 was considered statistically significant. Three trials were performed for each intervention application, with triplicate sampling.

5.3. Results

5.3.1. Quantification of *E. coli* O157:H7 on Beef by qPCR After Lactic Acid Intervention

To determine the most accurate method to quantify viable EHEC on beef steak after lactic acid intervention, five different detection methods were compared. Figures 5.2 A and B correlate quantification of *E. coli* O157:H7 on beef after lactic acid intervention by enumeration on LB agar as compared to the quantification of DNA or RNA by qPCR or RTqPCR, respectively. At 0 and 30 min, detection methods by plating, PMA with qPCR and rRNA and mRNA with RTqPCR determined that viable cells were not significantly reduced by lactic acid intervention. Interestingly, only in samples treated with PMA combined with deoxycholate was there significant reduction in the log gene copy #/cm^2 of *E. coli* O157:H7 determined by qPCR. However, reductions observed at 0 and 30 min indicate that the combination treatment not only prevented all dead cell amplification, but it also prevented the amplification of sub-lethally injured cells when compared to plate count data which explains why reductions were significant as compared to plating.

5.3.2. Quantification of *E. coli* O157:H7 on Beef by qPCR After Peroxyacetic Acid Intervention

Similarly, after peroxyacetic acid intervention on steaks inoculated with *E. coli* O157:H7, five different detection methods were compared to determine the most accurate method to quantify viable cells. Figures 5.2 A and B correlate quantification of *E. coli* O157:H7 on beef after peroxyacetic acid intervention by means of enumeration on LB agar as compared to DNA or RNA quantification in qPCR or RTqPCR, respectively. After peroxyacetic acid intervention, plate counts, PMA qPCR and rRNA and mRNA RTqPCR detection methods determined viable

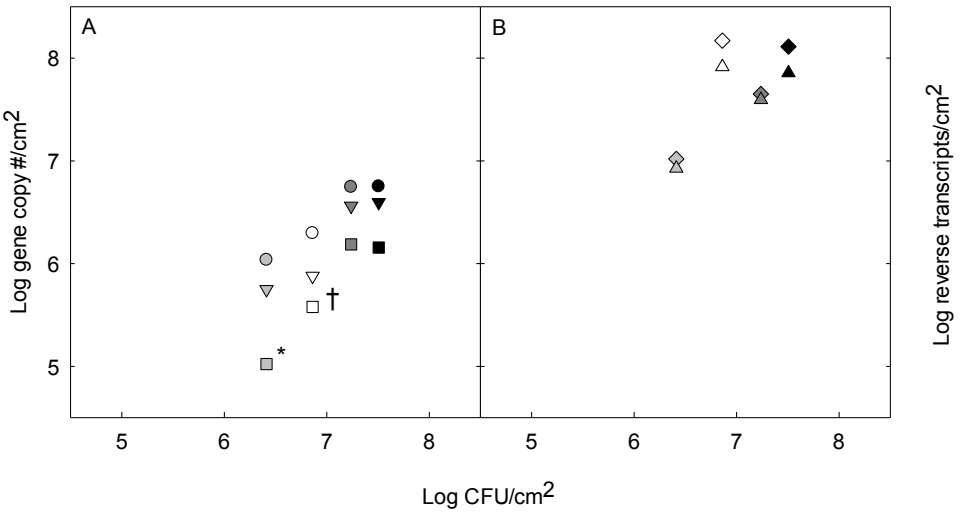

Figure 5.2. (**A**) Correlation of cell counts on LB agar & qPCR DNA quantification of *E. coli* O157:H7 recovered from beef after lactic acid (LA) intervention & PMA or PMA + deoxycholate (DC) treatment. Circle, control; triangle, PMA; square, PMA + DC; black, control 0 min; white, LA 0 min; dark grey, control 30 min; light grey, LA 30 min. Treatments plotted at log CFU/cm^2 of respective control or LA sample at 0 or 30 min. Values are means of three trials with technical repeat (n=3), standard error of means of LB ≤ 0.32; DNA ≤ 0.72. †Significantly different log gene copy #/cm^2 from Control at 0 & 30 min (p<0.05) *Significantly different log gene copy #/cm^2 from Control, Control + PMA, Control + PMA + DC and LA at both 0 & 30 min (p<0.05) (**B**) Correlation of cell counts on LB agar & RTqPCR RNA quantification of *E. coli* O157:H7 recovered from beef after LA intervention. Diamond, rRNA; triangle, mRNA; black, control 0 min; white, LA 0 min; dark grey, control 30 min; light grey, LA 30 min. Treatments plotted at log CFU/cm^2 of respective control or LA sample at 0 or 30 min. Values are means of three trials (n=3) with technical repeat, standard error of means of rRNA ≤ 0.68; mRNA ≤ 0.65.

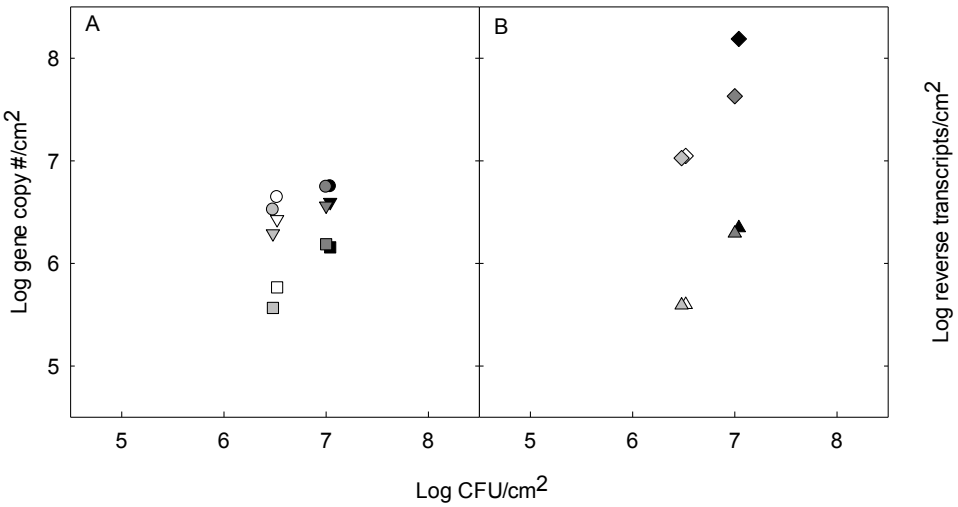

Figure 5.3. (**A**) Correlation of cell counts on LB agar & qPCR DNA quantification of *E. coli* O157:H7 recovered from beef after peroxyacetic acid (PA) intervention and PMA or PMA with deoxycholate treatment. Circle, control; triangle, PMA; square, PMA with deoxycholate; black, control 0 min; white, PA 0 min; dark grey, control 30 min; light grey, PA 30 min. Treatments plotted at log CFU/cm^2 of respective control or PA sample at 0 or 30 min. Values are means of three trials with technical repeat (n=3), standard error of means of LB ≤ 0.39; DNA ≤ 0.68. (**B**) Correlation of cell counts on LB agar & RTqPCR RNA quantification of *E. coli* O157:H7 recovered from beef after PA intervention. Diamond, rRNA; triangle, mRNA; black, control 0 min; white, PA 0 min; dark grey, control 30 min; light grey, PA 30 min. Treatments plotted at log CFU/cm^2 of respective control or PA sample at 0 or 30 min. Values are means of three trials with technical repeat (n=3), standard error of means of rRNA ≤0.35; mRNA ≤1.12.

cells not significantly reduced at either 0 and 30 min sampling times and in addition, the combination treatment of PMA with deoxycholate was also not significant, likely because this intervention did not cause the same degree of sub-lethal injury as the other two interventions. Also, results indicate the combination treatment prevented not only all amplification of dead cells but also the injured cell population when compared to cell counts by plating.

5.3.3. Quantification of *E. coli* O157:H7 on Beef by qPCR After Hot Water Intervention

The same five different detection methods were compared to determine the most accurate method to quantify live cells after hot water intervention on beef steaks inoculated with *E. coli* O157:H7. The correlation of quantification of *E. coli* O157:H7 on beef after hot water intervention by means of enumeration on LB agar as compared to DNA or RNA quantification in qPCR or RTqPCR is presented in Figures 5.3 A and B, respectively. Enumeration by plating, PMA qPCR quantification and rRNA and mRNA RTqPCR quantification determined that the number of viable *E. coli* O157:H7 on steaks at both 0 and 30 min were not significantly reduced by hot water intervention. However, PMA with deoxycholate treatment and qPCR quantification determined there was a significant reduction in log gene copy #/cm^2 at 0 and 30 min. The significant reduction is likely attributed the degree of sub-lethal injury that the intervention caused, and that this population of cells was sensitive to the combination of PMA with deoxycholate treatment, which then lead to the prevention of amplification in qPCR.

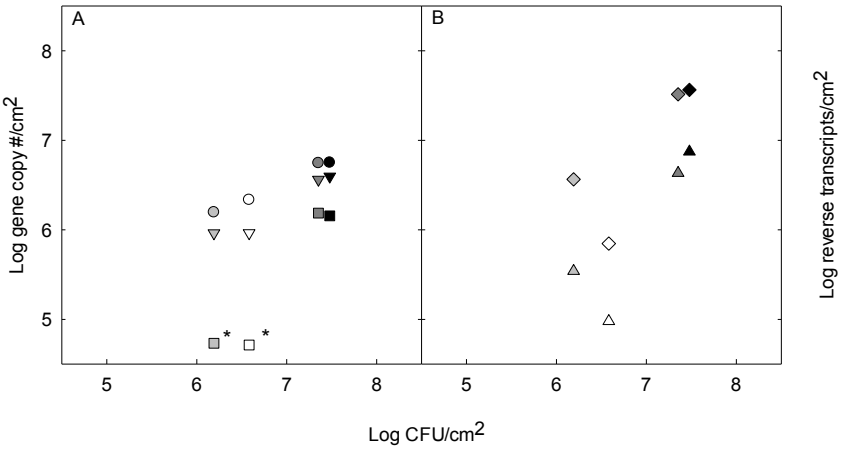

Figure 5.4. (**A**) Correlation of cell counts on LB agar & qPCR DNA quantification of *E. coli* O157:H7 recovered from beef after hot water (HW) intervention & PMA or PMA with deoxycholate treatment. Circle, control; triangle, PMA; square, PMA with deoxycholate; black, control 0 min; white, HW 0 min; dark grey, control 30 min; light grey, HW 30 min.. Treatments plotted at log CFU/cm^2 of respective control or HW sample. Values are means of three trials with technical repeat (n=3), standard error of means of LB ≤ 0.54; DNA ≤ 0.93. *Significantly different log gene copy #/cm^2 from Control, Control + PMA, Control + PMA + DC, HW, HW + PMA at 0 and 30 min (p<0.05) (**B**) Correlation of cell counts on LB agar & RTqPCR RNA quantification of *E. coli* O157:H7 recovered from beef after HW intervention. Diamond, rRNA; triangle, mRNA; black, control 0 min; white, HW 0 min; dark grey, control 30 min; light grey, HW 30 min. Treatments plotted at log CFU/cm^2 of respective control or HW sample at 0 or 30 min. Values are means of three trials with technical repeat (n=3), standard error of means of rRNA ≤ 1.48; mRNA ≤ 1.20.

5.3.4 *E. coli* O157:H7 Recovered on LB and VRB from Beef Steaks at 30 min after Intervention and after Treatment of Cells with PMA or PMA with Deoxycholate

qPCR quantification indicated that the combination of PMA and deoxycholate treatment caused sensitivity to viable EHEC cells after intervention when compared to control and cell count enumeration data by plating. Therefore, samples that were subjected to PMA and

Table 5.1. Enumeration of *E. coli* O157:H7 (Log CFU/cm^2) Recovered on LB and VRB agars from Beef Steaks held for 30 min after Interventions of Lactic Acid (LA), Peroxyacetic Acid (PA) and Hot Water (HW) and after Treatment of cells with PMA or PMA with Deoxycholate deoxycholate treatment in addition to intervention were also enumerated on LB and VRB at 0

Sample	Intervention					
	Lactic Acid		Peroxyacetic Acid		Hot Water	
	LB	VRB	LB	VRB	LB	VRB
Control	7.24 ± 0.08	6.85 ± 0.27	7.00 ± 0.40	6.70 ± 0.70	7.35 ± 0.09^{a}	7.03 ± 0.18^{a}
Control + PMA	6.65 ± 0.30	6.45 ± 0.17	6.98 ± 0.45	6.63 ± 0.79	7.57 ± 0.16^{a}	7.11 ± 0.46^{a}
Control + PMA + DC	6.57 ± 0.09	6.15 ± 0.70	6.41 ± 0.16	6.22 ± 0.25	6.46 ± 0.22^{ab}	6.09 ± 0.48^{ab}
Intervention	6.41 ± 0.14	5.27 ± 0.69	6.48 ± 0.13	6.08 ± 0.57	6.19 ± 0.54^{ab}	5.65 ± 0.62^{ab}
Intervention + PMA	5.91 ± 0.54	5.44 ± 0.45	6.29 ± 0.61	5.75 ± 1.17	6.16 ± 0.64^{ab}	5.88 ± 0.77^{ab}
Intervention + PMA + DC	5.58 ± 0.47	4.85 ± 0.94	5.70 ± 0.41	5.62 ± 0.31	5.20 ± 0.72^{b}	4.85 ± 0.68^{b}

Values are means ± SD, n=3. Values with superscripts are significantly different among values in the same column; those that do not share a common superscript are significantly different (p<0.05).

(data not shown) and 30 min to confirm this result (Table 5.1). This data showed that viability of cells is not affected in all interventions after treatment of samples with PMA. Following the combined treatment of both PMA and deoxycholate, a trend was observed of a reduction in viable cell count after all interventions, indicating that injured cells were killed by treatment. However, only hot water intervention in addition to the combined treatment of PMA with deoxycholate had a significant reduction in viable cell counts from the control.

5.4 Discussion

This is the first study comparing the five different detection methods for the quantification of viable EHEC on beef after intervention. An advantage of this study is that it was practical: it used a real food system and interventions used in the meat industry today. However, a limitation of this study is that cell count reductions were within the same order of magnitude as error bars and this made the meaning of the results more difficult to determine. Detection methods mRNA and rRNA RTqPCR, DNA qPCR and plate count methods quantified EHEC within 1 log or so of each other. Overall, enumeration by plating on LB agar was similar to rRNA RTqPCR quantification, but generally yielded higher viable cell counts than DNA qPCR and mRNA RTqPCR quantification. However, the correlation of results from DNA qPCR and plate counts decreased as dead cell concentration within the sample increased; this is because qPCR does not discriminate between viable and dead cells, whereas plating does (Sidari et al. 2011).The variability between DNA and RNA quantification can be attributed to amplification of different target genes in qPCR or RTqPCR as well as different sample preparation protocols (Keer and Birch 2003). Furthermore, rRNA provided more robust result to mRNA as mRNA is present as a lower percentage of total RNA within cells and that mRNA is more susceptible to degradation as compared to rRNA (Kushner 2002).

The application of lactic acid, peroxyacetic acid and hot water interventions to beef steaks inoculated with a five strain cocktail of *E. coli* O157:H7 created a heterogeneous population of viable, sub-lethally injured, and dead cells which created a suitable sample suspension to test detection methods for live cell discrimination. For controls in all three interventions at 0 and 30 min, mean viable cell count of inoculated EHEC on beef steaks was 7.27 log CFU/cm^2 on LB with an average injured population of 0.33 log CFU/cm^2. After intervention, there was an increase in injured cell count on steaks treated with lactic acid and hot water to an average of ~ 0.9 and 0.6 log CFU/cm^2 over 0 and 30 min, respectively, which suggests these treatments resulted in sub-lethally injury of cells. The average cell injury from peroxyacetic acid treatment of 0.40 log CFU/cm^2 was within range of control samples. Furthermore, comparison of intervention counts to the control data also indicated that the percentage of dead cells within samples was 68%, 45% and 90% for lactic acid, peroxyacetic acid and hot water, respectively. This determined that lactic acid and hot water interventions were more lethal to EHEC on beef than peroxyacetic acid, and this result is consistent with previous research indicating that lactic acid and hot water are more effective interventions on meat surface than peroxyacetic acid (Gill and Bryant 2000; Youssef et al. 2012; Castillo et al. 1998; Ellebracht et al. 2005; King et al. 2005). However, no intervention alone caused significant reduction of viable EHEC cells on beef from controls.

Interestingly, at 0 min sampling there was more variation among viable cell quantification methods for respective interventions than at 30 min sampling. With lactic acid, there was no reduction at 0 min for either rRNA or mRNA RTqPCR but with peroxyacetic acid or hot water intervention, reduction of both rRNA and mRNA in RTqPCR was observed. This may be due to the time required for lactic acid to enter into cytoplasm and kill cells, since the antimicrobial

effect specifically includes penetration of cells in an undissociated form and lowering intercellular pH (Alakomi et al. 2000). However, with peroxyacetic acid and hot water treatments instantaneous reduction of EHEC after application was observed; this is likely due to immediate oxidizing effect of H_2O_2 with peroxyacetic acid intervention since mechanisms of action include oxidization of sulphur bonds in cell membrane, enzymes and proteins and in addition, immediate membrane disruption and irreversible denaturation of protein occurs with hot water intervention (Finnegan et al. 2010; Lee and Kaletunc 2002). At 30 min sampling, lactic acid and peroxyacetic acid were not different using RNA RTqPCR quantification methods, which averaged 0.64 log reverse transcript/cm^2; however, plating indicated that lactic acid was more lethal than peroxyacetic acid. This indicates that conventional plating is a more sensitive indicator of viable cells than RTqPCR RNA. Moreover, compared to both acid interventions hot water had greater reduction in not only plate counts but over double log reverse transcript/cm^2 for both RNA RTqPCR quantification methods, indicating that this intervention was the most lethal for *E. coli* O157:H7 on beef steaks.

PMA treatment does not affect viability of cells (Yang et al. 2011; Elizaquivel et al. 2012; Nocker et al. 2006). PMA qPCR quantification prevented some, but not all, amplification of DNA from dead cells in sample suspensions as it was found to be excluded by up to 87% of dead cells when correlated to LB plate count reductions from control after intervention. The reduction in DNA amplification that was observed with PMA qPCR did not reflect variation in reduction from severity of intervention as the higher kill of hot water intervention was not reflected by PMA treatment. This is likely due to a high initial cell concentration in samples and the increased dead cell concentration that can interfere with viable cell quantification in PMA qPCR and decrease the correlation of plate count enumeration and PMA qPCR quantification of live

cells (Yañez et al. 2011; Løvdal et al. 2011; Pan and Breidt 2007; Wagner et al. 2008). This could be because with a high initial cell density containing a high concentration of dead cells, PMA crosslinking during light activation can be inhibited (Løvdal et al. 2011). Others have also suggested that incubation of samples for a longer period of time and at higher temperatures will increase PMA penetration into cells (Nkuipou-Kenfack et al. 2013). Løvdal et al. (2011) used PMA qPCR in an attempt to discriminate between live and dead *L. innocua* cells in media after applying a severe lethal heat treatment of 80°C for 15 min; in comparison to plate counts, large discrepancies were observed between these methods as plate counts were below detection limit whereas PMA qPCR quantified 8 log cells/mL. Furthermore, in a food system correlation of live cell detection methods can vary depending on food matrix, amount of microbial contamination and severity of interventions applied. Pachelowicz et al. (2013) found that viable *Campylobacter* cell counts on raw chicken carcasses were not in accordance between PMA qPCR and conventional plate count method wherein PMA was excluded from dead cells and especially in higher cell density samples, whereas Josefson et al. (2010) found that viable *Campylobacter* cell counts on raw chicken carcasses were highly correlated between qPCR PMA and conventional plating.

A recent study also compared different methods for detection of live *E. coli* O157:H7 on lettuce: culture based method (plating) as compared to more rapid molecular detection techniques of gene transcript (mRNA) quantification in RTqPCR and qPCR with PMA (Ju et al. 2016). As the surface of lettuce leaves do not provide sufficient nutrition for survival, enumeration by plating determined that *E. coli* O157:H7 declined by 10^7 in 96 h in low relative humidity conditions. Molecular methods determined there was no reduction of *E. coli* O157:H7 in qPCR quantification alone and only a 10^2 and 10^3 reduction by the viable detection methods

assessed: mRNA RTqPCR and PMA qPCR quantification, respectively. Much like our study, their results indicate that PMA qPCR leads to false positive result and they determined that this is likely due to the presence of a high concentration of dead cells within a sample. Also, they determined that mRNA RTqPCR amplified transcripts from dead cells, and therefore this method could also lead to false positive result. In comparison, our study also confirms that PMA qPCR and mRNA RTqPCR quantification do not provide accurate estimates of bacterial viability in comparison to culture based methods. The other viable cell detection method assessed in the present study, that was not included in work by Ju et al. (2016), was qPCR quantification after PMA with deoxycholate treatment.

Interestingly, this work indicates that combination treatment of PMA with deoxycholate does affect the viability of cells. The use of deoxycholate prior to PMA treatment to increase cell membrane permeability is a relatively new area of research and what has been investigated is not within food systems. In suspensions where *E. coli* cells were killed after heat treatment with no viable cells remaining, treatment with PMA prior to qPCR analysis resulted in a difference in the mean cycle threshold (Ct) values of -1.5 cycles from controls whereas treatment with PMA with deoxycholate resulted in much higher Ct values with a mean increase of 7.30, indicating that PMA with deoxycholate significantly decreases amplification of dead cells in qPCR as compared to PMA alone (Yang et al. 2014). Yang et al. (2011) found that *E. coli* cells that were killed by over 80°C heat treatment were permeable to PMA and while the majority of cells killed by under 72°C heat treatment were not, the addition of 1% deoxycholate treatment rendered a large portion of these cells permeable to the dye. Furthermore, Wang et al. (2014) found that after lactic acid intervention, membranes of dead and injured *E. coli* cells that were impermeable to PMA were rendered permeable to the dye after treatment with 1% deoxycholate treatment and in

this study, the injured cells were resuscitated in nutrient rich broth for 2 h prior to deoxycholate treatment which restored injured cell membrane barrier properties. This could be a possible solution to injured cell sensitivity to PMA with deoxycholate treatment but since our study was performed in a more complex system rather than in broth or buffer, this could prove to be more complicated and warrants further investigation. In the present investigation, the comparison of 3 different interventions on *E. coli* O157:H7 inoculated beef steaks with PMA and deoxycholate treatment consistently resulted in reduced log gene copy #/cm^2, which was significant in two of three interventions. Therefore, our results indicate that if PMA with deoxycholate treatment with qPCR is used for pathogen detection in meat, it could kill injured cells present in samples and lead to false negative detection. Since the inability to discriminate between DNA in live and dead cells in qPCR is a problem that extends beyond EHEC in meat, this combination treatment could be a solution in these other areas of research. This includes determination of viable probiotics in the gut or viable lactic acid bacteria or bifidobacteria in fermented foods (Tabasco et al. 2014; Villarreal et al. 2013; Cocolin et al. 2011; Rantsiou and Cocolin 2006). However, it is important to note that concentration of treatment should be adjusted to target microorganism, as Gram positive microorganisms have different resistance to deoxycholate than Gram negative microorganisms (Nkuipou-Kenfack et al. 2013).

This study compared five different methods to determine most accurate quantification of viable *E. coli* O157:H7 detection on beef steaks after intervention. PMA treatment with qPCR did not prevent all dead cell amplification in samples after intervention and therefore this treatment does not resolve the issue of false positive result in qPCR; however, it does prevent amplification of some dead cell DNA and therefore increases the accuracy of viable cell quantification compared to qPCR alone. In contrast, treatment of cells with PMA with

deoxycholate did prevent all dead cell DNA amplification; however, it does so at the cost of sub-lethally injured cells as deoxycholate supported PMA penetration into injured cells which may have otherwise recovered; this makes DNA inaccessible for qPCR quantification and would lead to false negative result. Both rRNA and mRNA quantification in qPCR had more variability and was not as sensitive for quantifying viable cells as plate counts. While PMA with deoxycholate may not be suitable for pathogen detection in food, it could be used in other areas of research that aim to exclude all dead cells at the expense of injured cells. On the other hand, if research aims to amplify all viable cells including the injured cell population at the cost of also amplifying some dead cell DNA, PMA or rRNA with qPCR detection methods could be used. Overall, conventional plating provided most sensitive and reliable detection of viable *E. coli* O157:H7 on beef.

6. Conclusions and Future Directions

6.1. General Discussion

EHEC's ability to cause severe disease in humans with only 10 cells or fewer makes it an important foodborne pathogen (Willshaw et al. 1994). Since there is no treatment for EHEC infection, control and detection on food are the only means currently available to prevent sickness. Illness outbreaks are linked to the consumption of foods such as spinach and beef (Rangel et al. 2005). Therefore, these at risk foods were chosen as food models for projects in this thesis on EHEC control and detection. Novel intervention methods for controlling EHEC on food, and improved EHEC detection methods on food, are necessary to increase the safety of our food supply and decrease the risk of foodborne illness. However, it is important that new control and detection methods are also tested within a food system to ensure that they are effective and compatible with food matrix and composition as well as the required food storage conditions, so that they may be practically used in an industry setting.

6.1.1. Control

A novel method of controlling EHEC on food is the use of antimicrobial allyl isothiocyanate (AITC), a compound that is naturally found in high levels in mustard seed in the form of its precursor compound sinigrin (Antonious et al. 2009; Delaquis and Mazza 1995; Lin et al 2000b). While AITC is versatile as an intervention in that it can be applied in various forms, the use of mustard powder as a vector for AITC release is particularly interesting. The AITC molecule is highly volatile which presents challenges use, as it can cause irritation to exposed workers and rapid decomposition (Dussault et al. 2014; Martinez-Abad et al. 2013). However, use in the form of mustard meal could help manage the antimicrobial; volatile AITC remains in the form of stable sinigrin until water is added and mustard's endogenous enzyme myrosinase

catalyzes release of the antimicrobial from its precursor (Graumann and Holley 2009; Shofran et al. 1998). Another limitation is that the sensory quality of food can be affected by AITCs pungent odor and spicy flavor but, concentrations of mustard meal added to foods such as ground beef patties have also corresponded to acceptable taste, smell and color of food by panelists, and these concentrations have corresponded to lethal effect on pathogens (Chacon et al. 2006a). Furthermore, while pure AITC concentrations required for lethal effect on produce are within compliance of allowed levels in food by regulatory authority, on meat, these levels are not within allowable levels as higher amounts are required due to interaction with proteins in meat matrix (USDA 2014). The use of mustard meal; however, is not regulated in food and therefore could be used to deliver AITC to food in concentrations that cause lethal effect and retain sensory quality (Chacon et al. 2006a; Nadaraja et al. 2005b). Furthermore, use of a natural source of AITC such as mustard on at-risk foods would be beneficial since it would provide a clean food label rather than require listing as a synthetic chemical preservative (Khan and Abourashed 2009).

To explore mustard AITC intervention in a way that has not been done before, one study of this thesis involved the investigation on the use of AITC gas released from mustard meal to eliminate *E. coli* O157:H7 and *L. innocua* on spinach and lettuce. After HPLC analysis, it was discovered that sinigrin was stable at 4°C throughout 4 d storage, and therefore AITC could not be released and not surprisingly, mustard meal did not reduce *E. coli* O157:H7 or *L. innocua* on spinach, lettuce or filter paper at this temperature during 4 d storage. However, since AITC remains a potent antimicrobial at refrigerated temperature (Lin et al. 2000a; Piercey et al. 2012), absence of activity at 4°C was attributed to inactivity of myrosinase enzyme in mustard meal at this temperature, thereby preventing AITC release into headspace. At 15 and 30°C, sinigrin was significantly reduced over 4 d which corresponded to a maximum concentration of AITC in

headspace of 30 mg/L; this amount of AITC has been found effective in reducing *E. coli* O157:H7 on spinach, lettuce and tomatoes at 4°C (Obaidat et al. 2009a, 2009b). However, in this project *E. coli* O157:H7 was not killed at elevated temperature, but at the same time, these temperatures were not practical to use for intervention on these products due requirement of storage at 4°C. Therefore, I reject my hypothesis that mustard meal could be used to control EHEC on spinach and lettuce at refrigerated temperature. Rather, mustard meal AITC could possibly be used as a preservation method to reduce other microorganisms, such as spoilage microbiota, on foods stored at room temperature.

6.1.2. Detection

Current validated enrichment times for methods of *E. coli* O157:H7 detection on beef and leafy greens are 7-24 h, inclusive (Health Canada 2011). This thesis also involved investigation into enrichment times to determine if they were sufficient to detect all cells within a sample, with focus on sub-lethally injured cells with extended lag phases. Enrichment is still a required step that is used in conjunction with various types of detection methods, including conventional plating and molecular detection methods, as it increases target microorganism to a concentration at which it can be detected as well as resuscitates injured cells. However, enrichment poses a problem by increasing overall detection time; even with rapid molecular methods of detection such as qPCR, time is increased from hours to days when coupled with enrichment (Ge and Meng 2009; Brehm-Strecher et al. 2009). Therefore, decreasing enrichment times would be beneficial as this would allow for more rapid pathogen detection.

Lag phases of microorganisms are extended after sub-lethal injury or stress to cells, and this can affect time to detection (Stephens et al. 1997, Smelt et al. 2002, Li et al. 2006). To determine necessary EHEC enrichment times, the current work in this thesis involved the

assessment of individual *E. coli* O157:H7 cell lag phases of uninjured and sub-lethally heat injured cells with spectrophotometer measurement (with a high detection limit of 10^6 cells) as well as correlation to qPCR measurement (with a lower detection limit of 10^3 cells). It was found that while the majority of uninjured cells are detected within current validated enrichment times with both detection methods, it is the cells sub-lethally injured by heat intervention, causing longer lag phases, which pose a problem for decreasing enrichment times. For spectrophotometer detection at 24 h, up to 39% of heat injured cells were not detected and in qPCR, up to 27% heat injured cells would go undetected at this time. As sub-lethally injured cells can recover and grow, it is important that they are detected on food since EHEC has a low infectious dose (Kiranmayi et al. 2010). As another means to assess necessary enrichment time, the present work also quantified low initial *E. coli* O157:H7 cell count samples (an estimated 1-10 cells/mL and 10-100 cells/mL per sample) in qPCR after incubation of 12 or 24 h at 37°C in LB broth with or without sub-lethal heat treatment. While at 24 h, there was no significant difference between uninjured and injured cell growth in either sampling group, with reduced incubation time of 12 h, sub-lethally injured cells were significantly lower compared to uninjured cells in both sampling groups. Furthermore, with a lower initial cell count (1-10 cells/mL), heat injured cells reach concentrations near detection limit of qPCR at 12 h sampling. Therefore, it was found that longer enrichment times should be used as opposed to shorter enrichment times to increase the probability of detecting sub-lethally injured cells. Further decreasing enrichment times from current validated methods increases the probability of not detecting sub-lethally injured cells which could lead to false negative result, therefore, the hypothesis that EHEC enrichment times can be decreased is rejected.

The required lengthy enrichment step prior to qPCR analysis was the first major limitation of qPCR detection explored. The second major limitation of qPCR detection, the inability to differentiate between live and dead cell DNA in a sample, was also investigated in the work of this thesis. Moreover, this research was the first to compare five different viable EHEC detection methods in a food model system; PMA or PMA with deoxycholate in conjunction with qPCR, in comparison to qPCR mRNA and rRNA quantification and conventional plate count enumeration. While the photo-inducible DNA binding dye, PMA, prevented some dead cell amplification in qPCR, it did not prevent all; this was also found by others (Patcholewicz et al. 2013, Løvedal et al. 2011). PMA in combination with membrane emulsifying deoxycholate has been previously proposed to enhance PMA penetration into dead cells with intact membranes and therefore provide a more accurate quantification of viable cells in qPCR (Yang et al. 2011). These authors found that this method was successful to prevent dead *E. coli* cell DNA amplification in qPCR in broth and the current work in a food system also confirmed this result but also found other complications. While PMA with deoxycholate prevented all dead EHEC cell amplification in qPCR on beef steaks after interventions of lactic acid, peroxyacetic acid and hot water wash, the combination treatment also killed the sub-lethally injured cells within sample suspensions; this treatment was more complex within a food system. Moreover, instead of preventing false positive result, this treatment could cause false negative result since this population of sub-lethally injured cells may have otherwise recovered. Further optimization of this method is warranted, including the adjustment of deoxycholate concentration to target microorganism (Nkuipou-Kenfack et al. 2013), providing an injured cell resuscitation step prior to treatment (Wang et al. 2014) and testing treatment within different food systems and after a representative intervention application has been applied. In comparison,

RNA analysis provided viable cell quantification but was more time consuming, labor-intensive and was subject to sample degradation than DNA quantification. While PMA alone used as a pre-treatment could provide some improved accuracy of live cell DNA quantification in qPCR, overall it was the conventional plating method that was the most reproducible of the five methods assessed for viable EHEC cell quantification on beef steak after intervention. The hypothesis that PMA with deoxycholate could increase the accuracy of live cell detection in qPCR for pathogen detection on food is rejected.

6.1.3. Importance of Testing Intervention Models in Food Systems under Practical Storage Conditions

The majority of microbial experimentation is performed in media and while this allows for control of variables such as pH, nutrient composition, water activity and temperature, it does not reflect microbial behaviour in the relevant food matrix since media is far less complex than food systems (Smith et al. 2005). Interventions may appear effective in theory and prove to be successful in broth or buffer; however, it is important that they are tested in a food system since efficacy of intervention can be influenced by food matrix. Firstly, food matrix can have a protective effect on microorganisms as cells can hide in cracks or crevices of food and be physically protected from intervention application (FDA 2014b). Secondly, food matrix composition can also protect cells, as food constituents within the matrix can interact with bacteria and influence their resistance to lethal interventions. For example, bactericidal effect of high pressure on foodborne pathogens such as *E. coli* O157:H7, *Salmonella* spp., and *Campylobacter jejuni* is highly dependent on food matrices and therefore an evaluation of intervention against target microorganisms on the specified food is necessary to determine efficacy (Gänzle and Liu 2015; Huang et al. 2013; Liu et al. 2012; Morales et al. 2008). For

instance, pathogens are more resistant to high pressure processing when in ground beef as opposed to peptone water (Baccus-Taylor et al. 2015). This may be attributed to constituents within the meat matrix such as calcium and magnesium ions, which can stabilize the outer membrane of the cell (Garcia-Hernandez 2015) as well as the concentration and type of fat, although influence of fat on efficacy of high pressure intervention is not well understood (Escriu and Mor-Mur 2009). Lastly, food composition can also protect cells by interacting with antimicrobial that are applied directly to food, rendering them ineffective against the target microorganism. This is observed with non-specific antimicrobials which are beneficial in that they can target a wide range of microorganisms, but also because of this, they can also react with constituents of the food matrix. Chlorine is one such non-specific antimicrobial, and while effective on hard surfaces or buffer, its efficacy is reduced in the presence of organic matter since it readily reacts with unsaturated fatty acids and is neutralized (Rahman 2007; Fukayama 1986; FDA 2014b). Moreover, natural plant essential oils are another good example; a reduction of antimicrobial activity of cinnamon, clove, oregano or thyme oil against pathogens in foods with increased fat content as opposed to their lower fat content counterparts, including milk, cheeses and hot dogs (Cava et al. 2007; Smith-Palmer et al. 2001; Singh et al. 2004), a reduction in oregano and thyme oil activity against forborne pathogens due to increased complex carbohydrate (potato starch) content whereas simple carbohydrate (sugar) did not interfere with antimicrobial efficacy of essential oils (Gutierrez et al. 2008; Gutierrez et al. 2009) and with allyl isothiocyanate from mustard essential oil, a reduction in activity was observed due to interaction with proteins and amino acids, leading to the requirement of at least ten times more AITC for an observed antimicrobial effect in meat such as ground beef as compared to produce (Luciano et al. 2008; Nadaraja et al. 2005b). Overall, the components of a food's matrix create a complex

system that can greatly influence intervention efficacy against target pathogens due to interactions with various food components.

Furthermore, in addition to influencing cell survival during intervention, the complexity of food matrix can also affect sampling and detection in methods such as qPCR quantification. Food samples may contain various inhibitors and native microflora that are not present in broth or buffer samples (Wang et al. 2013a; Smith 2005). There are a wide range of qPCR inhibitors that can be found in food samples, including carbohydrates (di- and polysaccharides), proteins (collagen, haemoglobin, and proteinases) as well as minerals such as salt and calcium (Rossen et al. 1992; Schrader et al. 2012). The conditions in which food products are stored is also an important factor to consider in the development of new antimicrobial interventions. In the current work, AITC release from sinigrin in mustard meal is significant at higher temperatures of 15 and 30°C; however, at refrigerated temperature, little AITC was released over 4 d storage due to low enzyme activity; this renders mustard meal intervention impractical for spinach and lettuce decontamination, since produce requires storage at refrigerated temperature. Therefore, testing of new preservation methods on the intended food product and under required storage conditions is essential before it should be considered by industry, and in addition, potential food matrix inhibitors that may interfere with pathogen detection should be determined so that methods can be developed to eliminate or minimize their impact and increase accuracy of detection.

In summary, the current projects provided insight into control and detection of EHEC on food. Use of mustard meal as a vector for AITC gas release can be beneficial for foods stored at room temperature; however, for successful use this method must be optimized and food composition, storage time and temperature should be considered. Secondly, current validated enrichment times used prior to EHEC detection methods were insufficient for detection of all

sub-lethally injured single cells. Longer enrichment times would increase the probability that all viable cells will be detected in a sample, whereas shortening enrichment times increases the risk of not detecting all viable cells within a sample, especially the sub-lethally injured cell population, which could lead to a false negative result. Lastly, the issue of a false positive result due to dead cell amplification in qPCR remains an issue; treatment of PMA with deoxycholate prior to detection killed injured cells and therefore was counter-productive, while PMA alone did not prevent amplification of all dead cells and of the 5 detection methods it was conventional plating that provided the most reproducible, reliable and accurate quantification of viable EHEC on food.

6.2. Future Directions

This thesis provided insight on the use of mustard meal as an intervention to release volatile AITC from sinigrin; mustard meal should not be used at refrigerated temperature since myrosinase is inactive and rather should be used at elevated temperature. Preservation of food from spoilage organisms at room temperature by use of mustard meal could be a useful intervention and further research is warranted.

This thesis proposed that enrichment time should not be decreased from current validated methods as this would increase the probability of not detecting the sub-lethally injured population that have extended lag phase. Current enrichment times should be explored in food systems to determine if food matrix further complicates the enrichment of such cells.

This thesis provided insight into the use of PMA with deoxycholate treatment prior to qPCR quantification to increase the accuracy of live cell detection. EHEC on beef steaks injured by intervention were killed by the combination treatment however, and requires further

optimization in a food system if it is to be considered to prevent dead cell amplification and increase accuracy of viable pathogen detection in qPCR.

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