USE OF ORIENTAL MUSTARD AND ALLYL ISOTHIOCYANATE TO CONTROL *SALMONELLA, CAMPYLOBACTER* AND *L. MONOCYTOGENES* IN POULTRY MEAT

By

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Dedication

IT IS AN HONOUR TO DEDICATE THIS THESIS TO:

MY LOVELY PARENTS

MY WIFE AND OUR CHILDREN "JUDY AND AOUN"

MY BROTHERS AND SISTERS

THE SOUL OF JORDANIAN PILOT MARTYR MUATH KASASBEH

MY BELOVED HOMELAND "JORDAN"

Thesis format

The current thesis is comprised of nine chapters including a brief introduction to the importance of study subjects, the previous work, limitations and objectives (Chapter 1), a comprehnsive literature review about poultry meats and their safety, foodborne pathogens in poultry products, use of natural antimicrobials in food (mustard, AITC, organic acids and EDTA) and edible films and coatings (Chapter 2). The thesis ends with an overall summary of the results, an iterpretation of the importance of the results and recommendations for future research (Chapter 9). The thesis also includes six manuscripts at different stages of publication in peer-reviewed scientific journals, and these are presented as Chapters 3-8. Manuscripts were standardized for presentation in the thesis format and they are identified as follows:

Chapter 3 "Effects of changes in pH and temperature on the inhibition of *Salmonella* and *Listeria monocytogenes* by allyl isothiocyanate" was originally published in the journal Food Control 34: 414-419 in 2013 with authorship by Olaimat, A.N. and Holley, R.A.

Chapter 4 "Inhibition of *Listeria monocytogenes* and *Salmonella* by combinations of Oriental mustard, malic acid and EDTA" was originally published in the Journal of Food Science 79: M614-621 in 2014 with authorship by Olaimat, A.N. and Holley, R.A.

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Chapter 6 "Inhibition of *Listeria moncytogenes* on cooked cured chicken breasts by malic or acetic acid-κ-carrageenan/chitosan-based coatings containing allyl isothiocyanate or

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Chapter 8 "Inhibition of *Campylobacter jejuni* on fresh chicken breasts by κ-carrageenan/chitosan-based coatings containing allyl isothiocyanate or deodorized Oriental mustard extract" was originally published in the International Journal of Food Microbiology 187: 77-82 in 2014 with authorship by Olaimat, A.N., Fang Y. and Holley, R.A.

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ABSTRACT

In this project the factors influencing the stability and antimicrobial activity of allyl isothiocyanate (AITC) against *Campylobacter jejuni*, *Salmonella* or *Listeria monocytogenes* as well as factors that enhance sinigrin (glucosinolate in Oriental mustard) hydrolysis by these pathogens were investigated. The minimum inhibitory concentration (MIC) of AITC against 5 strains of each of *Salmonella* or *L. monocytogenes*, ranged from 60-100 ppm at 37 ºC. This was reduced to 10-40 ppm at 21 ºC and a further reduction to 5-10 ppm against strains of *L. monocytogenes* was observed at 4 ºC. This was attributed to greater stability of AITC as temperature was decreased.

C. jejuni strains were more susceptible to AITC with MICs of 0.63-1.25 ppm and 2.5-5 ppm at 37 and 42 ºC, respectively. AITC was more inhibitory at ≤ 21 ºC against *Salmonella* with acidic pH or against *L. monocytogenes* with neutral pH. *C. jejuni, Salmonella* and *L. monocytogenes* strains and mixtures had the ability to degrade sinigrin to form inhibitory concentrations of AITC, and sinigrin hydrolysis was significantly enhanced by higher incubation temperature (21 ºC > 10 ºC > 4 ºC), the presence of 10 mM ferric or ferrous irons, and the presence of < 0.25% glucose.

This project also investigated the antimicrobial activity of AITC or Oriental mustard extract alone or combined with ethylenediamine tetraacetic acid (EDTA), malic acid and acetic acid in edible antimicrobial coatings against *C. jejuni* and *Salmonella* on fresh, refrigerated, vacuum-packed chicken breasts or *L. monocytogenes* on refrigerated, cured roast chicken. Malic acid improved the antimicrobial activity of Oriental mustard extract against *L. monocytogenes*, while EDTA improved its activity against *Salmonella*. Incorporation of 25 to 50 µl/g AITC or 100 to 250 mg/g Oriental mustard extract in 0.5%κ-carrageenan/2%chitosan coatings, prepared

using 1.5% malic or acetic acid, reduced *L. monocytogenes* on cooked, cured, vacuum-packed chicken slices 4.2 to > 7.0 $\log_{10}$ CFU/g, compared to uncoated chicken by 70 d at 4 ºC. In addition, 0.2%κ-carrageenan/2%chitosan coatings (prepared using a 1% acetic acid solution) containing 250 mg/g mustard extract or 50 µl/g AITC reduced *Salmonella* numbers on vacuum-packed chicken breasts 3.0 $\log_{10}$ CFU/g by 21 d at 4 ºC. Further, 0.2%κ-carrageenan/2%chitosan coatings containing 50 or 100 µl/g AITC reduced numbers of *C. jejuni* on fresh, vacuum-packed chicken breasts > 5.0 $\log_{10}$ CFU/g (*C. jejuni* cells were not detected) after 5 d storage at 4 ºC, while coatings containing 200 to 300 mg/g Oriental mustard extract or 25 µl/g AITC reduced *C. jejuni* numbers by 3.6 to 4.6 $\log_{10}$ CFU/g. Numbers of lactic acid and aerobic bacteria on poultry meat products were significantly reduced by the coatings. It is clear that κ-carrageenan/chitosan coatings containing either AITC, mustard extract alone or combined with EDTA, malic or acetic acid significantly reduced *C. jejuni* and *Salmonella* on fresh, refrigerated, vacuum-packed chicken breasts and *L. monocytogenes* on refrigerated, cured roast chicken, and consequently enhanced their safety.

Chapter 1

Introduction

Meat and meat products, particularly poultry meats, are the major food categories linked to bacterial foodborne illness outbreaks (Painter et al., 2013; CSPI, 2009). However, when foodborne illnesses are considered together with consumption rates, poultry products were more likely to cause illnesses than red meats (beef and pork), fruits and vegetables and dairy products (CSPI, 2014). In the US from 1998 to 2010, meat and poultry products were linked to 1,714 reported outbreaks including 33,372 illnesses. Among these categories, ground beef and chicken were considered the highest risk groups followed by beef products (except steak, ground or roast beef), steak and turkey. Chicken and turkey together were responsible for 582 foodborne outbreaks with 11245 illnesses (CSPI, 2013). In another study, it was found that most bacterial illnesses that occurred in the US during 1998–2008 were attributed to dairy (18%) and poultry (17.9%) products, but the latter was associated with more deaths (262 deaths, 30.4% of bacterial illnesses) than other food categories. Most poultry-associated deaths were caused by *L. monocytogenes* (63%) or *Salmonella* spp. (26%) (Painter et al., 2013). These included three large listeriosis outbreaks with 192 illnesses, 26 deaths, and 10 miscarriages which were linked to turkey delicatessen meat contaminated in the processing plant after cooking (Gottlieb et al., 2006; Mead et al., 2006; Olsen et al., 2005). In Canada, a significant listeriosis outbreak occurred in 2008 which caused 57 illnesses with 24 deaths linked to delicatessen meat (Clark et al., 2010; Farber et al., 2011). In Canada, *Campylobacter* and *Salmonella* are the second and third leading bacterial pathogens, and account for 145,000 and 87,510 foodborne illnesses, respectively, every year (Thomas et al., 2013). Similarly, the most frequent foodborne illnesses reported in the US in

2013 were caused by *Salmonella* and *Campylobacter*, with proportions of 38% and 35%, respectively (CDC, 2014b). In the EU in 2012, *Campylobacter* infection was the most frequently occurring zoonotic disease and 44% of the *Campylobacter* outbreaks in which information on the implicated food vehicle was provided, were linked to broiler meat (EFSA and ECDC, 2014). Scharff (2010) estimated that the annual costs associated with foodborne outbreaks linked to *Salmonella*, *Campylobacter* and *L. monocytogenes* in the US were $15 billion, $19 billion and $9 billion, respectively.

Nowadays consumers are asking for healthier and safer meat products that contain reduced salt, fat, cholesterol, nitrite, with health-promoting bioactive components including natural compounds such as spices and herbs (Jiménez-Colemenero et al., 2001; Weiss et al., 2010). Mustard, the major spice used in meat manufacturing, contains high levels of secondary metabolites, glucosinolates, that can be hydrolyzed by the enzyme thioglucoside glucohydrolase (myrosinase) in the presence of water to yield isothiocyanates which have antioxidant and antimicrobial properties (Fahey et al., 2001; Rask et al., 2000; Vig et al., 2009). In deodorized mustard (devoid of myrosinase), glucosinolates can be converted into highly antimicrobial isothiocyanates by bacterial myrosinase-like enzyme action present in *E. coli* O157:H7, *Staphylococcus carnosus* and *Pediococcus pentosaceus* (Luciano and Holley 2010, 2011; Luciano et al., 2011). These findings generated the question of how widespread the distribution of this activity might be among other pathogens like *Salmonella, Campylobacter* and *L. monocytogenes*. Herzallah et al. (2011) found that *S.* Typhimurium and *L. monocytogenes* converted sinigrin into allyl isothiocyanate (AITC) by myrosinase-like activity(ies). However this study was limited to testing a single strain of each organism and no studies have examined the presence of myrosinase-like activity(ies) in *Campylobacter*.

Factors including pH value, temperature, iron compounds and glucose concentration affect the activity of plant myrosinase and consequently, sinigrin decomposition rate and products formed. Prakash et al. (2013) found that 1 mM Fe^{2+} completely inhibited the activity of myrosinase from cauliflower while 1 mM Fe^{3+} or 50 mM glucose reduced myrosinase activity by 50% compared to the control. In an earlier study, Borek et al. (1994) found that the addition of Fe^{2+} to a solution buffered at pH 6.0 or 4.0 promoted the formation of allylnitrile and reduced the formation of AITC, whereas Fe^{3+} inhibited the enzymatic reaction. The optimum temperature of plant myrosinase activity ranged from 37 to 60 ºC based upon its source (Al-Turki and Dick, 2003; Hochkoeppler and Palmieri, 1992; Sharma and Garg, 1996; Springett and Adams, 1989). These factors may also affect the activity of bacterial myrosinase during degradation of glucosinolates. The optimum temperature for bacterial myrosinase from *E. cloacae* ranged from 25 to 40 ºC with higher stability between 25-30 ºC. However, ferric and ferrous chloride at 1 mM or 1.8% glucose reduced its activity by 18% to 68% at 37 ºC (Tani et al., 1974). It should be noted that none of these studies examined the effects of temperature, iron compounds or glucose concentration on the activity of the myrosinase-like enzyme(s) from *Salmonella* or *L. monocytogenes*.

AITC is unstable in aqueous solution and is easily decomposed to new compounds, particularly under alkaline conditions or at elevated temperature (Tsao et al., 2000; Ohta et al, 1995). Therefore, temperature and pH are the major factors influencing AITC stability and these factors also may influence its antimicrobial activity. Luciano and Holley (2009) reported that AITC was more inhibitory to *E. coli* O157:H7 at low pH. There is only one study which examined the effect of temperature on antimicrobial activity of AITC against *E. coli* O157:H7, and that was done in ground beef 4 ºC (Nadarajah et al., 2005). Unfortunatly, each of these

studies characterized the antimicrobial activity of AITC against *E. coli* O157:H7 under different experimental conditions. Further, no studies have examined the interactive effects of pH and temperature on AITC stability and antimicrobial activity.

It has been reported that yellow mustard powder is effective against *E. coli* O157:H7 in meat products (Cordeiro et al., 2014b; Cordeiro et al., 2013; Graumann and Holley, 2008; 2009; Luciano et al., 2011; Nadarajah et al., 2005; Nilson and Holley, 2012). These studies have focused on red meat products which differ in composition from raw and processed poultry, and this may affect the antimicrobial efficiency of mustard. Further, hydrolysis of the glucosinolate, sinalbin, in yellow mustard produces ρ-hydroxybenzyl isothiocyanate (ρ-HBITC), while hydrolysis of the glucosinolate, sinigrin, in brown and Oriental mustard by myrosinase yields AITC. Although both products formed are antimicrobial, AITC had greater stability and antimicrobial activity than ρ-HBITC (Luciano and Holley, 2010; 2011). Based on these studies, it is likely that brown or Oriental mustard would be more effective than yellow mustard in inactivation of foodborne pathogens when they are used at the same glucosinolate concentration. However, it must be kept in mind that sinigrin occurs in Oriental mustard at a level of 0.8% (w/w), whereas sinalbin represents 2.5% of the weight of yellow mustard (Zrybko et al. 1997). Lara-Lledó et al. (2012) found that a polymeric film containing Oriental mustard extract with 5% (w/w) sinigrin was more inhibitory than a film with 6% (w/w) sinalbin in a yellow mustard extract against *L. monocytogenes* on vacuum-packed bologna slices at 4 ºC. But when yellow and Oriental mustard powder were incorporated at the same mustard concentration in dry sausage (the sinalbin level was higher than that of sinigrin), yellow mustard was more active against *E. coli* O157:H7, and this was attributed to the formation of greater amounts of ρ-HBITC than AITC (Cordeiro et al., 2014b). Nonetheless, the high cost of purified sinalbin standards

made it worthwhile to examine the hydrolysis of sinigrin from Oriental mustard for its ability to eliminate *Salmonella* and *Campylobacter* from fresh raw chicken and *L. monocytogenes* from cooked cured chicken products.

Since incorporation of mustard powder may affect the physicochemical (water holding capacity, texture, oxidative stability, water activity and pH) and organoleptic properties (color and flavor) of poultry meat, the antimicrobial film approach is a more practical application. Edible film and coatings formulated with food additives including colorants, flavors, nutrients, spices, anti-browning, and antimicrobial agents can improve the shelf-life and stabilize the organoleptic characteristics of food. They can also serve as selective barriers to moisture transfer, oxygen uptake, lipid oxidation and the loss of volatile aromas and flavors (Kester and Fennema, 1986). When food is packaged with edible films and coatings containing natural antimicrobials, the latter are gradually released at the surface of food, and lower antimicrobial concentrations are required to inhibit pathogenic bacteria (Sánchez-Ortega et al., 2014; Sangsuwan et al., 2009). Chitosan and κ-carrageenan are oppositely charged polysaccharides which are compatible when combined. In combination they have good coating properties, improved action as a gas barrier, and facilitated delayed release of incorporated bioactive compounds (Pinheiro et al., 2012a, b).

Combinations of plant extracts or their essential oils with other natural antimicrobials may achieve desired antibacterial effects at low concentrations and consequently minimize undesirable changes in physicochemical and organoleptic properties. Ethylenediamine tetraacetic acid (EDTA) has been approved as GRAS in the US (FDA, 2011) and is used in a number of food products as a chelating agent to prevent oxidation, rancidity, discoloration and off-flavor as well as stabilize vitamins and minerals (Ko et al., 2010; Wreesmann, 2014). Organic acids are

attractive antimicrobials due to their acceptance in foods and their low cost since they are either naturally present in fruits and vegetables or are synthesized by microorganisms (Miller et al., 1996). Several studies have shown synergistic interactions of plant extracts or their essential oils with organic acids or EDTA (Gadang et al., 2008; Zhou et al., 2007). However, only two studies have examined the combined effect of mustard with acetic acid and these showed that 10% or 20% (w/v) mustard in combination with 1% (v/w) acetic acid had greater antimicrobial activity against *E. coli* O157:H7, *L. monocytogenes*, and *S.* Typhimurium compared with mustard alone when stored at 5 or 22 ºC (Rhee et al., 2002; 2003). It is possible that combinations of Oriental mustard extract or AITC with organic acids or EDTA could show enhanced antimicrobial effects against foodborne pathogens such as *L. monocytogenes* and *Salmonella.*

It may be that AITC from sinigrin in Oriental mustard has application as an antimicrobial for control of *Salmonella* and *Campylobacter* on fresh raw chicken and *L. monocytogenes* on cooked, cured chicken. The overall objective of this project was to develop an efficient mustard-based antimicrobial coating to eliminate pathogens from processed or fresh poultry meat products. The objectives of the current project were to:

- Determine the stability plus inhibitory and bactericidal effects of AITC against *Salmonella, Campylobacter* and *L. monocytogenes* at different pH values and temperatures.
- Screen *Salmonella, Campylobacter* and *L. monocytogenes* for their ability to degrade sinigrin and study the effects of temperature, iron compounds and glucose concentrations on myrosinase like activity(ies) in these pathogens.
- Determine the antimicrobial effects of Oriental mustard extract and AITC alone or combined with malic acid, acetic acid and EDTA directly incorporated in edible κ-carrageenan/chitosan

coatings against *L. monocytogenes* on cooked, cured chicken meat and against *Salmonella* and *Campylobacter* on fresh, refrigerated, packaged chicken.

Chapter 2

Review of Literature

2.1 Poultry products

Meat and meat products are excellent sources of protein, essential amino acids, vitamins and minerals, particularly iron (Bender, 1992). However, these products contain significant levels of saturated fatty acids which increase levels of low-density lipoprotein (LDL) and cholesterol in humans, and consequently increase the risk of coronary heart disease (CHD), which is the largest cause of death in developed countries (Micha et al., 2010). In the human diet, approximately 25% of the saturated fatty acids are supplied by meat products; therefore, the dietary guidelines for Americans and Canadians recommended that consumption of red and processed meat should be moderated (Health Canada, 2011a; U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2005). Furthermore, these guidelines recommended substituting in part red meat with poultry products which have less saturated and more polyunsaturated fatty acids (PUFA) than red meat. Poultry meat is one of the most popular foods worldwide because of sensory, dietary, and economic considerations. In addition, it is a highly digestible, tasty, low-calorie food, and is often recommended by nutritionists over other meats (Mulder and Schlundt, 1999).

Meat processing is the largest food manufacturing sector in Canada. In 2012, there were 2,645 and 531 regulated chicken and turkey producers, respectively; in addition to 5,000 commercial poultry and egg producers with combined production of 1.2 billion kg of chicken and turkey meat. In the same year, Canada exported > 14.4 million chicks and poults (young

turkeys) to 24 countries, with a value of $44 million, and 171 million kg of poultry meat and edible bi-products (fresh, chilled, frozen) to 72 countries, worth > $373 million (Agriculture and Agri-Food Canada, 2013). Since 1977 Canadian consumption of red meats decreased while consumption of chicken meat almost doubled and reached 30.1 kg/person in 2013. This increase was attributed in part to a change in the country's population which increased by 39% from 24.5 million in 1980 to approximately 34 million in 2010. In addition, an increase in the awareness and perception that chicken meat has lower fat and is likely healthier than other meats, as well as the development of poultry products containing reduced fat with acceptable sensory characteristics, increased the popularity of chicken among Canadians (Lupescu, 2014).

However, meat products are considered an ideal medium for growth of spoilage and pathogenic organisms because of the high moisture, high content of nitrogenous compounds, presence of minerals and fermentable glycogen and pH favorable for most enteric organisms. Meat products become contaminated with microorganisms through handling, manufacturing, packing and distribution. Improper cooking, refrigeration and storage are the main causes of meat borne illness (Al-Mutairi, 2011; Fratmico et al., 2005). Greig and Ravel (2009) reported that beef, pork, chicken, other meats, turkey and other poultry products were responsible for 498, 197, 365, 167 and 105 reported (published in electronic reports or journals) worldwide outbreaks, respectively, from 1988 to 2007. In the US, the Center for Science in the Public Interest (CSPI, 2009) reported that meat and meat products were responsible for 1599 outbreaks (34.5%) with 43,000 cases (36.7%) between 1998 and 2007. These categories included beef and beef dishes (428 outbreaks), luncheon and other meats (143 outbreaks), pork and pork dishes (200 outbreaks), and poultry and poultry dishes (538 outbreaks). In addition, 290 outbreaks were linked to meals containing meat (meat may have been responsible for these outbreaks) such as

chicken served with salad, pork with coleslaw and ground beef with potatoes. More recently, CSPI (2014) noted that poultry products were responsible for 413 outbreaks with 11,333 illnesses in the US from 2002 to 2011. In another study, Painter et al. (2013) estimated the annual number of foodborne illnesses, hospitalizations and deaths in the US based on the outbreaks that occurred during 1998–2008. Poultry products caused 17.9% of foodborne illnesses, 16.2% of hospitalizations and 30% of deaths. Poultry was the single food category that caused the greatest mortality. In England and Wales, Adak et al. (2005) estimated the percentage of illnesses and deaths by food category using data from the national surveillance database between 1996 and 2000. Poultry, red meat and complex foods (meal dishes containing mixed foods) were the major three food groups most often linked to illnesses (29%, 17, and 26%, respectively) and deaths (28%, 24% and 26%, respectively). These data show that poultry is the main category of meat products that is linked to foodborne outbreaks.

2.2 Foodborne pathogens

Foodborne illness is a major public health concern worldwide in terms of numbers of persons affected and economic costs, yet information reported is often incomplete. Greig and Ravel (2009) examined 4093 foodborne illness outbreaks which occurred internationally (208 in Canada) from 1988 to 2007 where both the agent responsible and food vehicle were known. From these data it appeared that 240 outbreaks occurred each year in Canada during this period. CSPI (2009) noted that 4,638 outbreaks, linked to specific foods, involving 117,136 cases occurred from 1998 to 2007 in the US alone. In an updated analysis, the CSPI (2014) reported that 10,409 foodborne outbreaks occurred in the US from 2002 to 2011. The European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC, 2014) reported that 5,363 foodborne outbreaks involving 55,453 illnesses, 5,118 hospitalizations

and 41 deaths occurred in the European Union in 2012. However, under-diagnosis and under-reporting of foodborne illnesses are common and present challenges for surveillance and the detection of outbreaks (Centers for Disease Control and Prevention, CDC, 2011). It has been estimated that 4 million cases of foodborne illnesses occur in Canada every year (Thomas et al., 2013). In the US, Scallan et al. (2011) estimated that 48 million persons acquire foodborne illness with 128,000 hospitalizations and 3,000 deaths each year. In an earlier study, Scharff (2010) had estimated that foodborne pathogens cause 82 million cases of illness in the US each year with a cost of $ 152 billion related to medical treatment and quality-of-life losses. However, the costs of investigations, illness prevention and losses to industry were not included in this study. An accurate estimate is probably between 42-82 million cases. It should be noted that the most significant zoonotic pathogens in foods of animal origin are those that frequently colonize or infect animals asymptomatically. These include *Salmonella* spp., *Campylobacter* spp., *L. monocytogenes* and enterohemorrhagic *Escherichia coli* (EHEC) which can spread easily from infected animals within and between herds during livestock production (EFSA and ECDC, 2014).

2.2.1 Foodborne pathogens in poultry products

Contamination of poultry meat with foodborne pathogens is an important public health concern that is difficult to resolve, because it can occur in different locations and result from different activities including handling and pre- or post- cooking treatment. Broilers may become contaminated with pathogens by two main routes. The first is vertical transmission which occurs by transfer of bacteria from the hen to the chick via the fertile egg and the second involves horizontal transmission, which is the transfer of bacteria from environmental sources during growth at the farm. These sources include insects, rodents, wild birds, old litter, drinking water,

improperly disinfected houses, equipment, transport vehicles and worker activities (Cox et al., 2012; FAO/WHO, 2009; Newell and Fearnley, 2003; Pearson et al., 1996). When a bird is colonized with organisms such as *Campylobacter* or *Salmonella*, they will be excreted in large numbers in the feces. Therefore, contact with bird feces is the major way organisms can spread throughout a flock. This contamination may occur while the birds are on the farm or during transportation to the slaughter facility. Processing operations including scalding, plucking and evisceration may increase the potential for contamination (FAO/WHO, 2009; Newell and Fearnley, 2003). Poultry products are frequently found to be contaminated with foodborne pathogens such as *Salmonella*, *Campylobacter*, *Staphylococcus aureus*, *E. coli*, *L. monocytogenes*, *Yersinia enterocolitica*, *Aeromonas* and *Clostridium perfringens*. However, *Salmonella*, *Campylobacter*, and to a lesser extent *L. monocytogenes*, are the major pathogens linked to foodborne illnesses caused by poultry products (Mulder and Schlundt, 1999).

2.2.1.1 *Campylobacter jejuni*

Campylobacter is a Gram-negative, slender, spirally-curved, 0.2–0.9 μm diameter and 0.2–5.0 μm long bacterium that is motile by a single polar flagellum at one or both poles. The genus *Campylobacter* belongs to the family *Campylobacteriaceae* which is comprised of 18 species, 6 sub-species and 2 biovars, which include more than 90 biotypes and serotypes. However, *C. jejuni* and *C. coli* are thermophilic bacteria and are the species most often associated with foodborne infections in humans (Adedayo and Kirkpatrick, 2008; Humphrey et al., 2007; Keener et al., 2004; Nachamkin et al. 2008). It has been estimated that *C. jejuni* is responsible for 90% of campylobacteriosis in humans (WHO/FAO, 2009). *C. jejuni* is a fastidious organism that is able to survive in a wide range of environments. It grows at 37 to 42 °C with optimum growth at 42 °C, but it is unable to grow below 30 °C. In contrast, the organism survives longer at

refrigerator than at room temperature. Further, it can grow over a pH range of 4.9 to 9.0 with optimum growth at pH 6.5 to 7.5. *C. jejuni* strains are sensitive to dehydration and they are microaerophilic, requiring a special atmosphere consisting of 5% oxygen, 10% carbon dioxide, and 85% nitrogen for growth in laboratory media (Adedayo and Kirkpatrick, 2008; Keener et al., 2004; Nachamkin et al. 2008).

Unlike other foodborne pathogens, *Campylobacter* lack many regulatory genes that could enable them to overcome environmental stresses (Murphy et al., 2006; Park, 2002; Parkhill et al., 2000). For example, bacteria such as *E. coli* O157:H7 and *Salmonella* possess multiple alternative sigma factors that can coordinate gene transcription in response to environmental stresses. RpoS is a sigma factor which is important for adaptive responses in many Gram-negative pathogens (Hwang et al., 2011). The lack of *rpoS* in *Campylobacter* may increase its sensitivity to a variety of stresses. It has been found that resistance of *C. jejuni* NCTC 11351 to heat and oxidative stresses was not induced during the stationary phase (Kelly et al., 2001). In environments with suboptimal growth conditions such as low moisture, *Campylobacter* may convert to a viable but non-culturable (VBNC) state which is characterized by a change of cellular morphology from a spiral rod to a coccoid form, with changed cell wall structure and decreased metabolic activity. However, *Campylobacter* may become re-culturable after entry into the intestinal tracts of chickens, mice and rats or upon access to embryonated eggs (Cappelier et al., 1999; Humphrey et al., 2007; Li et al., 2014a).

Campylobacteriosis is an acute zoonotic bacterial infection of the human gastrointestinal tract or blood. The minimum infective dose of *Campylobacter* was found to be as low as 500 CFU and symptoms may occur within 4 to 10 d (Humphrey et al., 2007). The main symptoms of campylobacteriosis are abdominal pain, bloody diarrhea, fever, headache, dizziness and muscle

pain. Severe complications such as reactive arthritis (joint inflammation), Guillain-Barré syndrome (GBS) or Miller Fisher syndrome (MFS) may also occur (Blaser and Engberg, 2008). GBS is an autoimmune neural disorder involving paralysis caused by antibodies generated in an immune response to the *C. jejuni* lipo-oligosaccharide which affects the peripheral nervous system (Yuki and Koga, 2006). It has been estimated that only 0.1% of campylobacteriosis cases may develop GBS symptoms, however, this may increase to 0.5% in patients infected with *C. jejuni*, Penner type HS:19 (Nachamkin, 2002). MFS is a variant of GBS which is characterized by abnormal muscle coordination, paralysis of the eye muscles and absence of tendon reflexes which may progress to GBS (Yuki and Koga, 2006).

Campylobacter is the leading cause of human gastroenteritis and is responsible for 400 to 500 million cases of human infection worldwide each year (Ruiz-Palacios, 2007). Scallan *et al*. (2011) reported that more than 845,000 campylobacteriosis illnesses with 119 deaths occur in the US annually, while Scharff (2010) estimated the number of illnesses it caused to be 2.1 million/year in the US. In another study, Taylor et al. (2012a) reported that *Campylobacter* was responsible for 262 outbreaks with 9135 illnesses, 159 hospitalizations and three deaths in the US from 1997 to 2008. In a Canadian study, Greig and Ravel (2009) examined international reports and found that *Campylobacter* caused 191 outbreaks worldwide from 1988 to 2007. In the EU, the number of campylobacteriosis illnesses has increased since 2008 with 214,268 cases reported in 2012, and it was noted that campylobacteriosis remained the most frequently occurring zoonotic disease (EFSA and ECDC, 2014). In Canada, *Campylobacter* is the second most frequent bacterial cause of foodborne illnesses and it was estimated to be responsible for 145,000 illnesses every year (Thomas et al., 2013). The incidence of campylobacteriosis reported reached 361 cases per million population in 2008 (Public Health Agency of Canada, 2010).

However, it was estimated that the actual number of campylobacteriosis illnesses is substantially greater than these numbers due to underreporting (Thomas et al., 2006). This is because campylobacteriosis cases occur sporadically in small groups and most patients fully recover within several days without seeking medical treatment. It has been reported that sporadic infections account for > 99% of campylobacteriosis in the US (CDC, 2008). The principal reservoir of *Campylobacter* spp. is the digestive tract of wild and domesticated mammals and birds including broilers, broiler breeder flocks, cattle, pigs, sheep and dogs. Nonetheless, poultry products are the major source of campylobacteriosis (Cork, 2010; Suzuki and Yamamoto, 2009). In the EU, approximately 25% of fresh chicken meats tested were positive for *Campylobacter* (EFSA and ECDC, 2014). Suzuki and Yamamoto (2009) summarized research describing the prevalence of *Campylobacter* in retail poultry meats and by-products in various countries and found the pathogen was present in 72% and 58% of samples in the US and Canada, respectively. Bohaychuk *et al.* (2006) found *Campylobacter* in 62% of raw chicken legs in Edmonton, AB, Canada. In another study, Medeiros et al. (2008) found that 9.7% of raw chicken samples from food service operations specializing in poultry dishes in Ottawa, ON, Canada were positive for *Campylobacter*. Raw poultry is often contaminated by feces during slaughter (Keener et al., 2004). Sanitizing agents including chlorine, organic acids, chlorine dioxide, trisodium phosphate or acidified sodium chlorite reduced *Campylobacter* numbers on poultry carcasses by 0.5 to 1.5 $\log_{10}$ CFU/g during washing; however, this is a negligible reduction compared to the 5.0 to 8.0 $\log_{10}$ CFU/g of poultry feces that may enter the processing area (Keener et al., 2004).

2.2.1.2 *Salmonella*

Salmonella spp. are Gram-negative, non-sporeforming, facultatively anaerobic, rod-shaped bacteria with 0.7-1.5 μm diameter and 2-5 μm length, which belong to the family

Enterobacteriaceae. *Salmonella* are grouped into two species, *Salmonella bongori* and *Salmonella enterica*. The latter is divided into 6 subspecies (*enterica, salamae, arizonae, diarizonae, houtanae and indica*), of which *S. enterica* is the largest group and contains 2610 different serotypes, and is most frequently responsible for causing foodborne illness (Public Health Agency of Canada, 2011; Su and Chiu, 2007). The most medically important serotypes of *Salmonella enterica* subspecies *enterica* are Typhi, Paratyphi, Enteriditis, Typhimurium and Choleraesuis (Su and Chiu, 2007). *Salmonella* can grow over a temperature range of 5 to 47 °C with optimum growth at 37 °C. However, some serotypes like *S. enterica* Typhimurium can grow at 2 to 54 °C. *Salmonella* also can grow over a pH range from 4.0 to 9.0 with optimum growth at pH 7.0 and at a water activity (a_w) as low as 0.94; however, it can survive at $a_w < 0.2$ in dried foods (Bhunia, 2008; D'Aoust et al., 2001).

Salmonella spp. (except *S. enterica* Typhi and Paratyphi) are zoonotic in origin and cause salmonellosis in humans which can lead to acute or chronic symptoms which range from diarrhea to septicemia. Although healthy people can recover quickly from this illness, it can be fatal during pregnancy, for infants, the elderly and immunocompromised patients (D'Aoust et al., 2001). *Salmonella enterica* may cause acute gastroenteritis or more serious infections (bacteremia) (Ryan, 2004). Invasive infections are characterized by fever, headache, brachycardia, a faint rose-colored rash on the abdomen and chest, anorexia, abdominal pain, myalgias, malaise, diarrhea, constipation, hepatosplenomegaly, segmental ileus, meningismus, and neuropsychiatric manifestations. Typhoid fever caused by *S. enterica* Typhi and Paratyphi is not normally zoonotic in origin and is transmitted from humans to humans by contact and by contaminated water (rarely by food) (Fleckenstein and Patel, 2005; Public Health Agency of Canada, 2011; Ryan, 2004). Gastroenteritis (salmonellosis) is usually characterized by nausea,

vomiting, abdominal cramps, diarrhea, headache and fever, and is caused by non-typhoidal *Salmonella*, mainly *S.* Typhimurium, *S.* Enteritidis, *S.* Heidelberg and *S.* Newport (Fleckenstein and Patel, 2005; Ryan, 2004). Bacteremia may involve septic shock, mesenteric lymphadenitis, osteomyelitis in long bones and vertebrae, pneumonia, pulmonary abscess, brain abscess, subdural and epidural empyema, meningitis, infection of the aorta, liver, spleen and biliary and urinary tract infection. Bacteremia usually occurs in 3 to 10% of *Salmonella enterica* infections and susceptible individuals have a higher risk for developing bacteremia (Fleckenstein and Patel, 2005; Public Health Agency of Canada, 2011; Ryan, 2004). The infective doses of typhoidal and non-typhoidal *Salmonella* are approximately 5 and 3 $\log_{10}$ CFU/ml, respectively. However, pregnant women, elderly and immunocomprised persons may become infected with a lower infective dose (Fleckenstein and Patel, 2005; Ryan, 2004). The mortality rate of non-typhoidal *Salmonella* infection is < 2%; however, in developing countries, the mortality rate can reach as high as 24% (Chimalizeni et al., 2010; Public Health Agency of Canada, 2011).

In recent years, the incidence of salmonellosis has increased worldwide and internationally *Salmonella* is considered to be one of the most common causes of foodborne illness. It is believed that the increased consumption of poultry meat and table eggs has contributed to this increase in salmonellosis (Foley et al., 2011). Worldwide annual typhoidal and non-typhoidal illnesses were estimated at 17 million and 1.3 billion cases resulting in 3 million and 500,000 deaths, respectively (Fleckenstein and Patel, 2005; Chimalizeni et al., 2010). Greig and Ravel (2009) noted there were 1918 non-typhoidal salmonellosis outbreaks (47% of total outbreaks) reported internationally from 1988 to 2007. In the US, Scallan et al. (2011) reported that more than 1.2 million cases of salmonellosis with 452 deaths occur annually. However, in another study, Scharff (2010) estimated that 1.6 million salmonellosis cases occur in the US every year.

Although salmonellosis in the EU decreased by 32% compared with the numbers reported in 2008; 91,034 confirmed salmonellosis illnesses with 61 deaths were reported in 2012 (EFSA and ECDC, 2014). In Canada, *Salmonella* is the third leading bacterial pathogen and accounts for 87,510 foodborne illnesses every year (Thomas et al., 2013). In 2008 the incidence of laboratory-confirmed salmonellosis was 223 cases per million population (Public Health Agency of Canada, 2010).

Salmonella is widespread in the environment and is commonly found in farm effluents and human sewage. Domestic animals infected by *Salmonella* can become asymptomatic carriers which creates a challenge for the food animal industry in its attempts to reduce meat contamination. The consumption of undercooked meat products has been recognized as a major risk factor for infection by *Salmonella* (Humphrey and Jorgensen, 2006). Recent multistate salmonellosis outbreaks that have been reported in the US are shown in Table 2.1. The organisms are also problematic in the food industry, internationally. Panisello et al. (2000) reported *Salmonella* spp. were responsible for 67% of foodborne outbreaks associated with poultry in England and Wales from 1992 to 1996. In the EU, the prevalence of *Salmonella* in 51,093 fresh broiler meat units was 4.1 % (EFSA and ECDC, 2014). Bohaychuk et al. (2006) found *Salmonella* in 30% of raw chicken legs for sale at retail in Edmonton, AB, Canada.

Table 2.1: Salmonellosis outbreaks linked to poultry or poultry meat products in the US from 2011 to 2014. (CDC, 2014a)

***Salmonella* spp.**	**Year**	**Cases (deaths)**	**Vehicle**
S. Infantis, *S.* Newport, *S.* Hadar	2014	363	Live poultry
S. Heidelberg	2014	9	Chicken
S. Heidelberg	2013	634	Chicken
S. Typhimurium	2013	356	Live poultry
S. Infantis, *S.* Lille, *S.* Newport, *S.* Mbandaka	2013	158	Live poultry
S. Heidelberg	2013	134	Chicken
S. Hadar	2012	46	Live poultry
S. Montevideo	2012	93 (1)	Live poultry
S. Infantis, *S.* Newport, *S.* Lille	2012	195 (2)	Live poultry
S. Heidelberg	2011	190	Chicken Livers
S. Heidelberg	2011	136 (1)	Ground Turkey
S. Altona, *S.* Johannesburg	2011	68	Live poultry
S. Hadar	2011	12	Turkey Burgers

2.2.1.3 *Listeria monocytogenes*

L. monocytogenes is Gram-positive, motile, facultatively anaerobic, non-sporeforming, rod-shaped, and has a diameter of 0.5 μm and is 0.5-2 μm in length. The genus *Listeria* contains 9 other species; *L. innocua, L. welshimeri, L. seeligeri, L. ivanovii, L. grayi, L. marthii* and *L. rocourtiae, L. weihenstephanensis,* and *L. fleischmannii* (Halter et al., 2013; Bertsch et al., 2013; Graves et al., 2010; Leclercq et al., 2009). However, only *L. monocytogenes* and *L. ivanovii* are considered pathogenic (Dussurget, 2008). *L. monocytogenes* represents a major concern with regards to food safety due to its ability to grow over a wide range of environmental conditions. It can tolerate extreme pH values, low temperatures and high NaCl concentrations. The organism is psychrotrophic and can grow at 0.5 to 45 °C with an optimum temperature range of 30 to 37 °C. It grows from pH 4.3 to 9.6 with optimal growth at pH 7.0 and can also grow at 10% NaCl (Dortet et al., 2009; Dussurget, 2008; Lado and Yousef, 2007). In addition, *L. monocytogenes* is able to form biofilms on surfaces of stainless steel, rubber and plastics which may protect the organism from environmental stresses and increase its resistance to cleaners and sanitizers that are used in the food industry (Oliveira et al., 2010; Gandhi and Chikindas, 2007).

L. monocytogenes is an opportunistic foodborne pathogen that can cause severe disease in humans. Symptoms of listeriosis can vary from constipation, cramps, diarrhea, headache, persistent fever, vomiting and flu-like illness (e.g., chills, fatigue, muscle and joint pain) to severe complications leading to meningitis, septicemia, spontaneous abortion or infection of the newborn and/or death (FAO/WHO, 2004). *L. monocytogenes* has remarkable ability to invade and replicate in phagocytic and epithelial cells of the host using various sensors. Internalized bacteria lyse the enclosing vacuole and are released in the cytoplasm where they replicate and polymerize actin to move within and to neighboring cells and are internalized in a double

membrane vacuole that is also lysed and a second infectious cycle can begin (Dussurget, 2008). The infective dose of *L. monocytogenes* is still unknown but it was estimated to be approximately > 3 $\log_{10}$ CFU for healthy individuals and 2 to 3 $\log_{10}$ CFU in susceptible individuals (Drevets and Bronze, 2008). Unlike other foodborne illnesses, the incubation period for listeriosis can be long and ranged from 1 to 67 d (Goulet et al., 2013).

Although listeriosis outbreaks are rare, the case-fatality rate is high, at approximately 20-30% (FAO/WHO, 2004). Scallan et al. (2011) estimated that *L. monocytogenes* causes 1662 illnesses with 19% deaths annually in the US. Similarly, in the EU 1642 listeriosis illnesses were reported in 2012 with a mortality rate of 17.8% (EFSA and ECDC, 2014). Generally, the incidence of human listeriosis ranges from 1 to 13 reported cases per million population (Todd and Notermans, 2011). In Canada, the incidence rate of listeriosis increased from 1.8 cases in 1996 to 4.2 cases per million population in 2007 and this was followed in 2008 by a further increase to 7.2. This was largely attributed to the national outbreak of listeriosis linked to deli-meat in Canada which resulted in 57 illnesses with 24 (~40%) deaths (Clark et al., 2010; Farber et al., 2011). At least 9 listeriosis outbreaks (Table 2.2) have been documented in Canada from 1981 to 2008 (Clark et al., 2010; Farber et al., 2011).

L. monocytogenes has been isolated from a range of environments including soil, sewage, silage, water, waste effluents and feces of humans and animals such as cattle, goats, sheep and poultry (Farber and Peterkin, 1991; Jeyaletchumi et al., 2010). *L. monocytogenes* is commonly found in fish, dairy and meat processing plants (Fox et al., 2010). Although *L. monocytogenes* has been isolated from all food categories (Dortet et al., 2009), the greatest number of listeriosis outbreaks have been associated with refrigerated, ready-to-eat foods (Clark et al., 2010; Farber et al., 2011; White et al., 2002). Cooked, ready-to-eat meats including sausages, paté, rillettes, hot

dogs, ham, salami, chicken wraps and deli turkey breast have been associated with listeriosis. Cooked ready-to-eat foods with a long shelf-life are vulnerable because of the ability of *L. monocytogenes* to grow at refrigerator temperature. While cooking of meats readily eliminates *L. monocytogenes* in meat, its presence in the products is primarily due to post-processing contamination. Glass and Doyle (1989) studied the behavior of *L. monocytogenes* on processed vacuum-packed meat products including ham, bologna, weiners, sliced chicken, sliced turkey, fermented semi-dry sausage, bratwurst and cooked roast beef during storage at 4.4 °C. The organism grew extensively on bratwurst, chicken and turkey products, with a 3.5 to 5 $\log_{10}$ CFU/g increase observed by 4 weeks compared to < 3 $\log_{10}$ CFU/g increase on the other products tested. Therefore, the FDA has maintained a policy of "zero-tolerance" for *L. monocytogenes* in ready-to-eat foods (FDA, 2003). In other countries, including Canada and the EU, a limit of 2 $\log_{10}$ CFU/g *L. monocytogenes* cells is permitted for ready-to-eat foods that do not support growth of *L. monocytogenes* (EFSA, 2014; Health Canada, 2011c).

In 2000, an outbreak of listeriosis in 11 US states which consisted of 30 illnesses with 4 deaths and 3 miscarriages was reported. Processed turkey meat was implicated as a source of the outbreak and the company involved recalled 16 million pounds of processed meat (Olsen et al., 2005). In the US in 2002, processed turkey meat contaminated with *L. monocytogenes* was responsible for another multistate outbreak where there were 54 illnesses with 8 deaths and 3 miscarriages (Gottlieb et al., 2006). The Food Safety and Inspection Service in the US reported 890 meat and poultry recalls involving nearly 323 million pounds of products from 1994 to 2009. *L. monocytogenes* was associated with 266 (30%) of the recalls which involved more than 147 million pounds (45% of all recalled meat by weight). Poultry and processed poultry products

were associated with 21.3% of recalls (24.4% of meat recalled by weight) (Brougher and Greene, 2011).

Table 2.2: Listeriosis outbreaks in Canada from 1981 to 2008.

Location (year)	**Cases (deaths)**	**Vehicle**	**References**
Nova Scotia (1981)	41 (17)	Coleslaw	Schlech et al. (1983)
Ontario (1996)	2	Imitation crab meat	Farber et al. (2000)
British Columbia and Manitoba (2000)	8	Not determined	Clark et al. (2010)
Manitoba (2001)	25	Soft cheese	Pagotto et al. (2006)
British Columbia (2002)	47	Soft cheese	Pagotto et al. (2006)
British Columbia (2002)	86	Soft cheese	Pagotto et al. (2006)
Quebec (2002)	17	Heat-treated cheese	Government of Canada (2009)
Quebec (2008)	38 (2)	Soft cheese	Government of Canada (2009)
Alberta, British Columbia, Manitoba, New Brunswick, Ontario, Quebec and Saskatchewan (2008)	57 (24)	Deli-meat	Farber et al. (2011)

2.3 Natural antimicrobials

Natural antimicrobials are substances that are naturally present in food or purposefully added to food products as ingredients, or incorporated in food packaging and contact surfaces, or food processing environments to inhibit pathogenic or spoilage microorganisms. The increased demand to ensure food safety and nutritional and quality attributes of food (flavor, odor, color, texture and nutritional value) has increased the interest to use natural preservatives derived from plants, animals or microbial sources to inactivate microorganisms in foods. For example, lactoperoxidase from milk, lysozyme from egg white, saponins and flavonoids from herbs and spices, bacteriocins from lactic acid bacteria (LAB), and chitosan from shrimp shells (Davidson et al., 2013; Holley and Patel, 2005; Tajkarimi et al., 2010; Tiwari et al., 2009) have useful antimicrobial properties and can be used in foods as natural antimicrobials. A natural antimicrobial should be effective at low concentrations with low cost, be organoleptically acceptable, active against a wide range of pathogenic and spoilage organisms and non-toxic (Davidson et al., 2013; Holley and Patel, 2005).

It has been estimated that there are 250,000 to 500,000 plant species present on earth, however; only 10% of these have been studied for their potential use in medicine or food (Borris, 1996). Plant extracts with antimicrobial properties have shown considerable promise in a range of applications in the food industry. In addition, several plant extracts are categorized in the US as Generally Recognized As Safe (GRAS) (Negi, 2012). Herbal plants and their derived essential oils contain high levels of secondary metabolites (pro- or post-inhibitins) that may eliminate or inhibit growth of bacteria, yeast, and molds. Pro-inhibitins are preformed antimicrobial compounds which naturally exist in plant tissues such as polyphenols, flavonoids, tannins, alkaloids, terpenoids, or lectins, while post-inhibitins are antimicrobial metabolites synthesized

by plants in response to microbial infections. These include sulphoxides in onion and isothiocyanates in mustard which are stored in the form of inactive precursors (glucosinolates) and can be activated by hydrolases or oxidases (Holley and Patel, 2005). Glucosinolates in mustard can be converted by naturally occurring plant myrosinase in the presence of moisture to yield isothiocyanates (ITCs) which are strong antimicrobials (Holley and Patel, 2005). Besides their antimicrobial activity, plants also may be used for different purposes to enhance human health, acting as traditional medicines as well as functional foods and dietary supplements (Negi, 2012). Spices including parsley, black pepper, garlic, nutmeg and mustard are used in processed meats to improve flavor and enhance the aroma of finished products, as well as for their antimicrobial and antioxidant effects (Brown, 2009).

2.4 Oriental mustard

There are three botanical types of mustard: yellow, brown (black) and Oriental. Yellow mustard is the most common mustard variety planted in North America. Brown and Oriental mustard varieties are grown on limited acreage and have lower seed yields than yellow mustard. Canada is the world's leading exporter of mustard seed, producing 43% of the mustard seed traded internationally. World mustard seed production increased by 66% from 1993 to 2004 and reached 703,000 metric tons in 2004 (Smith and Jimmerson, 2005). Development of a spicy flavor from hot mustard is the limiting factor in the use of mustard in food systems; however, thermal treatment produces deodorized (cold, de-heated) mustard which has a bland flavor without spiciness (but still contains intact glucosinolates) and this enables the use of mustard in food systems as a thickener, stabilizer, emulsifier and antioxidant (Luciano et al., 2011). Mustard has functional characteristics including oil-in-water emulsification, absorption of liquids (including oil), prevention of phase separation, prevention of syneresis, flavor enhancement,

increased water and fat binding capacity, enables cost reduction and label simplification, has no cholesterol, yields improvement in color, stability and viscosity plus acts as a natural antioxidant (Minn-Dak Growers Ltd, 2002).

Mustard contains high levels of glucosinolates which have antioxidant and antimicrobial properties when hydrolyzed by thioglucoside glucohydrolase (myrosinase EC.3.2.1.147) in the presence of water to form glucose and an unstable intermediate, thiohydroximate-*O*-sulfonate which undergoes further transformation and yields primarily ITC, nitrile and thiocyanate, but epithionitrile, oxazolidine-2-thione and other less common products can be formed, depending upon the nature of the glucosinolate and the conditions during its hydrolysis (Fig. 2.1) (Fahey et al., 2001; Rask et al., 2000; Vig et al., 2009).

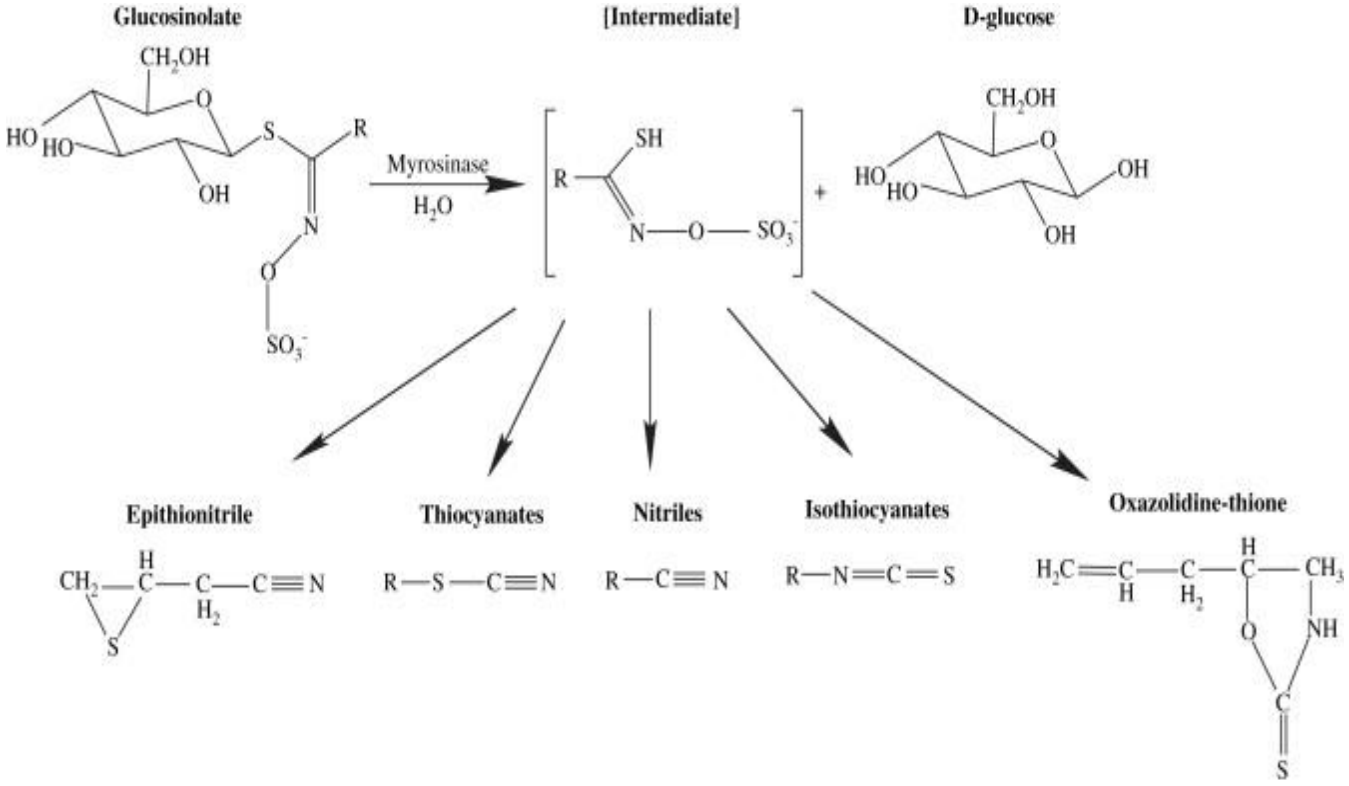

Fig. 2.1: General outline of glucosinolate degradation by myrosinase (Vig et al., 2009)

Glucosinolate metabolites (especially the ITCs) are antimicrobial against a number of pathogens. A series of studies found that mustard powder is effective against *E. coli* O157:H7 in meat products (Cordeiro et al., 2013; Graumann and Holley, 2008; 2009; Luciano et al., 2011; Nadarajah et al., 2005; Nilson and Holley, 2012). Graumann and Holley (2009) found that incorporation of 6% non-deheated (hot or spicy) mustard powder in dry-cured Westphalian ham reduced numbers of *E. coli* O157:H7 by 5 $\log_{10}$ CFU/g during 45 d compared to the control which required 80 d for the same reduction. Nadarajah et al. (2005) found that 5%, 10% and 20% hot mustard flour reduced *E. coli* O157:H7 numbers after 21 d by 0.5, 3.0 and 5.4 $\log_{10}$ CFU/g, respectively, in ground beef under nitrogen-flushed packaging. Luciano et al. (2011) reported that 6% commercially deodorized, hot or autoclaved mustard flour reduced numbers of *E. coli* O157:H7 in dry fermented sausage by 5 $\log_{10}$ CFU/g after 38, 31 and 18 d processing, respectively. In another study, Graumann and Holley (2008) found that hot mustard powder at 2, 4, and 6% in dry sausage reduced *E. coli* O157:H7 levels by 3.4, 4.4, and 6.9 $\log_{10}$ CFU/g, respectively, within 30 d of ripening. They also reported that 6% deodorized mustard reduced numbers of *E. coli* O157:H7 to undetectable levels (<0.20 $\log_{10}$ CFU/g) within 24 d. The unexpected results in the last two studies showed that autoclaved or cold mustard caused more rapid reductions in numbers of *E. coli* O157:H7 in dry fermented sausage, even though the plant myrosinase (which normally degrades the glucosinolates and forms ITC) was inactivated. Luciano and Holley (2010; 2011) explained how the cold or deodorized mustard was able to kill *E. coli* O157:H7. They found that *E. coli* O157:H7, *Staphylococcus carnosus* and *Pediococcus pentosaceus* were able to degrade sinigrin and sinalbin (from deodorized mustard) and form isothiocyanates. Herzallah et al. (2011) also found that other microorganisms including *L.*

monocytogenes, *Enterococcus faecalis*, *Staphylococcus aureus* and *S.* Typhimurium converted sinigrin into allyl isothiocyanate (AITC) by myrosinase-like activity(ies).

In the presence of myrosinase, hydrolysis of the glucosinolate, sinigrin, in brown and Oriental mustard forms AITC, while hydrolysis of the glucosinolate, sinalbin, in yellow mustard produces ρ-hydroxybenzyl isothiocyanate (ρ-HBITC) which is antimicrobial but unstable in aqueous solutions and forms ρ-hydroxybenzyl alcohol (ρ-HBA) and ρ-hydroxybenzyl cyanide (ρ-HBC) which are only weakly antimicrobial, as well as SCN- which has no inhibitory activity against *E. coli* O157:H7. In contrast, AITC was effective against *E. coli* O157:H7 (Luciano and Holley, 2010; 2011). Based on these studies, brown or Oriental mustard could be expected to be more effective than yellow mustard in elimination of foodborne pathogens. Lara-Lledó et al. (2012) found that a polymeric film containing Oriental mustard extract with 5% (w/w) sinigrin was more inhibitory than a film containing yellow mustard with 6% (w/w) sinalbin against *L. monocytogenes* on vacuum-packed bologna slices at 4 ºC. But when yellow and Oriental mustard powder were incorporated into sausage at the same concentration, yellow mustard was more inhibitory against *E. coli* O157:H7 (Cordeiro et al., 2014b). This was attributed to the lower concentrations of sinigrin in Oriental mustard (0.8%) than levels of sinalbin (2.5%) in yellow mustard (Zrybko et al. 1997) which would yield less AITC than ρ-HBITC. It should be noted that Oriental and yellow mustards also have high phenolic contents which may be responsible in part for their antimicrobial activity, and preliminary work showed that the total phenolic content of yellow mustard was > 2-fold greater than in Oriental mustard (Wu, 2013).

Factors including the amount, type and composition of mustard powder, type of microorganism, substances which are added to processed products (proteins, lipids, salts, and phenols), pH, iron concentration and storage temperature could influence the antimicrobial

outcome. For example, it has been reported that 10% or 20% (w/v) mustard in combination with 1% (v/w) acetic acid had greater antimicrobial effects against *E. coli* O157:H7, *L. monocytogenes*, and *S.* Typhimurium compared with mustard alone when stored at 5 or 22 ºC (Rhee *et al*., 2002; 2003). Luciano and Holley (2009) reported that AITC was more effective as an antimicrobial against *E. coli* O157:H7 at low pH. The minimum inhibitory concentration of AITC at pH 4.5 and 5.5 was 25 µl/l compared to 500 µl/l at pH 8.5. Lemay et al. (2002) reported that essential oil of mustard had good antimicrobial efficacy against *E. coli* after 2 or 7 d in cooked, acidified chicken meat (pH 5.0).

2.5 Allyl isothiocyanate

AITC is a natural colorless and volatile compound which contains an organosulfur group (CH_2CHCH_2NCS) and can be formed when myrosinase hydrolyzes the glucosinolate, sinigrin, in damaged tissues of *Brassicaceae* members which include mustard, horseradish, cabbage, wasabi and others (Fenwick et al., 1982). Of 27 essential oils tested, only garlic and horseradish oils were able to inhibit the growth of all tested organisms including *Pseudomonas aeruginosa, L. monocytogenes, S. aureus, E. coli,* and *S.* Enteritidis. Horseradish oil also had the greatest inhibitory effect against the tested organisms compared to the other essential oils (Nedorostova et al., 2009). The antimicrobial activity of horseradish oil is largely due to AITC which is the major component of its ingredients (Matan et al., 2006).

2.5.1 Food applications

AITC from natural sources is presently used as a food preservative in Japan, and as a GRAS flavoring agent in the US (Kim et al., 2002). In addition to its activity as an anti-cancer agent (Xiao et al. 2003; Zhang, 2010), AITC showed potent antimicrobial activity against

spoilage bacteria and foodborne pathogens *in vitro* (Dufour et al., 2012; Luciano et al., 2011; Wilson et al., 2013; Zou et al., 2013) and in food products. In fermented sausages, AITC at 750 and 1000 ppm reduced numbers of *E. coli* O157:H7 by 6.5 $\log_{10}$ CFU/g after 21 and 16 d processing, respectively. Formulation of dry sausage with 500 ppm AITC reduced *E. coli* O157:H7 by 4.75 $\log_{10}$ CFU/g after 28 d processing, and the organism was not recovered from this product after 40 d (Chacon et al., 2006). The effect of AITC against *E. coli* O157:H7 in packaged (100% N_2) ground beef stored at 4, 10 or -18 ºC using filter paper inserts wetted with 0.5 ml or 1 ml of an AITC solution (AITC:corn oil = 7:3 v/v) was studied by Nadarajah et al. (2005). They found that AITC reduced *E. coli* O157:H7 numbers by 3.0 $\log_{10}$ CFU/g after 21 d at 4 ºC and 1.0 $\log_{10}$ CFU/g after 8 and 35 d at 10 or -18 ºC, respectively; with final AITC concentrations in the package headspaces of 444, 456 and 112 µg/ml at 10, 4, and -18 ºC after 8, 21, and 35 d, respectively. In another study, incorporation of 400 ppm AITC in dry-cured Westphalian ham reduced numbers of *E. coli* O157:H7 by 5 $\log_{10}$ CFU/g in 45 d compared to the control which required 80 d for the same reduction (Graumann and Holley, 2009). The vapor form of AITC is more antimicrobial than liquid AITC. AITC vapor at a release rate of 1.2 µg/h reduced *L. monocytogenes* and *S.* Typhimurium in modified-atmosphere packaged fresh chicken by 0.77 and 1.3 $\log_{10}$ CFU/g, respectively, by 21 d storage at 4 ºC (Shin et al., 2010).

Low concentrations of AITC showed substantial ability to control foodborne pathogens on fresh produce. AITC at 60 µl/ml incorporated in chitosan coatings reduced *Salmonella* on cantaloupe surfaces by > 5 $\log_{10}$ CFU/cm^2 at 24 h and room temperature (Chen et al., 2012). AITC vapor at 8.0 µl/l of air reduced *E. coli* O157:H7 by > 4.0 $\log_{10}$ CFU/leaf by 4 d on intact or damaged lettuce stored at 0 and 4 ºC. In other research, 16 µl/l AITC was required to reduce *E. coli* O157:H7 numbers by > 4.0 $\log_{10}$ CFU/leaf on intact spinach at 0 and 4 ºC and by 2.6 or 3.4

$\log_{10}$ CFU/leaf on damaged spinach at 0 and 4 ºC, respectively (Obaidat and Frank, 2009a). In another study, AITC vapor at 8.3 µl/l of air showed higher activity than cinnamaldehyde and carvacrol against *Salmonella* and *E. coli* O157:H7 on sliced and whole tomatoes (Obaidat and Frank, 2009b). AITC reduced *Salmonella* and *E. coli* O157:H7 on sliced tomatoes by 1.0 to 3.5 $\log_{10}$ CFU at 4 or 10 ºC after 10 d. On whole tomato surfaces, *Salmonella* and *E. coli* O157:H7 numbers were reduced by > 5.0 and 3.0 $\log_{10}$ CFU/tomato, respectively, at 4 ºC or by 2.2 and 1.0 $\log_{10}$ CFU/tomato, respectively, at 10 ºC.

Polylactic acid with 500 µl AITC coated on glass jars reduced viable *Salmonella* in a cocktail mixture in liquid egg white by 3.0 and 7.0 $\log_{10}$ CFU/ml after 7 and 21 d, respectively, at 10 ºC (Jin and Gurtler, 2011). In addition, polylactic acid polymer or chitosan coatings with 60 µl/ml AITC reduced viable *Salmonella* in a cocktail on egg shells by 1.2 and 1.7 $\log_{10}$ CFU/cm^2, respectively, at 22 ºC (Jin et al., 2013). Further, AITC has been used to control other pathogenic and spoilage organisms in several food products, for example inactivation of yeasts and molds on cheese (Gonçalves et al., 2009; Winther and Nielsen, 2006), *Vibrio parahaemolyticus* on tuna meat (Hasegawa et al., 1999), *P. aeruginosa* on fresh catfish fillets (Pang et al., 2013), *L. innocua* on frozen shrimp (Guo et al., 2013), lactic acid bacteria and yeasts on acidified cucumbers (Pérez-Diaz and McFeeters, 2010), fungi on fruits (Mari et al., 2008; Wu et al., 2011), total aerobic bacteria on cooked rice (Kim et al., 2002) and *Leuconostoc* and *Lactobacillus* on Kimchi, a traditional Korean fermented vegetable (Ko et al., 2012).

2.5.2 Mode of action

The mode of action of AITC is related to its chemical structure, since the central carbon atom of ITC (R−N=C=S) is highly electrophilic and reacts readily under mild conditions with

oxygen, sulfur or nitrogen (Hyldgaard et al., 2012). The antimicrobial activity of AITC may be related to several simultaneous intracellular actions. AITC has the ability to inactivate sulphydryl-enzymes by oxidative cleavage of disulfide bonds and this was shown when AITC at 10 to 100 µl/l significantly inhibited thioredoxin reductase and acetate kinase enzymes from *E. coli* which play important roles in cell growth and proliferation (Luciano and Holley, 2009). AITC also penetrates the plasma membrane to reach the cytoplasm of bacterial cells. Therefore, AITC may cause DNA damage by generating reactive oxygen species intracellularly which are converted to H_2O_2 by superoxide dismutase metabolism and yield DNA damage (Yonezawa et al., 1999).

AITC may change intracellular structures and affect the cellular energy generating processes. This was evident when it damaged the cell membranes of *E. coli* and *S.* Montevideo which led to leakage of cellular metabolites (Lin et al., 2000) and reduced the intracellular ATP concentrations of *L. monocytogenes* by 33.6-fold compared to untreated cells (Ahn et al., 2001). The combination of nisin and AITC changed the membrane fatty acid composition, increased membrane depolarisation and reduced the cytoplasmic particle size causing low granularity in *L. monocytogenes*, *S. aureus*, *S* Typhimurium and *Shigella boydii*. These changes increased membrane fluidity and blocked metabolic processes (such as electron transport, nutrient import, energy generation, macromolecular synthesis and signal transduction), leading to leakage of intracellular components through loss of cell membrane integrity and eventually caused the loss of cell viability (Zou et al., 2013). Recently, the mechanism of action of AITC was examined using *E. coli* HB101_pUCD607_*lux* (a complementary bioluminescence-based biosensor). The availability of intracellular ATP and/or NADPH in AITC-treated samples was <10% compared to healthy control samples (100%). NADPH is a coenzyme that transports chemical energy

within cells necessary for metabolism and are required in many vital cell processes. The significant decrease in ATP and NADPH observed within treated cells was due to substantial impairment of metabolism and the disruption of the barrier function of the cell membrane (Chan et al., 2013).

2.5.3 Bacterial resistance mechanisms

The mechanism used by rat cells to tolerate exposure to ITCs was related to the degradation of these compounds via conjugation with glutathione by glutathione-*S*-transferase (GST), and their transformation to cysteinyl glycine and cysteine by gamma glutamyl transpeptidase (GGT) (Brusewitz et al., 1977). This mechanism also was found in cyanobacteria where GSTs from *Thermosynechococcus longates* BP-1 and *Synechococcus longates* PCC 6301 were able to catalyse the conjugation of AITC and other ITCs to glutathione at high rates (Wiktelius and Stenberg, 2007). Sellam et al. (2006) found that ITCs differentially stimulated expression of the gene *AbGst1* encoding a glutathione transferase in *Alternaria brassicicola*, exhibiting high transferase activity, which increased resistance to ITCs. Recombinant *AbGst1*p expressed in *E. coli* exhibited high transferase activity with AITC and benzyl isothiocyanate (BITC) as substrates. On the other hand, the resistance of C. *jejuni* strains to AITC and BITC was not correlated with the presence of a GGT encoding gene, antibiotic resistance or the origin of the biological sample. However a *ggt* mutant of *C. jejuni* displayed decreased survival compared to the wild-type when exposed to ITCs (Dufour et al., 2012). These results suggest that ITC resistance probably depends on several factors; however, it is clear that GGT is involved in ITC resistance of some organisms.

Efflux systems may also be another mechanism used by bacteria to tolerate ITC exposure. Zou et al. (2012) studied the expression of efflux-related genes (*acrA, acrB, ompD* and *tolC*) in *S.* Typhimurium exposed to AITC and they found that the relative expression levels of *acrA* and *acrB* genes were increased 1.31- and 1.61-fold when *S.* Typhimurium cells were treated with AITC at 0.69 mg/ml, while the *ompD* and *tolC* genes were down-regulated. BaeSR is a two-component system which consists of an inner-membrane-bound sensor histidine kinase (BaeS), and a cytoplasmic response regulator (BaeR) which are responsible for an efflux pump in *E. coli* O157:H7 (Raffa and Raivio, 2002). Cordiero et al. (2014a) found that the MICs of AITC with *baeS* or *baeR* deletion mutants of *E. coli* O157:H7 were similar to the MIC of the wild-type strain, but colony numbers were lower than those of the wild-type strain at the MIC or higher concentrations of AITC. Furthermore, the MIC and MBC of AITC against a *baeSR* deletion mutant of *E. coli* O157:H7 were 26 and 206 ppm, respectively, compared to 51 and 412 ppm, respectively, against the wild-type *E. coli* O157:H7 strain. This suggested some involvement of the BaeSR efflux system in ITC tolerance by this organism.

2.6 Organic acids

Although microorganisms have specific minimum, maximum and optimum pH values for growth, in general, there is a preference for environments with neutral pH (6.5 to 7.5). Therefore, increases in the acidity of foods by the addition of organic acids or enhancing natural fermentations can reduce the number of undesirable bacteria. However, success depends on the species of unwanted microorganism, the type and concentration of organic acid, time of exposure, the buffering capacity of food, and other environmental conditions including temperature and initial pH (Doores, 2005).

Organic acids are weakly dissociated in a pH-dependent manner. Therefore, their antimicrobial activity depends on the dissociation constant (pKa) of the acid, that is, the pH at which 50% of the acid is dissociated. Since the un-dissociated form of the molecule is responsible for its antimicrobial activity, it is advantageous to use them near their pKa value, which ranges from pH 3.0 to 5.0 (Doores, 2005; Mani-López et al., 2012). Consequently, decreasing the pH increases the proportion of protonated acid, decreasing its polarity and increasing its diffusion across the membrane into the bacterial cytoplasm where it dissociates at the higher cytoplasmic pH (Mani-López et al., 2012; Taylor et al., 2012b). The antimicrobial activity of organic acids results from acidification of the cytoplasm with subsequent reduction of energy production and its regulation and/or accumulation of the dissociated acid anion to toxic levels (Taylor et al., 2012b). Antimicrobial activity of organic acids increases with increasing the carbon chain length up to 12 carbons. For example, acetic acid and sorbic acid have similar pKa values but showed different inhibitory effects. This was attributed to the longer carbon chain in sorbic acid which enhances its lipophilicity (its partitioning between an organic solvent and water) and consequently sorbic acid has a higher affinity for the hydrophobic components in the cell membrane (Ullah et al., 2012; van Beilen et al., 2014).

Acetic acid is formed as a metabolic by-product of *Acetobacter* spp. which are found naturally in fermented products, such as pickles and sauerkraut and the organisms are used to make vinegar because they are tolerant to this acid. Acetic acid is monocarboxylic with a pungent odor and taste, which limits its use in food systems. Since it is highly soluble in water, it can be used as a flavoring agent and is essential in formulations of condiments such as mustard, ketchup, salad dressings, mayonnaise and pickled products including sausages (Doores, 2005; Mani-López et al., 2012). Malic acid is dicarboxylic with a high water solubility and strong acid

flavor, but it does not have the same residual acid taste as other organic acids. It is naturally found in apples, pears, plums, and cherries and is used as a food additive in jams, jellies, candies and beverages to give a sour taste. Malic acid has two pKa values which are 3.40 and 5.11 because it is dicarboxylic (Doores, 2005).

In addition to their antimicrobial effectiveness, other beneficial secondary characteristics of organic acids influence their value to the food industry. Use of organic acids in the meat industry is attractive because of their natural origin, antimicrobial and antioxidant effects, flavoring properties and low cost. Furthermore, organic acids were classified by the FDA as generally recognized as safe (GRAS) for meat products (Doores, 2005; Mani-López et al., 2012).

Organic acids have been used in poultry processing to reduce microbial contamination. In general, their use at 1 to 3% as aqueous solutions in meat products reduced bacterial numbers by 1 to 2 $\log_{10}$ CFU without negative sensory changes (Smulders and Greer, 1998). In poultry scald water at 52 ºC, 0.1% acetic acid decreased levels of *S.* Typhimurium and *C. jejuni* by 0.5 to 1.5 $\log_{10}$ CFU/g. At a level of 1.0%, acetic acid caused instantaneous bacterial death (Okrend et al., 1986). Dipping chicken legs in 2% malic acid or acetic acid solutions for 5 min reduced numbers of *L. monocytogenes* by 1.1 $\log_{10}$ CFU/g after 6 d at 4 ºC (González-Fandos and Herrera, 2013; 2014).

2.7 Ethylenediamine tetraacetic acid

Ethylenediamine tetraacetic acid (EDTA) is a synthetic compound used in a wide range of applications. In food products, it is used as a chelating agent to prevent oxidation, rancidity, discoloration and off-flavor as well as to stabilize vitamins and minerals (Ko et al., 2010; Wreesmann, 2014). The maximum acceptable daily intake (ADI) of EDTA in the US is 1.9 mg/d

per kilogram bodyweight (Wreesmann, 2014). Calcium and disodium EDTA have been approved as GRAS in the US to be used in food to maintain color, flavor, and texture (FDA, 2011). EDTA inhibits bacterial growth by chelating the major cations involved in membrane stability and bacterial growth including Mg^{2+}, Ca^{2+}, and Fe^{2+} (Ko et al., 2010; Vaara, 1992). Furthermore, EDTA has the ability to enhance the efficacy of antimicrobials and antibiotics, particularly against Gram-negative bacteria. Hydrophobic antimicrobials fail to penetrate the outer membrane of Gram-negative bacteria due to the presence of the outer lipopolysaccharide (LPS) layer. The use of EDTA permeabilizes the outer membrane by chelation of Mg^{2+}, Ca^{2+}, and Fe^{2+} which are involved in LPS–LPS and LPS–protein interactions and thus destabilizes the outer membrane (Alakomi et al., 2000; 2003; Rowbury, 2011).

2.8 Edible film and coatings

Edible coatings are food-grade solutions which can be applied to food by spraying, spreading or dipping to form a thin layer on the food surface after drying, while edible films are obtained from food grade "filmogenic" materials that are usually cast over a surface, dried and then placed in contact with food surfaces (Han and Gennadios, 2005; Sánchez-Ortega et al., 2014). Edible films and coatings provide a novel way to improve the safety and shelf-life of food by serving as selective barriers to moisture transfer, oxygen uptake, lipid oxidation, and loss of volatile aromas and flavors (Kester and Fennema, 1986).

The major film and coating-forming materials are proteins, polysaccharides and lipids which can be used alone or in combination. Polysaccharide films and coatings can be prepared from cellulose, starch, pectin, seaweed extracts (alginates, carrageenan, and agar), gums (acacia, tragacanth, and guar), pullulan and chitosan (Han and Gennadios, 2005; Sánchez-Ortega et al.,

2014). Chitosan is a derivative of chitin and has good film-forming and biocompatibility properties when combined with other polar compounds due to its high densities of amino and hydroxyl groups (Chen et al., 2012). Chitosan-based films and coatings were the most commonly applied types used on meat products in recent years (Sánchez-Ortega et al., 2014). They have been used with sausages (Siripatrawan and Noipha, 2012), roast beef (Beverlya et al., 2008), sliced turkey (Guo et al., 2014; Jiang et al., 2011), chicken breast fillets (Higueras et al., 2013; Petrou et al., 2012) and fish (Günlü and Koyun, 2013; Gómez-Estaca et al., 2010).

In addition to its film-forming properties, chitosan is an antimicrobial agent. Its antimicrobial activity is attributed to its ability to chelate ions from the LPS layer of the bacterial outer membrane, and/or cause electrostatic interactions between its groups and the negative charges of the membrane. Both mechanisms increase cell permeability and release intracellular components (Sánchez-Ortega et al., 2014). The antimicrobial activity of chitosan is affected by its type, the degree of acetylation, its molecular weight and the environmental conditions during use, including pH, temperature and the presence of interfering compounds such as protein and lipid (Davidson et al., 2013; Sánchez-Ortega et al., 2014). It has been found that a lower chitosan molecular weight and degree of acetylation yielded greater antimicrobial activity (Chung et al., 2004; Liu et al., 2001). Low molecular weight increases mobility, attraction and ionic interactions of chitosan, and consequently facilitates its binding to the cell membrane, while a low degree of acetylation increases its solubility, charge development and increases the release of free amino and hydroxyl groups present in chitosan which enhances antimicrobial activity (Goy et al., 2009).

Carrageenan is a natural, water soluble hydrocolloid composed of galactose and anhydro-galactose units linked by glycosidic bonds and is extracted from red seaweed. Carrageenan is

widely used in the food industry due to its ability to facilitate textural changes in food resulting from its gelling, thickening, emulsifying and stabilizing characteristics. It has been used to improve the texture of cottage cheese, puddings and dairy desserts, and as a binder and stabilizer in the meat processing industry for manufacturing sausages, patties and low-fat hamburgers (Li et al., 2014b). κ-Carrageenan is considered a good film-forming material. Park (1996) reported that κ-carrageenan produced a clear film with excellent mechanical and structural properties and a tensile strength higher than films prepared using other types of carrageenan. Pinheiro et al. (2012a, b) found that a combination of chitosan and κ-carrageenan showed good coating properties, had improved action as a gas barrier and facilitated delayed release of incorporated bioactive compounds.

Chapter 3

Effects of Changes in pH and Temperature on the Inhibition of *Salmonella* and *Listeria monocytogenes* by Allyl Isothiocyanate

3.1 Abstract

The minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of mustard allyl isothiocyanate (AITC) against five strains each of *Salmonella* and *Listeria monocytogenes* individually, and when combined by genus were studied in Mueller-Hinton broth at 21 or 37 ºC as well as the interactive effects of pH (5.0 to 9.0) at temperatures of 4 to 21 ºC on cell viability when held under these conditions for up to 10 d. The stability of 200 ppm AITC was monitored during these trials. The MIC and MBC values of AITC ranged from 60-100 ppm and 120-180 ppm, respectively, at 37 ºC and ranged from 10-40 ppm and 200-600 ppm, respectively, at 21 ºC against both pathogens. AITC had no antimicrobial activity at low temperatures (4 or 10 ºC) and alkaline pH over 10 d, but at neutral pH, AITC reduced *L. monocytogenes* by 4.14 and 8.45 $\log_{10}$ CFU/ml at 4 or 10 ºC, respectively. At acidic pH, AITC was more effective against *Salmonella* which was reduced by 2.56 and 6.48 $\log_{10}$ CFU/ml at 4 and 10 ºC, respectively. However, AITC was more effective at combinations of 21 ºC and neutral pH against *L. monocytogenes* (cells were not detected at and beyond 3 d) and at combinations of the higher temperature and acidic pH against *Salmonella* (cells were not detected at and beyond 6 d). Mustard AITC was more stable at low pH and temperature indicating that it can be an effective antimicrobial at combinations of low or neutral pH values and room or refrigerator temperatures (4 to 10 ºC) against these foodborne pathogens.

3.2 Introduction

Foodborne illness is a major public health concern worldwide because of its associated morbidity, unnecessary mortality and significant economic cost. In Canada, it has been estimated that 11 to 13 million cases of foodborne illnesses occur every year (Health Canada, 2011b). An updated estimate of yearly foodborne illnesses in the US by the Centers for Disease Control and Prevention (CDC) indicated that about 48 million persons endure foodborne illness with 128,000 hospitalizations and 3,000 deaths each year (Scallan et al., 2011). The European Food Safety Authority (EFSA, 2013), European Centre for Disease Prevention and Control (ECDC) reported that 5,648 foodborne outbreaks involving 69,553 illnesses, 7,125 hospitalisations and 93 deaths occurred in the European Union in 2011.

In recent years, the incidence of non-typhoidal salmonellosis has increased worldwide, and now *Salmonella* is considered to be one of the most common causes of foodborne illness internationally (FAO/WHO, 2002). *Listeria monocytogenes* is an opportunistic foodborne pathogen that can cause severe forms of infection in humans with a high mortality rate (Nørrung, 2000). Scallan et al. (2011) reported that more than 1.2 million cases of salmonellosis with 452 deaths and 1662 cases of listeriosis with 19% deaths occur in the US annually.

Salmonella can grow over a temperature range of 5 to 47 °C with optimum growth at 37 °C and a pH range from 4 to 9 with optimum growth at pH 7 (Bhunia, 2008; D'Aoust et al., 2001). *L. monocytogenes* has been shown to grow at temperatures ranging from −0.4 to 45 ºC (Junttila et al., 1988) and pH values ranging from 5 to 9 (Kallipolitis and Ingmer, 2001).

The interest in antimicrobials derived from natural sources has increased in the past few years due to the usually accepted safe status of these compounds. Spices including parsley, black

pepper, garlic, nutmeg and mustard are used in processed foods, particularly meat products, to improve flavor and enhance the aroma of finished products, as well as for their antimicrobial and antioxidant effects (Brown, 2009). Mustard contains significant levels of glucosinolates which have antioxidant and antimicrobial properties when hydrolyzed by thioglucoside glucohydrolase (myrosinase, EC.3.2.1.147) in the presence of moisture to yield isothiocyanates, nitriles, D-glucose and sulphate. Of the glucosinolate metabolites, isothiocyanates are known to be antimicrobial and exert these effects by inhibiting cellular metabolic reactions and destabilizing the bacterial cell membrane (Luciano and Holley, 2009; Vig et al., 2009; Fahey et al., 2001). Allyl isothiocyanate (AITC) is one of the most common isothiocyanates that are found in cruciferous plants either in the free form or as glucosinolates. AITC from natural sources is permitted for use as a food preservative in Japan, and as a GRAS flavoring agent in the US (Isshiki et al., 1992; Delaquis and Mazza, 1995; Kim et al., 2002). In addition to these applications it was hypothesized that AITC could be used in the treatment of human prostate cancers since it acts as a cancer chemopreventive (Zhang, 2010; Xiao et al. 2003).

Although several studies reported that AITC was effective against various foodborne pathogens such as *E. coli* O157:H7, *L. monocytogenes, S.* Typhimurium, *Bacillus cereus, Staphylococcus aureus* and *Campylobacter jejuni, in vitro* (Siahaan et al., 2013; Zou et al., 2013; Zou et al., 2012; Dufour et al., 2012; Shin et al., 2010; Lin et al., 2000) or in meat products including ground beef, fresh chicken, fermented sausage and Westphalian ham (Graumann and Holley, 2009; Luciano and Holley, 2009; Chacon et al., 2006; Nadarajah et al., 2005), its high volatility, strong odor, poor water solubility and reactions with naturally occurring food nucleophiles limit its application in food systems (Chacon et al., 2006; Kim et al., 2008). AITC is unstable in aqueous solution and is easily decomposed to new compounds, particularly under

alkaline conditions or at elevated temperature (Tsao et al., 2000; Ohta et al., 1995). Therefore, temperature and pH are the major factors influencing AITC stability and these factors also may influence its antimicrobial activity. While some work has examined the effects of pH and temperature on the antimicrobial activity of AITC (Luciano and Holley, 2009; Nadarajah et al., 2005) these studies have been highly focused and not systematic. Nadarajah et al. (2005) studied the effect of AITC against *E. coli* O157:H7 in packaged (100% N_2) ground beef stored at 4, 10 or -18 ºC using filter paper inserts wetted with 0.5 ml or 1 ml of an AITC solution (AITC:corn oil = 7:3 v/v). They found that AITC reduced *E. coli* O157:H7 numbers by 3 $\log_{10}$ CFU/g after 21 d at 4 ºC and 1 $\log_{10}$ CFU/g after 8 and 35 d at 10 or -18 ºC, respectively; with final AITC concentrations in the package headspaces of 444, 456 and 112 µg/ml at 10, 4, and -18 ºC after 8, 21, and 35 d, respectively. Luciano and Holley (2009) reported that AITC was more effective as an antimicrobial against *E. coli* O157:H7 at low pH and 37 ºC. The MIC at pH 4.5 and 5.5 was 25 µL/L compared to 500 µL/L at pH 8.5. Previous studies have described the antimicrobial activity of AITC against foodborne pathogens under different experimental conditions so it is impossible to identify an optimum environment for its antimicrobial activity. In addition, no studies have examined the interactive effects of pH and temperature on AITC stability and antimicrobial activity. This study characterized i) the minimum inhibitory and bactericidal effects of AITC against two 5 strain cocktails of *Salmonella* and *L. monocytogenes* at different temperatures, and ii) the effect of different pH and temperature combinations on stability and the *in vitro* antimicrobial activity of AITC against *Salmonella* and *L. monocytogenes*.

3.3 Materials and Methods

3.3.1 Chemicals

Allyl isothiocyanate, 94%, was purchased from Acros Organics (Morris Plains, NJ, USA). Acetonitrile, potassium hydrogen phthalate, potassium dihydrogen phosphate and sodium tetraborate, sodium hydroxide (NaOH), hydrochloric acid (HCl) were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). All chemicals were of analytical grade except for acetonitrile which was HPLC grade.

3.3.2 Preparation of buffers and chemical solutions

Buffers used in the *in vitro* study had pH values of 5.0, 7.0, and 9.0. The pH 5.0 buffer was prepared by adjusting 0.05 M potassium hydrogen phthalate with 0.10 M NaOH. The pH 7.0 buffer was prepared by adjusting 0.05 M potassium dihydrogen phosphate with 0.10 M NaOH. The pH 9.0 buffer was prepared by adjusting 0.0125 M sodium tetraborate with 0.10 M HCl. HPLC solvents used were acetonitrile and double-distilled water. Both solvents were sterilized using 0.45 mm Millipore nylon filters (Fisher Scientific, Nepean, ON, Canada), and degassed for 30 min in a Bransonic bath (Branson, 5510, Grass Valley, CA, USA) before use.

3.3.3 Bacterial strains

The 5 strains of each of *Salmonella* and *L. monocytogenes* used in this study were from the culture collection of the Food Science Department, University of Manitoba, and were: *L. monocytogenes* 2-138, 2-243, GLM-1, GLM-3, GLM-5, and *Salmonella (S)* Heidelberg 271, *S.* Typhimurium 02 8423, *S.* Copenhagen PT 99, *S.* Enteritidis CRIFS 1016, and *S.* Kentucky 64701.

3.3.4 Culture preparation

All bacterial cultures were stored in Brain Heart Infusion (BHI) Broth (Oxoid Ltd., Basingstoke, England) with 25% glycerol at -80 ºC. Strains were subcultured in BHI broth 3 times and then one loopful from the third transfer of *L. monocytogenes* strains was streaked on *Listeria* Selective agar base with *Listeria* selective supplement (LSA, Oxoid Ltd.), and *Salmonella* serovars were streaked on Xylose lysine deoxycholate (XLD) agar (Oxoid Ltd.) and incubated at 37 ºC for 24 h. A working culture was prepared by transferring a single colony of each of the five *L. monocytogenes* or *Salmonella* strains from the selective agars to BHI broth which was incubated overnight at 37 ºC. Then, 0.1 % (v/v) of this culture was transferred to fresh BHI broth and incubated overnight at 37 ºC. Either freshly prepared single cultures or mixtures of *Salmonella* or *L. monocytogenes* strains, prepared by combining equal volumes of each the 5 freshly cultured strains in a sterile container were used in the experiments.

3.3.5 MIC and MBC of AITC against *Salmonella* and *L. monocytogenes*

The MIC of AITC against *Salmonella* and *L. monocytogenes* strains was determined using Mueller-Hinton (MH) broth (pH 7.2) (Oxoid Ltd.) in 12 ml screw-capped tubes with AITC and cultures. AITC stock solutions of 8000, 2000 or 100 ppm in MH broth were prepared after initial dilution of AITC in 2% ethanol to facilitate its dispersion. Tubes containing 9.9 ml MH broth with 10 to 200 ppm AITC (for incubation at 37 ºC) or 5 to 800 ppm AITC (for incubation at 21 ºC) were prepared. Tubes were vortex-mixed and 0.1 ml of 8 $\log_{10}$ CFU/ml of stationary phase *L. monocytogenes* or *Salmonella* cultures were added to yield 6 $\log_{10}$ CFU/ml in each tube and mixed. These were prepared in triplicate for each treatment and incubated with shaking at 200 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA) for 24 h at 21 or

37 ºC. The lowest concentration at which no visible growth occurred was identified as the MIC. To determine the MBC (AITC concentration which caused a 99.9% reduction of initial numbers), all concentrations showing no growth were diluted using 0.1 % (w/v) peptone water (Difco, Sparks, MD, USA) and plated in duplicate on LSA (*Listeria*) or XLD agar for *Salmonella* using an Autoplate 4000 plater (Spiral 168 Biotech, Norwood, MA, USA) and incubated at 37 ºC for 24 or 48 h at which time the colonies on plates were counted. The tubes with high AITC concentrations which were expected to contain low numbers of viable cells were spread-plated manually using a sterilized bent glass rod (1 ml/4 plates) to achieve a minimum detectable level of ≤ 1 CFU/ml.

3.3.6 Combined pH and temperature effects on *in vitro* AITC antimicrobial activity.

MH broth was dissolved in buffers previously adjusted to pH 5.0, 7.0 or 9.0. A cocktail of 5 strains of each pathogen was inoculated into the MH broth containing 200 ppm of AITC. Cultures were incubated with shaking at 200 rpm and 4, 10 or 21 ºC for 10 d. Samples were taken at 0, 1, 3, 6 and 10 d and plated on selective agar for each pathogen and incubated as described above prior to colony enumeration. The same procedure was repeated for control cells without AITC to measure the ability of *Salmonella* and *L. monocytogenes* to grow or survive at the different pH and temperature combinations. At these time intervals pH values were measured (Accumet Basic pH meter; Denver Instrument Co., Denver, CO, USA) to verify their stability during storage. All tests were performed in triplicate using different test tubes for each reading.

3.3.7 Effect of temperature and pH on stability of AITC

Initially, 200 ppm of AITC was added to MH broth. A total of 10 ml of broth was poured into 12 ml screw-capped tubes and incubated with shaking at 200 rpm for 48 h at 21 or 37 ºC,

and samples were taken at 0, 6, 12, 18, 24, and 48 h to measure AITC stability by reversed phase-liquid chromatography (RP-HPLC) as noted below. For this purpose, the same experiment as was done in section 2.6 was repeated using 200 ppm AITC, but without bacterial cultures.

3.3.8 AITC analysis by RP-HPLC

Analysis of AITC was based on the method used by Pechacek et al. (1997) with minor modifications. Samples at different time intervals were taken and filtered (0.22 µm, Millipore, Cork, Ireland) before HPLC analysis using a Waters 2695 unit (Waters Corporation, Milford, MA, USA) equipped with a C18 column (Gemini-NX, 150×4.60 mm, 5µ; Phenomenex, Torrance, CA, USA). A Waters model 486 detector (wavelength set at 244 nm), and a Waters 600E system controller were used. Waters LC-module 1Millennium software (version 32) was used to process the data. The binary mobile phase was composed of acetonitrile and water (60:40, v/v). The system was run with isocratic elution and the injection volume was 10 µl. The flow rate was kept constant at 1.0 ml/min for a total run time of 10 min. AITC was quantitatively determined using a calibration curve (25-250 ppm) established with the AITC standard under the same analytical conditions.

3.3.9 Statistical analysis

The microbiological data reported are average values of three replicates with at least two readings for each replicate (n=6), while AITC stability data are average values of two replicates with at least two readings for each replicate (n=4). All data are represented by means ± SD. Differences among treatments were analyzed by Tukey's test using JMP 9.0.2 software from SAS. Significant differences between treatments were concluded when p was < 0.05.

3.4 Results and Discussion

3.4.1 MIC and MBC of AITC against *Salmonella* and *L. monocytogenes* and AITC stability at different temperatures

The MIC and MBC values of AITC against both *L. monocytogenes* and *Salmonella* strains were highly affected by the temperature used and strain tested (Table 3.1). AITC was more inhibitory at 21 ºC where the MIC was 10 ppm against all *Salmonella* strains and ranged from 20 to 40 ppm against *L. monocytogenes* strains. Lara-Lledó et al. (2012) also found that the MIC of AITC against *L. monocytogenes* GLM4 was 75 ppm in BHI broth at 20 ºC. The MIC increased up to 10 times at 37 ºC, reaching 100 ppm against all *L. monocytogenes* strains and 60-100 ppm against *Salmonella* strains (Table 3.1). These results agree with previous findings of Liu and Yang (2010) who found that MICs of AITC against *S. enterica* and *L. monocytogenes* were 100 and 200 ppm in an oil-in-water emulsion incubated at 37 ºC, respectively. Kyung and Fleming (1997) found that the MIC of AITC against *L. monocytogenes* and *S.* Typhimurium was 200 and 50 ppm, respectively, in Tryptic Soy Broth incubated at 37 ºC. Palaniappan and Holley (2010) found the MIC of AITC against *S.* Typhimurium to be 0.31 mM (~31 ppm) in MH broth incubated at 37 ºC. In contrast, the MBC of AITC was higher at the lower temperature, increasing from 120-180 ppm at 37 ºC to 500-600 ppm at 21 ºC against *Salomonella* strains and from 120-160 ppm at 37 ºC to 200-300 ppm at 21 ºC against *L. monocytogenes* strains. Shin et al. (2004) found that the MBC of AITC against *S.* Typhimurium was 670 ppm at 37 ºC.

Although the MIC of AITC against *L. monocytogenes* strains was higher than for *Salmonella* strains at room temperature or 37 ºC, the MBC of AITC against *Salmonella* strains was higher than for *L. monocytogenes* at 21 ºC. At bactericidal concentrations, AITC causes

damage to the bacterial cell membrane, but also can cause DNA damage, has inhibitory effects on a range of metabolic enzymes, reacts with thiol-containing compounds and causes structural damage in the cytoplasm (Lin et al., 2000; Luciano and Holley 2009). Generally, Gram-negative bacteria are more resistant to neutral and anionic agents because the outer cell membrane is impermeable to these compounds and allows only limited diffusion of hydrophobic substances through its lipopolysaccharide-covered surface (Vaara, 1992). However, AITC is an exception to this general rule and resistance is not always determined by cell wall structure. Of several Gram-positive organisms, *Lactobacillus sakei* and a number of *Pediococcus* strains were highly resistant, *L. monocytogenes* showed some sensitivity but *Staphylococcus aureus* and *Staphylococcus carnosus* were quite sensitive to AITC. In contrast, the Gram-negative *E. coli* O157:H7, is highly sensitive to AITC (Chacon et al., 2006; Ward et al., 1998).

The results of AITC stability in sterile MH broth incubated at room temperature or 37 ºC for 48 h (Fig. 3.1) showed that AITC was reduced by more than 83% and 93 % after 6 and 12 h at 37 ºC, respectively, and it was not detected after 18 h. However, AITC showed more stability at 21 ºC where it was reduced by 26%, 49%, 69% and 86% after 6, 12, 24 and 48 h, respectively. It seems that AITC is more inhibitory to bacteria at room temperatures because its stability is higher at this temperature. These results are consistent with earlier work where it was shown that AITC is volatile and easily decomposed to new products at elevated temperatures in the presence of water (Tsao et al., 2000; Ohta et al., 1995). The same results were found by Cheng et al. (2004) where AITC was reduced by 84% after 12 h and was not detected after 24 h in sterile Gifu Anaerobic broth medium incubated at 37 ºC. Luciano and Holley (2009) also found that AITC rapidly decomposed in water at 37 ºC. Tsao et al. (2000) found that the half-life of AITC

in a sterile soil-water mixture (supernatant from 1:1 water/air-dried soil) was 43 d at 25 ºC which is greater than found in the present study.

3.4.2 Behavior of *Salmonella* or *L. monocytogenes* cocktails at different pH and temperature combinations

There were no changes in the initial pH values of 5.0, 7.0 or 9.0 (±0.1) during pathogen testing in buffered MH broth in the presence or absence of AITC during storage. Growth or survival of *Salmonella* or *L. monocytogenes* cocktails at different pH and temperature values are reported in Tables 3.2 and 3.3, respectively. The results showed that alkaline pH combined with 4, 10 or 21 ºC were most growth restrictive ($p<0.05$). The *Salmonella* cocktail survived at 4 ºC when pH values were 5.0, 7.0 and 9.0 and these resulted in population reductions of 0.49, 0.46 and 1.62 $\log_{10}$ CFU/ml, respectively, after 10 d. However at 10 or 21 ºC, *Salmonella* grew in MH broth adjusted to pH values of 7.0 or 5.0, but not at pH 9.0 over 10 d. The *L. monocytogenes* cocktail grew at 4, 10 and 21 ºC when combined with neutral pH ($p<0.05$), but under alkaline conditions their numbers were reduced by 0.43, 0.38 and 0.61 $\log_{10}$ CFU/ml at 4, 10 and 21 ºC, respectively, over 10 d. The pH and temperature combinations permitting the growth of *Salmonella* and *L. monocytogenes* found in the present study were similar to those reported in other studies (Ferreira and Lund, 1987; George et al., 1988; McDermid et al., 1996).

3.4.3 Combined pH and temperature effects on *in vitro* AITC antimicrobial activity and stability.

When the antimicrobial activity of 200 ppm AITC against the cocktails of *Salmonella* (Table 3.4) or *L. monocytogenes* (Table 3.5) in MH broth at different pH and temperature combinations was investigated over 10 d, AITC was found to be inactive at low temperatures (4

or 10 ºC) and at alkaline pH up to 10 d (p<0.05). But moderate antimicrobial activity was shown when alkaline pH was combined with room temperature, where *Salmonella* and *L. monocytogenes* numbers were reduced by 2.8 and 1.3 $\log_{10}$ CFU/ml, respectively. AITC reduced *Salmonella* and *L. monocytogenes* numbers by 2.2 and 4.1 $\log_{10}$ CFU/ml, respectively, at neutral pH, or by 2.6 and 1.2 $\log_{10}$ CFU/ml at acidic pH, respectively, after 10 d of incubation at 4 ºC. The results at higher pH are explained by the instability of AITC since it was not detected after 24 h at pH 9.0 when incubated at any temperature (Table 3.6). It is believed that AITC is susceptible to attack from nucleophiles such as water, OH−, and amino groups (Liu and Yang, 2010). Therefore, under alkaline conditions, AITC easily decomposed to form new compounds which were less bactericidal (Ohta et al., 1995).

On the other hand, it was found that viable *Salmonella* were not detected after 6 d at combinations of high temperature and acidic pH in the presence of AITC (p<0.05), while viable *L. monocytogenes* were not detected after 3 d at neutral pH and 21 ºC or after 10 d at neutral pH and 10 ºC. Luciano and Holley (2009) also reported that AITC had higher antibacterial activity against *E. coli* O157:H7 at low pH. The MIC found at pH 4.5 and 5.5 was 25 µL/L compared to 500 µL/L at pH 8.5. Furthermore, Shofran et al. (1998) found that the MIC of AITC varied from 60 to 140 ppm and 120 to 220 ppm over pH 5.0 to 7.0 against *E. coli* and *Staphylococcus aureus*, respectively.

In the present study, AITC at neutral pH was unstable and new compounds formed had bactericidal activity against *L. monocytogenes*, but not against *Salmonella*. Therefore, the latter was able to recover after 10 d at 21 ºC. This is likely since AITC was not detected by RP-HPLC after 6 d at neutral pH (Table 3.6). AITC was significantly more stable at acidic pH and 4 ºC or 10 ºC up to 10 d where it was reduced by 52.3% and 70.7%, respectively. At neutral pH AITC

was reduced by 63.5% at 4 ºC and was not detected after 6 or 10 d and 21 ºC or 10 ºC. Similar results were found in other studies (Tsao et al., 2000; Pechacek et al., 1997; Chen and Ho, 1998) and it is likely that AITC was more antimicrobial against *Salmonella* than the compounds formed by its degradation. As was found by Luciano and Holley (2009), the compounds generated by AITC decomposition were not able to reduce *E. coli* O157:H7 growth individually or even when combined with a sub-lethal concentration of AITC. However, interactive inhibitory effects of AITC with its degradation products at a pH value of 7.0 might explain the greater reduction of *L. monocytogenes* observed at this pH.

The results showed that temperature and pH affect AITC stability and thus its antimicrobial activity. AITC generates undesirable odors and flavors at concentrations that are antimicrobial. Consequently, it was encouraging to find that low concentrations of 10-40 ppm AITC were inhibitory against *Salmonella* or *L. monocytogenes* at 21 ºC. AITC also was more stable and effective against *Salmonella* at acidic pH; however, AITC decomposition at neutral pH may form new products that have antimicrobial activity against *L. monocytogenes*. These results suggest that optimizing conditions for the antimicrobial action of AITC may allow AITC to be effective at concentrations that are organoleptically undetectable.

3.5 Conclusions

AITC was more inhibitory against *Salmonella* or *L. monocytogenes* at room temperature because its stability is higher than at 37 ºC. AITC was more stable when 4 ºC or 10 ºC storage was combined with an acidic pH. However, AITC was an effective antimicrobial at ≤ 21 ºC against *Salmonella* when at acidic pH or against *L. monocytogenes* when at neutral pH. It is possible that at neutral pH, AITC decomposed to form new compounds which had bactericidal

activity against *L. monocytogenes*, but not against *Salmonella*. Refrigerator temperatures (4 ºC to 10 ºC) appeared to reduce AITC instability and prevent growth of the test microorganisms. Thus, these conditions may extend the interval during which AITC is antimicrobial, enhancing its efficacy while reducing its instability. Results from this study showed that AITC can be used as a preservative in food having acidic or neutral pH when stored at room temperature or 4 ºC to 10 ºC.

Table 3.1: MIC and MBC (ppm) of AITC against five individual *Salmonella* and *L. monocytogenes* strains and a mixture of these strains.

Strain	21 ºC		37 ºC	
	MIC [a]	MBC [b]	MIC [a]	MBC [b]
S. Enteritidis	10	600	80	180
S. Heidelberg	10	600	100	180
S. Copenhagen	10	500	60	120
S. Kentucky	10	600	80	160
S. Typhimurium	10	500	80	180
Salmonella 5 strain cocktail	10	600	80	160
L. monocytogenes GLM1	20	200	100	140
L. monocytogenes GLM3	20	200	100	160
L. monocytogenes GLM5	40	200	100	120
L. monocytogenes 2-138	40	300	100	140
L. monocytogenes 2-243	40	200	100	160
L. monocytogenes 5 strain cocktail	40	300	100	160

[a] The lowest concentration at which no visible growth occurred

[b] The lowest concentration causing a 99.9% reduction of the initial inoculum

Table 3.2: Change in viability of a 5 strain *Salmonella* cocktail (log_{10} CFU/ml) at different pH and temperature combinations

Time (d)	4 ºC			10 ºC			21 ºC		
	pH5	pH7	pH9	pH5	pH7	pH9	pH5	pH7	pH9
0	5.84±0.01^{a}†	5.84±0.01^{a}	5.84±0.01^{a}	5.84±0.01^{a}	5.84±0.01^{a}	5.84±0.01^{a}	5.84±0.01^{a}	5.84±0.01^{a}	5.84±0.01^{a}
1	5.70±0.04^{c}	5.82±0.11^{c}	5.64±0.05^{c}	6.55±0.08^{b}	6.50±0.56^{b}	5.82±0.03^{c}	8.44±0.13^{a}	8.68±0.28^{a}	5.38±0.05^{c}
3	5.45±0.07^{d}	5.64±.09^{d}	4.81±0.19^{e}	6.77±0.09^{c}	7.46±0.04^{b}	5.28±0.20^{d}	8.27±0.04^{a}	8.56±.09^{a}	6.84±0.22^{c}
6	5.27±0.08^{de}	5.82±0.06^{cd}	4.52±0.17^{e}	7.03±0.05^{bc}	8.71±0.25^{a}	4.60±0.21^{de}	8.11±0.88^{ab}	8.92±0.22^{a}	7.04±0.91^{bc}
10	5.35±0.03^{cd}	5.38±0.13^{cd}	4.22±0.05^{d}	7.08±018^{b}	8.39±0.12^{a}	4.35±.09^{d}	7.90±1.08^{ab}	8.46±0.25^{a}	5.72±0.59^{c}

†Means of microbial numbers at each sampling time in the same row with the same letters are not significantly different (p>0.05)

Table 3.3: Change in viability of a 5 strain *L. monocytogenes* cocktail (log_{10} CFU/ml) at different pH and temperature combinations

Time (d)	4 ºC			10 ºC			21 ºC		
	pH5	pH7	pH9	pH5	pH7	pH9	pH5	pH7	pH9
0	6.13±0.13^{a}†	6.13±0.13^{a}	6.13±0.13^{a}	6.13±0.13^{a}	6.13±0.13^{a}	6.13±0.13^{a}	6.13±0.13^{a}	6.13±0.13^{a}	6.13±0.13^{a}
1	6.20±0.23^{cd}	6.45±0.05^{c}	6.30±0.04^{cd}	6.32±0.07^{cd}	7.42±0.03^{b}	6.15±0.08^{d}	6.41±0.05^{c}	8.84±0.02^{a}	6.29±0.02^{cd}
3	6.23±0.04^{c}	6.96±0.15^{b}	6.13±0.06^{c}	6.29±0.06^{c}	8.76±0.06^{a}	5.63±0.23^{d}	7.15±0.13^{b}	8.88±0.10^{a}	6.13±0.18^{c}
6	6.21±0.08^{c}	7.42±0.35^{b}	5.93±0.15^{c}	6.36±0.03^{c}	8.69±0.04^{a}	4.95±0.29^{d}	7.47±0.37^{b}	8.85±0.16^{a}	5.81±0.25^{c}
10	6.27±0.11^{bc}	8.46±0.03^{a}	5.70±0.18^{bc}	6.39±0.19^{b}	8.45±0.25^{a}	5.75±0.33^{bc}	5.93±0.56^{bc}	8.46±0.21^{a}	5.52±0.09^{c}

†Means of microbial numbers at each sampling time in the same row with the same letters are not significantly different (p>0.05)

Table 3.4: Reduction in viable numbers of a 5 strain *Salmonella* cocktail (log_{10} CFU/ml) by 200 ppm AITC at different pH and temperature combinations

Time (d)	4 ºC			10 ºC			21 ºC		
	pH5	pH7	pH9	pH5	pH7	pH9	pH5	pH7	pH9
0	0.00±0.00^{a†}	0.00±0.00^{a}	0.00±0.00 ^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
1	0.06±0.06^{d}	0.46±0.08^{cd}	0.63±0.04^{bcd}	1.10±0.11^{b}	1.26±0.57^{b}	0.71±0.01^{bc}	3.59±0.01^{a}	3.67±0.31^{a}	0.43±0.06^{cd}
3	0.47±0.14^{e}	0.78±0.13^{e}	0.07±0.11^{e}	2.15±0.07^{d}	2.58±0.10^{cd}	0.45±0.34^{e}	7.39±0.87^{a}	4.31±0.11^{b}	3.39±0.18^{bc}
6	1.22±0.14^{ef}	1.76±0.24^{e}	0.07±0.10^{f}	3.77±0.75^{cd}	4.58±0.40^{bc}	0.16±0.28^{f}	8.11±0.88^{a*}	5.70±0.08^{b}	2.62±0.86^{de}
10	2.56±0.53^{c}	2.16±0.28^{c}	0.13±0.05^{d}	6.48±0.42^{a}	4.45±0.54^{b}	0.14±0.13^{d}	7.90±1.08^{a*}	-0.38±0.14^{d}	2.84±0.39^{c}

†Means of microbial reduction (control - treated samples) at each sampling time in the same row with the same letters are not significantly different (p>0.05)

* Microbial cells were not detected in the treated MH broth

Table 3.5: Reduction in viable numbers of a 5 strain *L. monocytogenes* cocktail (log_{10} CFU/ml) by 200 ppm AITC at different pH and temperature combinations

Time (d)	4 ºC			10 ºC			21 ºC		
	pH5	pH7	pH9	pH5	pH7	pH9	pH5	pH7	pH9
0	0.00 ± 0.00^{a}†	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
1	0.12 ± 0.32^{c}	0.73 ± 0.13^{c}	0.34 ± 0.33^{c}	0.01 ± 0.05^{c}	2.38 ± 0.25^{b}	-0.08 ± 0.10^{c}	1.89 ± 0.33^{b}	8.30 ± 0.54^{a}	0.56 ± 0.49^{c}
3	0.15 ± 0.09^{ef}	1.76 ± 0.11^{d}	0.31 ± 0.23^{ef}	0.42 ± 0.07^{e}	5.76 ± 0.59^{c}	-0.34 ± 0.30^{f}	7.15 ± 0.13^{b}	$8.88\pm0.10^{a*}$	0.23 ± 0.21^{ef}
6	0.44 ± 0.18^{d}	2.95 ± 0.33^{c}	0.25 ± 0.14^{d}	0.61 ± 0.10^{d}	7.87 ± 0.41^{b}	-1.20 ± 0.26^{e}	7.47 ± 0.37^{b}	$8.85\pm0.16^{a*}$	0.10 ± 0.23^{d}
10	1.19 ± 0.25^{d}	4.14 ± 0.53^{c}	-0.18 ± 0.22^{d}	3.07 ± 1.42^{c}	$8.45\pm0.25^{a*}$	0.02 ± 0.18^{d}	5.93 ± 0.56^{b}	$8.46\pm0.21^{a*}$	1.30 ± 0.62^{d}

†Means of microbial reduction (control - treated samples) at each sampling time in the same row with the same letters are not significantly different ($p>0.05$)

*Microbial cells were not detected in the treated MH broth

Table 3.6: Stability of 200 ppm AITC (% remaining) at different pH and temperatures

Time (d)	4 ºC			10 ºC			21 ºC		
	pH5	pH7	pH9	pH5	pH7	pH9	pH5	pH7	pH9
0	100±0.00 [a]†	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]
1	88.11±10.09 [a]	81.60±8.75 [a]	ND [d]	67.84±5.00 [ab]	53.12±6.59 [bc]	ND [d]	62.03±12.26 [abc]	35.57±5.50 [c]	ND [d]
3	72.63±5.26 [a]	69.64±14.05 [ab]	ND [d]	50.19±0.61 [bc]	33.56±2.25 [c]	ND [d]	35.8±2.93 [c]	10.21±2.32 [d]	ND [d]
6	54.71±2.41 [a]	52.61±6.48 [a]	ND [d]	40.41±1.03 [b]	19.35±1.42 [c]	ND [d]	7.18±0.01 [d]	ND [d]	ND [d]
10	48.28±13.94 [a]	36.50±3.34 [a]	ND [b]	29.28±2.29 [a]	ND [b]	ND [b]	2.24±0.21 [b]	ND [b]	ND [b]

†Means of remaining AITC (%) at each sampling time in the same row with the same letters are not significantly different (p>0.05)

ND: AITC was not detected (the detection limit was 2.5 ppm)

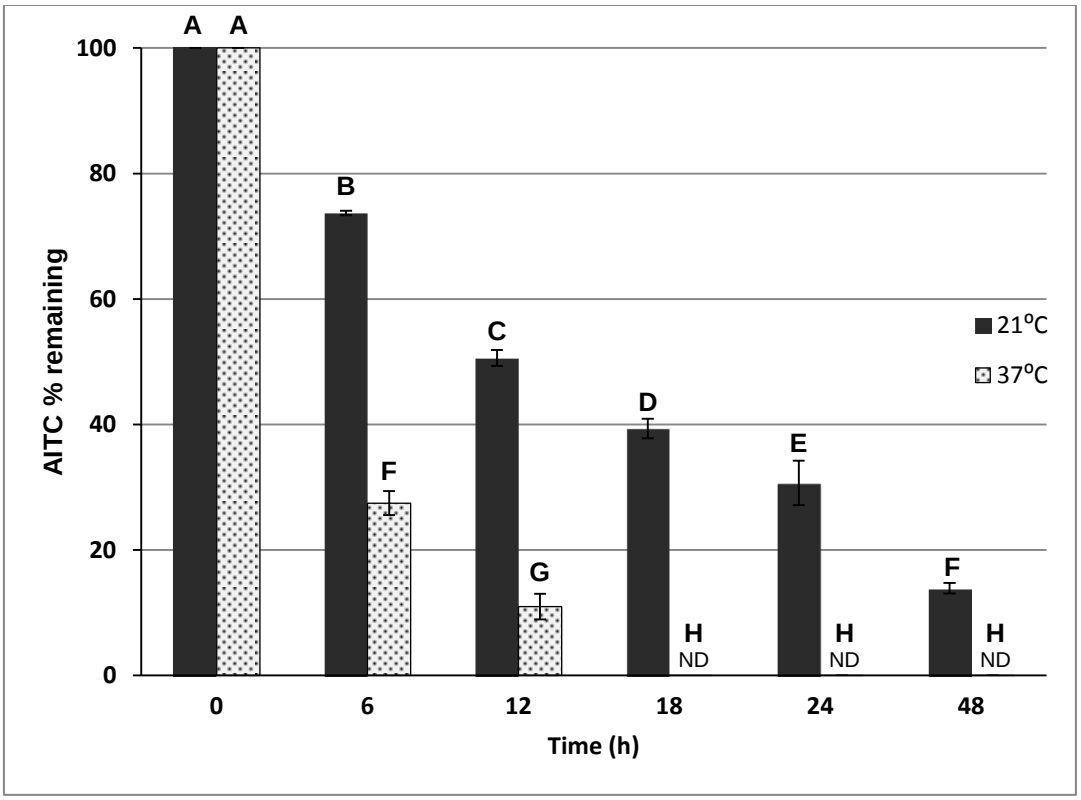

Fig. 3.1: Effect of incubation temperature on AITC stability at pH 7.2

Error bars represent the standard deviation of the mean.

100% AITC = 200 ppm

Columns with different letters are significantly different ($p<0.05$)

ND: AITC was not detected (the detection limit was 2.5 ppm).

Chapter 4

Inhibition of *Listeria monocytogenes* and *Salmonella* by Combinations of Oriental Mustard, Malic Acid and EDTA

4.1 Abstract

The antimicrobial activities of Oriental mustard extract alone or combined with malic acid and EDTA were investigated against *Salmonella* or *L. monocytogenes* at different temperatures. Five strain *Salmonella* or *L. monocytogenes* cocktails were separately inoculated in Brain Heart Infusion broth containing 0.5% (w/v) aqueous Oriental mustard extract and incubated at 4 to 21 ºC for 21 d. For inhibitor combination tests, *S.* Typhimurium 02:8423 and *L. monocytogenes* 2-243 were individually inoculated in Mueller Hinton broth containing the mustard extract with either or both 0.2% (w/v) malic acid and 0.2% (w/v) EDTA and incubated at 10 or 21 ºC for 10 to 14 d. Mustard extract inhibited growth of the *L. monocytogenes* cocktail at 4 ºC up to 21 d (2.3 $\log_{10}$ CFU/ml inhibition) or at 10 ºC for 7 d (2.4 $\log_{10}$ CFU/ml inhibition). *Salmonella* viability was slightly, but significantly reduced by mustard extract at 4 ºC by 21 d. Although hydrolysis of sinigrin in mustard extract by both pathogens was 2 to 6 times higher at 21 ºC than at 4 to 10 ºC, mustard was not inhibitory at 21 ºC, perhaps because of the instability of its hydrolysis product (allyl isothiocyanate). At 21 ºC, additive inhibitory effects of mustard extract with EDTA or malic acid led to undetectable levels of *S.* Typhimurium and *L. monocytogenes* by 7 d and 10 d, respectively. At 10 ºC, *S.* Typhimurium was similarly susceptible, but combinations of antimicrobials were not more inhibitory to *L. monocytogenes* than the individual agents. The bactericidal effects of Oriental mustard plus EDTA against *S.* Typhimurium at 21 or 10 ºC and mustard plus malic acid against *L. monocytogenes* at 21 ºC observed during *in vitro* tests may be

of value in formulating foods to reduce the risk associated with these foodborne pathogens.

4.2 Introduction

It has been estimated that 48 million persons suffer from foodborne illness with 128,000 hospitalizations and 3,000 deaths each year in the US (Scallan et al., 2011). Painter et al. (2013) reported that 13,352 foodborne outbreaks causing 271,974 illnesses were identified in the US from 1998 to 2008. *Salmonella* and *Listeria monocytogenes* are specific pathogens of concern where the former is considered one of the most common causes of foodborne illness worldwide and the latter is an opportunistic foodborne pathogen that can cause severe forms of infection in humans with high mortality. Scallan et al. (2011) estimated that *Salmonella* and *L. monocytogenes* cause 1.2 million illnesses (with 3.8% deaths) and 1662 illnesses (with 19% deaths), respectively, yearly in the US.

Oriental (brown) mustard powder from *Brassica juncea* is used as a spice and the less pungent yellow mustard (*Sinapis alba*) is also used as a filler, binder and emulsifier in cooked cured meat products after it is deodorized to eliminate the naturally present enzyme myrosinase by heating. Mustard can contain high levels of glucosinolates (sinalbin, sinigrin) which are degraded by endogenous plant myrosinase or by bacterial myrosinase-like activity (Luciano and Holley, 2011) to form antimicrobial isothiocyanates (ρ-hydroxybenzyl isothiocyanate, ρ-HBITC) from sinalbin in yellow mustard or allyl isothiocyanate (AITC) from sinigrin in Oriental mustard. A number of bacteria have been found to contain myrosinase(s) (Olaimat et al., 2014b [Chapter 5]; Herzallah et al., 2011) which convert these glucosinolates into auto-toxic isothiocyanates. Among them, AITC has significant bactericidal activity against several foodborne pathogens

including *E. coli* O157:H7, *Campylobacter jejuni, Salmonella* and *L. monocytogenes* plus others (Luciano et al., 2011; Dufour et al., 2012; Olaimat and Holley, 2013 [Chapter 3]).

Little information is available on the antimicrobial activity of Oriental mustard against *L. monocytogenes* or *Salmonella*. Lara-Lledó et al. (2012) found that Brain Heart Infusion (BHI) broth containing non-meat sausage ingredients (2.91% salt; 0.31% pickle cure containing salt, sodium nitrite, sodium bicarbonate, and glycerol; 0.1% glucose, and 0.05% sodium erythorbate) and Oriental mustard extract with 0.163% (w/w) sinigrin was not inhibitory to *L. monocytogenes* at 20 ºC. However, a polymeric film containing only Oriental mustard extract with 5% (w/w) sinigrin reduced *L. monocytogenes* in bologna after 52 d at 4 ºC to an undetectable level (≤ 1.6 $\log_{10}$ CFU/g). Since the glucosinolate, sinigrin, is not antimicrobial, but its bacterial myrosinase hydrolysis product, AITC, is bactericidal, work is needed to determine the effect of temperature changes on the degradation of sinigrin in mustard extract and its resulting antimicrobial activity at 4 to 21 ºC.

Ethylenediamine tetraacetic acid (EDTA) has been used in a number of food products as a chelating agent to prevent oxidation. It inhibits the growth of microorganisms by chelating Mg^{2+}, Ca^{2+}, and Fe^{2+} and depriving organisms of these cations which are essential for membrane stability and growth (Ko et al., 2010).

Organic acids and their salts are attractive antimicrobials due to their acceptance in foods and their low cost, often because they are either naturally present in fruits and vegetables or are synthesized by microorganisms (Miller et al., 1996). Although several studies have shown synergistic interactions of natural antimicrobials with organic acids or EDTA, there are only two which examined the combined effect of mustard with an organic acid. In that work, 10% or 20%

(w/v) mustard in combination with 1% (v/w) acetic acid had greater antimicrobial activity against *E. coli* O157:H7, *L. monocytogenes*, and *S.* Typhimurium compared with mustard alone when stored at 5 or 22 ºC (Rhee et al., 2002; 2003). As an alternative to acetic acid because of flavor considerations, malic acid has potential. Malic acid is dicarboxylic, found naturally in various fruiting plants and berries, and is approved for direct addition to foods as a GRAS ingredient (21CFR184) (Doores, 2005).

Therefore, the objectives of the first part of this study were to determine the antimicrobial activity of an aqueous Oriental mustard extract against 5 strain *L. monocytogenes* and *Salmonella* cocktails at 21, 10 or 4 ºC in BHI broth, and to study the ability of the pathogens to degrade sinigrin in the extract at these temperatures. In the second part of the work, the interactive effect of the mustard extract, malic acid and EDTA were examined using one culture each of *L. monocytogenes* and *Salmonella.*

4.3 Materials and Methods

4.3.1 Chemicals

Naturally hot (spicy or pungent) ground Oriental mustard (*Brassica juncea*) seed (pH 7.0) was obtained from Sakai Spice Canada Corp. (Lethbridge, AB, Canada). Malic acid, EDTA, sodium hydroxide (NaOH), hydrochloric acid (HCl) and HPLC grade acetonitrile were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). Tetrabutyl ammonium hydrogen sulfate (98%) was from J. T. Baker (Phillipsburg, NJ, USA).

4.3.2 Bacterial strains

Five strains of *L. monocytogenes* (2-138, 2-243, GLM-1, GLM-3, GLM-5) and *Salmonella*

(*S.* Heidelberg 271, *S.* Typhimurium 02:8423, *S.* Copenhagen PT 99, *S.* Enteritidis CRIFS 1016, and *S.* Kentucky 64701) were from the culture collection of the Food Science Department, University of Manitoba. These were previously tested for their ability to degrade pure sinigrin and resistance to AITC (Olaimat and Holley, 2013 [Chapter 3]; Olaimat et al., 2014b [Chapter 5]).

4.3.3 Culture preparation

Bacterial cultures were stored individually at -80 ºC in BHI broth (Oxoid Ltd., Basingstoke, England) with 25% glycerol. Strains were subcultured in BHI broth twice and then one loopful from the second transfer of *L. monocytogenes* strains or *Salmonella* serovars was streaked on *Listeria* selective agar base with *Listeria* selective supplement (LSA, Oxoid Ltd.) or on Xylose lysine deoxycholate (XLD) agar (Oxoid Ltd.), respectively, and incubated at 37 ºC for 24 h. A single colony of *L. monocytogenes* or *Salmonella* strains from the selective agars was transferred to BHI broth and incubated overnight at 37 ºC. Then 0.1 % (v/v) of this culture was transferred individually to fresh BHI broth and incubated overnight at 37 ºC.

4.3.4 Mustard extract preparation

Mustard extract was prepared as described by Lara-Lledó et al. (2012). To inactivate the plant myrosinase, a 2-cm layer of hot (spicy) Oriental mustard was placed in a tray (20×15 cm), covered with aluminum foil and autoclave-treated for 15 min at 115 ºC (similar temperature-time combinations are used commercially to inactivate the myrosinase in mustard). The resulting deodorized (non-spicy) mustard was mixed with distilled water (10% w/v) at room temperature and then stirred for 1 h at 350 rpm. The mixture was centrifuged for 20 min at 4200 *xg* at 4 ºC, and then passed through a Whatman no. 4 filter. The filtered mustard was boiled for 30 min at

100 ºC to assure inactivation of plant myrosinase and remove mucilage (the glycoprotein in the seed coat used by the plant to sequester moisture). The mixture was centrifuged and filtered as described before. The filtrate was frozen overnight at −20 ºC, then freeze-dried for 48 h and kept at 4 ºC.

4.3.5 Antimicrobial activity of Oriental mustard extracts against *Salmonella* or *L. monocytogenes*

Three ml of 5 strain *Salmonella* or *L. monocytogenes* cocktails were transferred to 300 ml BHI broth containing 0.0% (control, pH 7.2) or 0.5% (w/v) Oriental mustard extract (pH 6.6) to achieve 7.0 $\log_{10}$ CFU/ml and 10 ml was transferred to sterile 12 ml screw-capped tubes which were incubated at 4, 10 or 21 ºC for 21 d. Samples of each pathogen were taken at 0, 3, 7, 14 and 21 d, plated on the respective selective agar and incubated as described above prior to colony enumeration. Samples were also taken at 0 and 21 d, filtered (0.22 μm, Millipore, Cork, Ireland) and the decrease in sinigrin content (bacterial myrosinase hydrolysis formed AITC) was measured using HPLC.

4.3.6 Combined effects of Oriental mustard extract, malic acid or EDTA against *S.* Typhimurium or *L. monocytogenes*

Overnight cultures of *S.* Typhimurium 02:8423 and *L. monocytogenes* 2-243 were diluted to 5 $\log_{10}$ CFU/ml and then one ml of each strain was separately transferred to: 100 ml Mueller Hinton (MH, Oxoid Ltd.) broth (control) or 100 ml MH broth containing 0.5% (w/v) Oriental mustard extract; 0.2% (w/v) malic acid; 0.2% (w/v) EDTA or their combinations, and incubated at 21 or 10 ºC for 10 to 14 d. In addition, pH-adjusted controls including modified MH broth (pH 4.0) and modified 0.5% (w/v) Oriental mustard extract (pH 4.1) were also prepared using 1 M

HCl. Samples were taken at 0, 1, 3, 7, 10 and 14 d and plated on the respective selective agar for each pathogen and incubated as described above prior to colony enumeration. The bacterial detection level was reduced to ≤ 1 CFU/ml by spread plating 1 ml of each sample over 4 plates using a sterilized bent glass rod. In separate parallel experiments, bacteria were recovered on Plate Count Agar (Oxoid Ltd.) and differences in numbers compared to the genus-specific media were attributed to cells injured by the treatments. For treatments where cells were not detected by plating, a 5 ml sample was enriched by inoculation into 45 ml BHI broth, incubated at 37 ^{o}C overnight, plated on selective agar and examined for survivors.

4.3.7 HPLC analysis

Measurement of sinigrin degradation by *Salmonella* or *L. monocytogenes* was based on the method described by Luciano and Holley (2010, 2011). The quantity of sinigrin in the Oriental mustard extract was determined by integration of the sinigrin peak detected by HPLC using a calibration curve (100-1500 ppm) established with a sinigrin standard (Sigma-Aldrich, St. Louis, MO, USA).

4.3.8 Statistical analysis

The data reported are the average values of two experiments and are represented by means ± standard deviation (SD). The results were analyzed using JMP 10.0 software (SAS Inst. Inc., Cary, N.C., U.S.A.) and an analysis of variance (ANOVA) was conducted. Student *t*-tests ($P \leq 0.05$) were used to characterize changes in viability of pathogens in mustard treatments compared to the control at different temperatures and to analyze the differences among the treatment combination tests.

4.4 Results and Discussion

4.4.1 Sinigrin concentration in mustard extract

The sinigrin peak separated by RP-HPLC had a retention time of 4.3 min (Fig. 4.1). The Oriental extract, which represented 14.0% of the mustard powder, was found to contain 16% sinigrin (i.e. 2.1% of the mustard powder) which was lower than the 24% sinigrin in Oriental extract reported by Lara-Lledó et al. (2012). This was not surprising since the sinigrin concentrations in *Brassica* vegetables are influenced by several factors including the variety examined, method of cultivation, time of harvest and stresses such as insect damage or bruising during harvesting (Heaney and Fenwick, 1980).

4.4.2 Inhibition of *Salmonella* and *L. monocytogenes* cocktails by Oriental mustard extract

At 21 ºC, 0.5% Oriental mustard extract had no inhibitory effect against *L. monocytogenes* up to 21 d (Table 4.1). At 10 ºC, the mustard extract inhibited growth of *L. monocytogenes* up to 7 d by 2.4 $\log_{10}$ CFU/ml; however, after that growth of the pathogen in BHI broth containing mustard was not significantly different from the BHI broth control. At 4 ºC mustard significantly inhibited (prevented) growth of *L. monocytogenes* up to 21d, when numbers were 2.3 $\log_{10}$ CFU/ml lower than the control. These results agree with findings of Lara-Lledó et al. (2012) who reported that BHI broth containing sausage ingredients and 0.7% mustard extract was not inhibitory to *L. monocytogenes* at 20 ºC. However at 4 ºC, polymeric film containing Oriental mustard extract with 5% (w/w) sinigrin reduced numbers of *L. monocytogenes* on inoculated bologna to undetectable levels (<1.6 $\log_{10}$ CFU/g) after 52 d.

The mustard extract was also not inhibitory to the *Salmonella* cocktail at 21 or 10 ºC up to

21 d; however, at 4 ºC *Salmonella* numbers were significantly reduced (about 0.7 $\log_{10}$ CFU/ml) at 21 d (Table 4.2). In contrast at 25 ºC, Cordeiro et al. (2013) found that MH broth containing sausage ingredients and 4% autoclave-treated yellow mustard powder inoculated with the meat starter cultures *Pediococcus pentosaceus* and *Staphylococcus carnosus* reduced the number of *E. coli* O157:H7 by 2 $\log_{10}$ CFU/ml after 14 d. The difference in activity found between these two studies was due in part to differences in the tested organisms as well as to differences in the glucosinolate hydrolysis products from the two types of mustard used. Sinigrin in Oriental mustard is degraded by organisms to form AITC, while yellow mustard contains sinalbin and yields ρ-HBITC which has a greater antimicrobial activity, but is less stable in aqueous medium than AITC (Luciano and Holley, 2010; 2011).

4.4.3 Degradation of sinigrin in Oriental mustard by *Salmonella* or *L. monocytogenes* cocktails

When mustard extract was incubated under the same conditions at 4 ^{o}C, 10 ^{o}C and 21 ^{o}C, but without *Salmonella* or *L. monocytogenes* cocktails, no significant sinigrin degradation was observed. When the degradation of sinigrin in Oriental mustard extract by these bacteria was analyzed by RP-HPLC, it was found that by 21 d at 21 ^{o}C *Salmonella* and *L. monocytogenes* degraded 563 and 461 ppm sinigrin, respectively, with progressively less hydrolysis at lower temperatures (Fig. 4.2). Lara-Lledó et al. (2012) found that *L. monocytogenes* GLM-4 was able to degrade almost half (539 ppm) the pure sinigrin present in BHI broth containing sausage cure ingredients at 20 ºC. The observation that the myrosinase-like activity of *Salmonella* and *L. monocytogenes* (believed responsible for glucosinolate hydrolysis) was greater at 21 ºC than at lower temperatures was consistent with the the optimum temperature noted for myrosinase in *Enterobacter cloacae* which ranged from 25 ºC to 40 ºC (Tani et al., 1974), and that of

Sphingobacterium sp. strain OTG1 which ranged from 20 ºC to 30 ºC (Meulenbeld and Hartmans, 2001).

It was found that the conversion of glucosinolates to AITC by plant myrosinase was near 90% (Kawakishi and Muramatsu, 1966). If the conversion by bacterial myrosinase was similar here, the 563 and 461 ppm of singrin would be degraded by *Salmonella* and *L. monocytogenes* at 21 ºC to form 507 and 415 ppm of AITC, respectively. Since it was found by Olaimat and Holley (2013 [Chapter 3]) that the minimum inhibitory concentration of AITC against tested *Salmonella* strains was only 10 ppm and ranged from 20-40 ppm against *L. monocytogenes* strains at 21 ºC, inhibitory levels of AITC would have been produced. It is probable that the AITC formed did not accumulate to reach an inhibitory level because it is unstable in aqueous media and rapidly decomposes to new compounds which are less bactericidal.

4.4.4 Combined effects of Oriental mustard extract, malic acid and EDTA against a single *S.* Typhimurium or *L. monocytogenes* strain at 21 ºC or 10 ºC

The combination of either or both malic acid and EDTA with Oriental mustard extract showed improved antimicrobial activity against *Salmonella* or *L. monocytogenes* at 21 and 10 ºC (Tables 4.3 to 4.6). The two strains used in these tests were the most resistant of those examined to AITC, with AITC minimum inhibitory concentration (MIC) values of 10 ppm and 40 ppm against *S.* Typhimurium 02:8423 and *L. monocytogenes* 2-243, respectively, at 21 ºC (Olaimat and Holley, 2013 [Chapter 3]). Numbers of *S.* Typhimurium increased in MH broth (pH 7.2) or MH broth containing 0.5% mustard extract (pH 6.55) to reach 9.0 $\log_{10}$ CFU/ml at 21 ºC at 10 d and 8.1 to 8.5 $\log_{10}$ CFU/ml at 10 ºC by 14 d (Tables 4.3 and 4.4). Due to a pH reduction, *S.* Typhimurium grew more slowly in modified MH broth (pH 4.0), modified 0.5% (w/v) mustard

extract (pH 4.1) or in the presence of 0.2 (w/v) malic acid (pH 4.0) than with mustard (pH 6.6) at 21 ºC to reach 7.1, 6.2 and 8.6 $\log_{10}$ CFU/ml, respectively at 21 ºC at 14 d (Tables 4.3, 4.5), while there was no growth in these treatments at 10 ºC (Tables 4.4, 4.5). At 21 ºC, 0.2% (w/v) EDTA also reduced the pH and consequently reduced the numbers of *S.* Typhimurium to 1.0 $\log_{10}$ CFU/ml by day 7 and to not detectable by day 10 (Table 4.3). EDTA showed similar inhibitory effects against *S.* Typhimurium at 10 ºC (Table 4.4). The inhibitory effects of mustard extract plus malic acid or EDTA were additive and improved the individual antimicrobial action of all three ingredients at both 21 and 10 ºC, although mustard did not change the pH of reaction mixtures containing malic acid or EDTA (4.3) (Tables 4.3 and 4.4). Mustard extract plus EDTA or mustard with EDTA and malic acid prevented growth at both temperatures and gradually reduced *S.* Typhimurium numbers to an undetectable level between 7 d (21 ºC) and 10 d (10 ºC). EDTA and malic acid without mustard were just as effective as the former combinations at 21 ºC but not at 10 ºC (Tables 4.3 and 4.4).

Numbers of *L. monocytogenes* increased in MH broth (pH 7.2) to reach > 8 $\log_{10}$ CFU/ml within 1 day at 21 ºC and by 7 d at 10 ºC (Tables 4.6 and 4.7), while the numbers slowly increased to 5.5 $\log_{10}$ CFU/ml in modified MH broth (pH 4.0) by 14 d at 21 ºC and there were no changes at 10 ºC (Table 4.8). At 21 ºC, MH broth containing 0.5% Oriental mustard extract (pH 6.55) initially retarded growth of *L. monocytogenes,* preventing maximum numbers from being reached until day 7 (Table 4.6), while in modified 0.5% (w/v) mustard extract (pH 4.1) *L. monocytogenes* numbers were reduced 1.4 $\log_{10}$ CFU/ml by 14 d (Table 4.8). In contrast, at 10 ºC, the organism did not grow in the presence of mustard (pH 6.55) or modified mustard (pH 4.1) and numbers were reduced by ≤ 0.5 $\log_{10}$ CFU/ml after 10 d (Tables 4.7, 4.8). While mustard plus malic acid inactivated *L. monocytogenes* by day 14, EDTA paired with malic acid

or in combination with malic acid and mustard most rapidly (by day 10) inactivated the pathogen at 21 ºC. At these times, viable cells were not detected in these treatments after overnight enrichment in MH broth (Table 4.6). At 10 ºC mustard, malic acid and EDTA were inhibitory to about the same extent, with malic acid and EDTA reducing viability the greatest (by about 1.3 $\log_{10}$ CFU/ml). All antimicrobials prevented *L. monocytogenes* growth for 14 d and slightly reduced viable numbers when used alone or when combined, however at this temperature there was no significant additive effect when mustard was combined with either or both malic acid and EDTA (Table 4.7).

It is likely that the lowered pH of treatments containing either or both EDTA and malic acid contributed to the inhibitory effects observed; however, *S.* Typhimurium was able to overcome the effects of low pH caused by malic acid alone at 21^{o}C (Table 4.3). While injured cells were detected in the presence of mustard, EDTA and malic acid, their numbers were not significantly different from controls. Injured cells were ≤ 0.58 log CFU/ml in antimicrobial tests and ≤ 0.41 log CFU/ml in controls. Therefore, injury was not an important factor in terms of the bacterial responses observed.

EDTA alone or combined with either or both mustard extract and malic acid showed greater antimicrobial activity against *S.* Typhimurium than *L. monocytogenes*. This was likely due differences in the cell wall structure of the two organisms since EDTA disrupts the lipopolysaccharide structure in the outer membrane of Gram-negative bacteria (i.e. *Salmonella*) (Alakomi et al., 2000; 2003). In addition, EDTA enhanced the antimicrobial activity of mustard extract against *S*. Typhimurium. Similar findings with thymol or carvacrol were reported by Zhou et al. (2007) where the combination of these compounds with EDTA caused significantly greater reductions in numbers of *S*. Typhimurium than when these agents were used alone. In

addition, *S.* Typhimurium numbers were reduced by 2.0 $\log_{10}$ CFU/g on turkey frankfurters after 28 d storage at 4 ºC when 0.5% grape seed extract was combined with 6000 IU/g nisin and 1.0% malic acid in a whey protein isolate film (Gadang et al., 2008). It seems that other natural plant extracts were also more inhibitory against *S.* Typhimurium in combination with EDTA because of its ability to interfere with divalent cation stabilization of the outer membrane of Gram-negative bacteria (Alakomi et al., 2000; 2003).

In contrast, malic acid was more inhibitory against *L. monocytogenes* than *S.* Typhimurium. The antimicrobial action of organic acids is also influenced by bacterial cell wall structure where Gram negative bacteria are more resistant than Gram positives, since the former have an outer hydrophobic membrane that may block the entry of hydrophilic molecules of low molecular weight such as malic acid (Raybaudi-Massilia et al., 2009). Gadang et al. (2008) also found that malic acid improved the antimicrobial activity of grape seed extract against *L. monocytogenes* on turkey frankfurters after 28 d at 4 ºC. The antimicrobial activity of malic acid is attributed to its small molecular size, allowing it to more easily cross the cell membrane into the cytoplasm, then become ionized, reduce the internal pH and cause damage in the cell cytoplasm (Eswaranandam et al., 2004). This damage may be responsible for the enhanced susceptibility of *L. monocytogenes* when also exposed to Oriental mustard extract.

There was a significant influence of storage temperature on the reduction of viable *S.* Typhimurium by EDTA or *L. monocytogenes* by malic acid. At higher temperatures, the cell membrane has greater fluidity and less phospholipid rigidity than at low temperatures (Raybaudi-Massilia et al., 2009), and this may have enhanced both malic acid entry into the cell and lipopolysaccharide disruption by EDTA.

4.5 Conclusions

It was found that 0.5% Oriental mustard extract inhibited growth of the *L. monocytogenes* cocktail up to 21 d at 4 ºC and for 7 d at 10 ºC, but at 4 ºC it was slightly inhibitory toward *Salmonella* and this resulted in reduced viability. Mustard extract was unable to prevent growth of *Salmonella* and *L. monocytogenes* at 21 ºC for up to 21 d, although both organisms caused significant degradation of sinigrin which formed inhibitory AITC under these conditions. Further work to identify other inhibitory compounds active at 4 to 10 ^{o}C, possibly phenolics in mustard powder, is required. The combination of Oriental mustard extract with either or both malic acid and EDTA improved its activity against *S.* Typhimurium at 10 and 21 ºC, and against *L. monocytogenes* at 21 ºC. The two to 3 agent combinations used were bactericidal against *S.* Typhimurium and *L. monocytogenes* at 21 ^{o}C; however, there was less apparent lethality at 10 ^{o}C with *S.* Typhimurium, and at 10 ^{o}C mixtures were bacteriostatic toward *L. monocytogenes*. At 10 ºC there was only marginal value from combining mustard with the other inhibitors for use against *L. monocytogenes*.

Table 4.1: Antimicrobial activity of 0.5% (w/v) mustard extract against a 5 strain *L. monocytogenes* cocktail (log_{10} CFU/ml) at different temperatures [A B]

Day	4 ºC		10 ºC		21 ºC	
	Control	Mustard	Control	Mustard	Control	Mustard
0	6.70±0.04[a]	6.70±0.04[a]	6.70±0.04[a]	6.70±0.04[a]	6.70±0.04[a]	6.70±0.04[a]
3	7.86±0.02[b]	6.33±0.10[d]	8.71±0.15[a]	7.27±0.25[c]	8.86±0.05[a]	8.85±0.08[a]
7	9.18±0.09[a]	6.25±0.09[c]	9.32±0.21[a]	6.94±0.37[b]	9.15±0.06[a]	9.18±0.11[a]
14	8.54±0.01[a]	6.46±0.12[d]	8.60±0.06[a]	8.45±0.41[ab]	8.10±0.40[bc]	7.91±0.21[c]
21	8.69±0.36[a]	6.40±0.24[c]	8.74±0.05[a]	8.84±0.08[a]	7.58±0.42[b]	7.52±0.20[b]

[A] Values are the means of two experiments (each with two replicate analyses) ± standard deviation.

[B] Means of bacterial growth at each sampling time in the same row with the same letters are not significantly different ($p > 0.05$)

Table 4.2: Antimicrobial activity of 0.5% (w/v) mustard extract against a 5 strain *Salmonella* cocktail (log_{10} CFU/ml) at different temperatures [A B]

Day	4 ºC		10 ºC		21 ºC	
	Control	Mustard	Control	Mustard	Control	Mustard
0	7.20±0.11[a]	7.20±0.11[a]	7.20±0.11[a]	7.20±0.11[a]	7.20±0.11[a]	7.20±0.11[a]
3	6.81±0.17[e]	6.86±0.18[e]	7.65±0.49[d]	8.09±0.12[c]	9.80±0.07[a]	8.99±0.13[b]
7	6.90±0.18[c]	6.89±0.20[c]	8.97±0.29[b]	9.19±0.09[ab]	9.44±0.10[a]	9.32±0.10[a]
14	6.83±0.62[b]	6.58±0.39[b]	8.77±0.24[a]	8.54±0.63[a]	8.97±0.20[a]	8.63±0.77[a]
21	6.42±0.06[b]	5.75±0.70[c]	8.91±0.26[a]	9.22±0.05[a]	8.98±0.05[a]	9.20±0.26[a]

[A] Values are the means of two experiments (each with two replicate analyses) ± standard deviation.

[B] Means of bacterial growth at each sampling time in the same row with the same letters are not significantly different ($p > 0.05$)

Table 4.3: Combined effects of Oriental mustard extract with malic acid or EDTA against *S.* Typhimurium (log_{10} CFU/ml) at 21 ºC [A B].

Day[C]	Control	0.5% Mustard	0.2% Malic acid	0.2% EDTA	0.5% Mustard + 0.2% Malic acid	0.5% Mustard + 0.2% EDTA	0.2% EDTA + 0.2% Malic Acid	0.5% Mustard + 0.2% EDTA + 0.2% Malic Acid
0	3.47±0.19 [a]	3.47±0.19 [a]	3.47±0.19 [a]	3.47±0.19 [a]	3.47±0.19 [a]	3.47±0.19 [a]	3.73±0.92 [a]	3.73±0.92 [a]
1	8.00±0.37 [a]	7.78±0.84 [a]	4.95±1.00 [b]	3.25±0.35 [cd]	3.67±0.16 [c]	3.01±0.05 [cd]	2.20±0.05 [d]	2.37±0.38 [d]
3	8.97±0.15 [a]	8.65±0.48 [a]	7.22±0.51 [b]	2.96±0.02 [d]	5.96±0.42 [c]	2.41±0.15 [d]	0.38±0.53 [e]	0.35±0.50 [e]
7	9.07±0.18 [a]	8.97±0.02 [a]	8.17±0.12 [ab]	0.95±0.73 [c]	7.40±0.72 [b]	ND [*]	ND [*]	ND [*]
10	8.95±0.15 [a]	8.95±0.07 [a]	8.63±0.49 [a]	ND [*]	8.05±0.04 [b]	ND [*]	ND [*]	ND [*]
Initial pH	7.20	6.55	4.02	4.35	4.04	4.34	3.91	3.95
Final pH	5.66	5.58	4.31	4.13	4.16	4.22	3.88	3.89

[A] Values are the means of two experiments (each with two replicate analyses) ± standard deviation.
[B] Means of bacterial growth at each sampling time in the same row with the same letters are not significantly different ($p > 0.05$)
[C] Samples in this series were not taken at day 14.
ND: Not detected (detection level was ≤ 1 CFU/ml)
[*] Cells were not detected after overnight enrichment in MH broth

Table 4.4: Combined effects of Oriental mustard extract with malic acid or EDTA against *S.* Typhimurium (log_{10} CFU/ml) at 10 ºC [A B].

Day	Control	0.5% Mustard	0.2% Malic acid	0.2% EDTA	0.5% Mustard + 0.2% Malic acid	0.5% Mustard + 0.2% EDTA	0.2% EDTA + 0.2% Malic Acid	0.5% Mustard + 0.2% EDTA + 0.2% Malic Acid
0	3.73±0.92 [a]	3.73±0.92 [a]	3.73±0.92 [a]	3.73±0.92 [a]	3.73±0.92 [a]	3.73±0.92 [a]	3.73±0.92 [a]	3.73±0.92 [a]
1	4.49±0.55 [a]	4.15±0.59 [ab]	3.89±0.27 [ab]	3.87±0.35 [ab]	3.80±0.25 [ab]	3.28±0.50 [b]	3.36±0.72 [b]	3.12±0.05 [b]
3	5.43±1.06 [a]	5.00±0.97 [ab]	3.67±0.23 [cd]	3.52±0.23 [cde]	3.77±0.18 [bc]	2.98±0.19 [cde]	2.50±0.33 [de]	2.43±0.04 [e]
7	7.48±1.00 [a]	7.51±0.20 [a]	3.76±0.27 [b]	2.51±0.26 [bc]	3.31±0.19 [b]	1.11±0.27 [d]	1.55±1.13 [cd]	0.97±0.18 [d]
10	7.83±0.25 [a]	7.99±0.31 [a]	3.77±0.37 [b]	1.61±0.78 [c]	3.19±0.05 [b]	ND [*]	0.76±1.07 [cd]	ND [*]
14	8.47±0.55 [a]	8.12±0.40 [a]	3.62±0.29 [b]	0.73±1.03 [c]	2.85±0.14 [b]	ND [*]	0.19±0.27 [c]	ND [*]
Initial pH	7.20	6.55	4.02	4.35	4.04	4.34	3.91	3.95
Final pH	5.74	5.61	3.98	4.22	4.12	4.32	3.93	3.90

[A] Values are the means of two experiments (each with two replicate analyses) ± standard deviation.
[B] Means of bacterial growth at each sampling time in the same row with the same letters are not significantly different ($p > 0.05$)
ND: Not detected (detection level was ≤ 1 CFU/ml)
[*] Cells were not detected after overnight enrichment in MH broth

Table 4.5: Viability of *S.* Typhimurium (log_{10} CFU/ml) in modified MH broth (pH 4.0) or modified 0.5% (w/v) Oriental mustard extract (pH 4.1) at 10 or 21 ºC

Temperature	Day	Modified MH (pH=4.04) [A]	Modified Mustard (pH = 4.09) [A]
21 ºC	0	3.61±0.33	3.61±0.33
	1	4.37±0.40	3.71±0.16
	3	7.66±0.04	5.35±0.21
	7	7.06±0.34	7.19±0.10
	10	7.10±0.10	6.15±0.17
	14	6.79±0.14	5.60±0.09
10 ºC	0	3.61±0.33	3.61±0.33
	1	3.80±0.04	3.74±0.06
	3	3.67±0.10	3.54±0.07
	7	3.47±0.05	3.24±0.09
	10	3.41±0.07	3.27±0.13
	14	3.40±0.03	3.10±0.05

[A] pH was adjusted using HCl

Table 4.6: Combined effects of Oriental mustard extract with malic acid or EDTA against *L. monocytogenes* (log_{10} CFU/ml) at 21 ºC [A] [B].

Day	Control	0.5% Mustard	0.2% Malic acid	0.2% EDTA	0.5% Mustard + 0.2% Malic acid	0.5% Mustard + 0.2% EDTA	0.2% EDTA + 0.2% Malic Acid	0.5% Mustard + 0.2% EDTA + 0.2% Malic Acid
0	3.08±0.59 [a]	3.08±0.59 [a]	3.08±0.59 [a]	3.08±0.59 [a]	3.08±0.59 [a]	3.08±0.59 [a]	3.54±0.29 [a]	3.54±0.29 [a]
1	8.59±0.08 [a]	3.06±0.35 [b]	3.02±0.33 [b]	2.95±0.49 [b]	2.91±0.40 [b]	2.77±0.67 [b]	2.46±0.20 [b]	2.40±0.16 [b]
3	9.02±0.04 [a]	4.76±0.55 [b]	2.97±0.28 [c]	3.03±0.49 [c]	2.86±0.54 [c]	2.87±0.50 [c]	2.22±0.15 [c]	2.15±0.23 [c]
7	9.31±0.12 [a]	8.85±0.04 [b]	2.84±0.16 [c]	2.66±0.20 [c]	1.95±0.08 [d]	2.59±0.24 [c]	1.25±0.35 [e]	1.02±0.17 [e]
10	9.01±0.10 [a]	8.53±0.37 [a]	2.34±0.44 [b]	2.41±0.16 [b]	1.12±0.32 [c]	1.97±0.51 [b]	ND [*]	ND [*]
14	8.74±0.04 [a]	8.44±0.43 [a]	1.32±0.45 [b]	1.46±0.75 [b]	ND [*]	1.52±0.80 [b]	ND [*]	ND [*]
Initial pH	7.20	6.55	4.02	4.35	4.04	4.34	3.91	3.95
Final pH	5.81	5.72	4.07	4.16	4.21	4.26	3.80	3.97

[A] Values are the means of two experiments (each with two replicate analyses) ± standard deviation.
[B] Means of bacterial growth at each sampling time in the same row with the same letters are not significantly different ($p > 0.05$)
ND: Not detected (detection level was ≤ 1 CFU/ml)
[*] Cells were not detected after overnight enrichment in MH broth

Table 4.7: Combined effects of Oriental mustard extract with malic acid or EDTA against *L. monocytogenes* (log_{10} CFU/ml) at 10 ºC [A] [B].

Day	Control	0.5% Mustard	0.2% Malic acid	0.2% EDTA	0.5% Mustard + 0.2% Malic acid	0.5% Mustard + 0.2% EDTA	0.2% EDTA + 0.2% Malic Acid	0.5% Mustard + 0.2% EDTA + 0.2% Malic Acid
0	3.54±0.29 [a]	3.54±0.29 [a]	3.54±0.29 [a]	3.54±0.29 [a]	3.54±0.29 [a]	3.54±0.29 [a]	3.54±0.29 [a]	3.54±0.29 [a]
1	4.67±0.54 [a]	3.33±0.07 [b]	3.42±0.30 [b]	3.37±0.16 [b]	3.39±0.10 [b]	3.41±0.18 [b]	3.41±0.17 [b]	3.31±0.08 [b]
3	7.49±0.37 [a]	3.30±0.01 [b]	3.36±0.07 [b]	3.40±0.01 [b]	3.38±0.12 [b]	3.38±0.09 [b]	3.35±0.01 [b]	3.33±0.10 [b]
7	8.80±0.05 [a]	3.31±0.10 [bc]	3.36±0.11 [bc]	3.23±0.01 [bc]	3.45±0.26 [b]	3.38±0.16 [bc]	2.91±0.56 [c]	3.23±0.04 [bc]
10	8.75±0.02 [a]	3.04±0.16 [bc]	3.29±0.01 [bc]	3.36±0.03 [b]	3.37±0.16 [b]	3.33±0.02 [b]	2.68±0.71 [c]	3.17±0.05 [bc]
14	8.52±0.10 [a]	3.09±0.18 [b]	3.31±0.13 [b]	3.37±0.02 [b]	3.05±0.63 [b]	3.40±0.13 [b]	2.28±1.16 [b]	2.89±0.11 [b]
Initial pH	7.20	6.55	4.02	4.35	4.04	4.34	3.91	3.95
Final pH	5.59	6.42	4.13	4.31	4.14	4.22	3.86	3.90

[A] Values are the means of two experiments (each with two replicate analyses) ± standard deviation.
[B] Means of bacterial growth at each sampling time in the same row with the same letters are not significantly different ($p > 0.05$)

Table 4.8: Viability of *L. monocytogenes* (log_{10} CFU/ml) in modified MH broth (pH 4.0) or modified 0.5% (w/v) Oriental mustard extract (pH 4.1) at 10 or 21 ºC

Temperature	Day	Control (pH=4.04) [A]	Mustard (pH = 4.09) [A]
21 ºC	0	3.65±0.07	3.65±0.07
	1	3.72±0.11	3.64±0.01
	3	4.54±0.98	3.54±0.10
	7	5.10±0.38	3.29±0.10
	10	5.75±0.59	3.02±0.11
	14	5.52±0.41	2.30±0.11
10 ºC	0	3.65±0.07	3.65±0.07
	1	3.54±0.10	3.41±0.13
	3	3.63±0.06	3.46±0.12
	7	3.45±0.15	3.37±0.05
	10	3.65±0.03	3.62±0.13
	14	3.61±0.12	3.44±0.09

[A] pH was adjusted using HCl

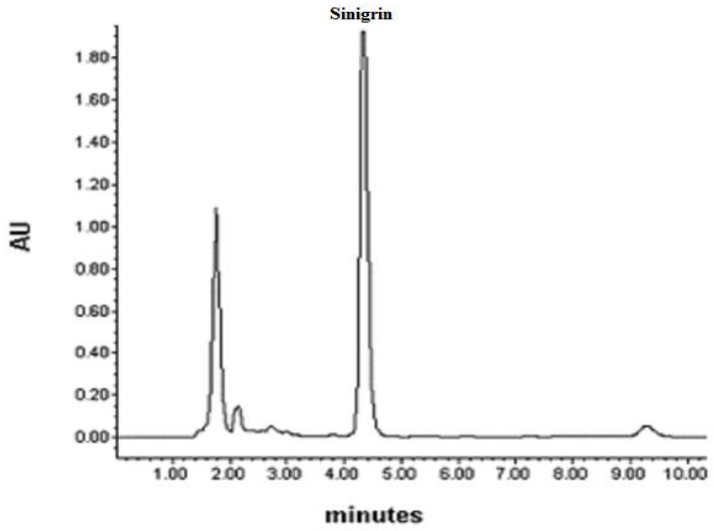

Fig. 4.1: HPLC chromatogram of sinigrin at 227 nm

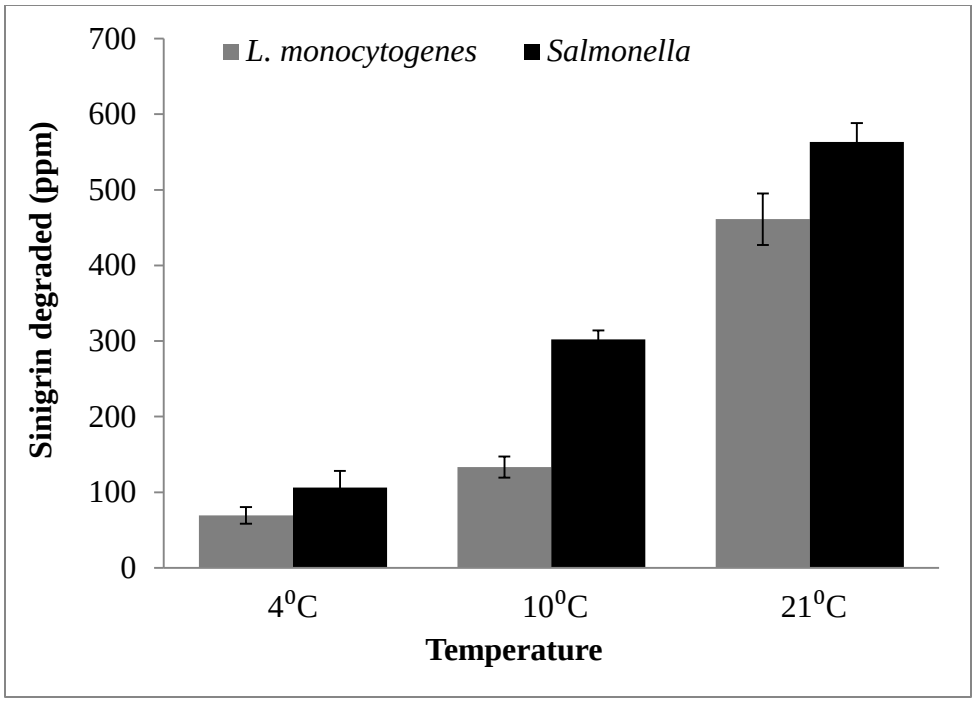

Fig. 4.2: Degradation of sinigrin (ppm) from Oriental mustard by *L. monocytogenes* or *Salmonella* cocktails after 21 d at 3 temperatures.

Chapter 5

Influence of Temperature, Glucose and Iron on Sinigrin Degradation by *Salmonella* and *L. monocytogenes*

5.1 Abstract

Factors including pH, temperature, glucose concentration and iron compounds affect the activity of plant myrosinase and consequently, endogenous glucosinolate degradation. These factors also may affect glucosinolate degradation by bacterial myrosinase, therefore this study examined the effect of temperature (4-21 ºC), glucose (0.05-1.0%) and iron (10 mM ferrous or ferric) on sinigrin degradation by *Salmonella* or *L. monocytogenes* cocktails in Mueller-Hinton (MH) broth and the effect of sinigrin degradation on bacterial viability. The degradation of sinigrin by both pathogens increased with higher temperatures (21 ºC > 10 ºC > 4 ºC). *Salmonella* and *L. monocytogenes* cocktails hydrolyzed 59.1% and 53.2% of sinigrin, respectively, at 21 ºC up to 21 d. Both iron compounds significantly enhanced sinigrin degradation by the pathogens. At day 7, sinigrin was not detected when the *Salmonella* cocktail was cultured with ferrous iron or when the *L. monocytogenes* cocktail was cultured in MH broth containing ferric iron. In contrast, ferric and ferrous iron inhibited the activity of 0.002 U/ml myrosinase from white mustard by 63% and 35% at day 1. *Salmonella* and *L. monocytogenes* cocktails were able to degrade >80% of sinigrin at 0.05 and 0.1% glucose; however, 0.25-1.0% glucose significantly reduced sinigrin degradation. Although both pathogens significantly degraded sinigrin, the allyl isothiocyanate (AITC) recoverable was ≤ 6.2 ppm which is not inhibitory to *Salmonella* or *L. monocytogenes*. It is probable that the gradual hydrolysis of sinigrin to form AITC either did not produce an inhibitory level of AITC or the AITC formed

was unstable in the aqueous medium and rapidly decomposed to new compounds which were less bactericidal against the pathogens.

5.2 Introduction

Spices and their essential oils are used in the food industry as preservatives for extending the shelf-life of foods, inhibiting foodborne pathogens, and increasing the overall quality of food products (Holley and Patel, 2005; Tajkarimi et al., 2010). Studies have shown that mustard powder or its components are effective against foodborne pathogens including *E. coli* O157:H7, *Campylobacter jejuni*, *Salmonella* and *L. monocytogenes* plus others (Dufour et al., 2012; Luciano et al., 2011; Olaimat and Holley, 2013 [Chapter 3]; Zou et al., 2013). Development of spicy flavor from hot mustard is the factor limiting the use of mustard in food systems; however, thermal treatment (115 ºC for 15 min) inactivates the plant myrosinase and produces deodorized (de-heated) mustard which has a bland flavor without spiciness and this enables its use in food as a thickener, stabilizer, emulsifier and antioxidant.

Glucosinolates are found in mustard and other plants of the family *Brassicaceae* as secondary metabolites which are subsequently hydrolyzed by endogenous myrosinase, a thioglucohydrolase (EC 3.2.1.147), when the plants tissues are damaged. The hydrolysis of glucosinolates forms glucose and an unstable intermediate, thiohydroximate-*O*-sulfonate which undergoes further transformation to yield primarily isothiocyanate, nitrile and thiocyanate but epithionitrile, oxazolidine-2-thione and other less common products can be formed depending upon the nature of the glucosinolate and the conditions during its hydrolysis (Fahey et al., 2001; Rask et al., 2000). Sinigrin, a glucosinolate found in Brussels sprouts, broccoli and Oriental mustard, can be hydrolyzed to form allyl isothiocyanate (AITC) which is antimicrobial, but

unstable in aqueous solution (Fahey et al., 2001; Luciano et al., 2011; Olaimat and Holley, 2013 [Chapter 3]).

Several studies have shown that glucosinolates also can be degraded by different bacterial species which possess myrosinase-like activity such as: *Lactobacillus agilis* (Palop et al., 1995), *Bacillus* and *Staphylococcus* (Brabban and Edwards, 1994), *Bacteroides thetaiotaomicron* (Elfoul et al., 2001), human faecal microflora (Krul et al., 2002), *Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, Bifidobacterium longum* (Cheng et al., 2004), *Pediococcus pentosaceus*, *Staphylococcus carnosus*, *Escherichia coli* O157:H7 (Graumann and Holley, 2008), *Lactobacillus curvatus* and *Lactobacillus plantarum* (Luciano et al., 2011), *S.* Typhimurium, *Enterococcus faecalis* and *L. monocytogenes* (Herzallah et al., 2011). It is currently unknown whether bacteria have more than one enzyme capable of hydrolyzing glucosinolates.

The properties of plant myrosinase have been investigated in different cruciferous vegetables, such as yellow mustard (Ohtsuru and Hata, 1972), rapeseed (Lönnerdal and Janson, 1972), wasabi (Ohtsuru and Kawatani, 1979), Brussels sprouts (Springett and Adams, 1989), oilseed rape (James and Rossiter, 1991), Oriental mustard (Tsao et al., 2000), crambe (Finiguerra et al., 2001) and black mustard seeds (Stoin et al., 2009). Factors including pH value, temperature, iron compounds and glucose concentration affect the activity of plant myrosinase and consequently, sinigrin decomposition rate and products formed. Addition of 1 mM Fe^{2+} completely inhibited the activity of myrosinase from cauliflower while 1 mM Fe^{3+} or 50 mM glucose reduced myrosinase activity by 50%, compared to controls without these additions (Prakash et al., 2013). The optimum temperature of plant myrosinase activity ranged from 37 ºC to 60 ºC based upon its source (Al-Turki and Dick, 2003; Hochkoeppler and Palmieri, 1992;

Sharma and Garg, 1996; Springett and Adams, 1989). These factors likely influence the ability of mustard to inhibit pathogens in food, and they may affect the activity of bacterial myrosinase during degradation of glucosinolates. However, little information is available on bacterial myrosinase activity and no studies have examined the effect of temperature, iron compounds or glucose concentration on the activity of the myrosinase-like enzyme(s) from *Salmonella* or *L. monocytogenes*. Thus, the objectives of the present study were to study the influence of these factors on the activity of *Salmonella* or *L. monocytogenes* myrosinase(s) and examine the effect of sinigrin degradation on bacterial viability.

5.3 Materials and methods

5.3.1 Materials

Sinigrin from horseradish, plant myrosinase (thioglucosidase from *Sinapis alba* seed, white [alternately called yellow] mustard) and glucose were from Sigma Chemical Co. (St. Louis, MO, USA). Tetrabutylammonium hydrogen sulfate HPLC grade (98%), HPLC grade acetonitrile, reagent grade potassium dihydrogen phosphate, ferric ammonium sulfate ($FeNH_4(SO_4)_2$), ferrous sulfate ($FeSO_4$), and sodium hydroxide (NaOH) were from Fisher Scientific Co. (Fair Lawn, NJ, USA).

5.3.2 Bacterial strains

Salmonella (*S.* Heidelberg 271, *S.* Typhimurium 02:8423, *S.* Copenhagen PT 99, *S.* Enteritidis CRIFS 1016, and *S.* Kentucky 64701), and *L. monocytogenes* strains (2-138, 2-243, GLM-1, GLM-3, GLM-5) were from the culture collection of the Food Science Department, University of Manitoba.

5.3.3 Culture preparation and Growth Conditions

Organisms were stored in Brain Heart Infusion (BHI) Broth (Oxoid Ltd., Basingstoke, England) containing 25% (v/v) glycerol at -80 ºC and were activated by streaking one loopful from each strain on BHI agar (Oxoid) which was incubated at 37 ºC for 24 h to 48 h. One colony of each *L. monocytogenes* strain was streaked on *Listeria* Selective Agar Base with *Listeria* Selective Supplement (LSA, Oxoid) and *Salmonella* serovars were streaked on Xylose Lysine Deoxycholate (XLD) agar (Oxoid), and incubated at 37 ºC for 24 h to 48 h. A single colony of *L. monocytogenes* or *Salmonella* strains from the selective agars was transferred to Mueller-Hinton (MH) broth and incubated overnight at 37 ºC. Organisms were suspended again in MH broth and incubated as before. Then, 0.1 % (v/v) of this culture was transferred to fresh MH broth and incubated overnight at 37 ºC. Separate mixtures of *Salmonella* or *L. monocytogenes* strains were prepared by combining 2 ml of each of the 5 freshly cultured organisms in a sterile container which was used in the experiments.

5.3.4 Effect of different temperatures, iron compounds and glucose concentrations on sinigrin degradation by *Salmonella* or *L. monocytogenes*

The effect of temperature on sinigrin degradation was investigated by transfer of 9.9 ml MH broth containing 0.13% sinigrin to sterile 12 ml screw-capped tubes which were inoculated with 0.1 ml of 8 $\log_{10}$ CFU/ml mixtures of equal numbers of 5 strain component *L. monocytogenes* or *Salmonella* cocktails at stationary phase. Tubes containing sinigrin and cultures were mixed to yield cultures containing 6 $\log_{10}$ CFU/ml of either *L. monocytogenes* or *Salmonella* which were incubated at 4, 10 or 21 ºC with shaking at 200 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA) for 21d.

Sinigrin degradation by *Salmonella* or *L. monocytogenes* cocktails was measured in the presence of iron (Fe^{2+} and Fe^{3+}) or glucose (0.05-1.0%) by transferring 9.9 ml buffered (potassium dihydrogen phosphate, pH 7.0) MH broth containing approximately 0.1% sinigrin and either 10 mM Fe^{2+} or 10 mM Fe^{3+} (9.4 ml MH broth containing 0.1% sinigrin + 0.5 ml of Fe^{2+} or Fe^{3+} solutions (0.2 M $FeSO_4$ or $FeNH_4(SO_4)_2$), respectively (adapted from Borek et al. (1994) or 0.05 to 1.0% glucose instead of iron to sterile screw-capped test tubes which were inoculated with 0.1 ml of 8 $\log_{10}$ CFU/ml of *L. monocytogenes* or *Salmonella* cocktails and incubated with shaking for 21d at 25 ºC. Experiments with $FeSO_4$ and $FeNH_4(SO_4)_2$) were repeated with $FeCl_2$ and $FeCl_3$. Sinigrin degradation by 0.002 U/ml myrosinase from white mustard was performed in the presence or absence of ferric and ferrous iron using the same conditions as outlined for bacteria.

Samples of 1 ml from all experiments were taken initially and at 3, 7, 14 and 21 d for bacterial enumeration and sinigrin analysis by reversed phase-liquid chromatography (RP-HPLC). The same procedure in each experiment was repeated for control cells without sinigrin as well as negative controls containing sinigrin without bacteria, and controls containing sinigrin with bacteria killed by heating at 100^{o}C for 1 min.

5.3.5 Sinigrin analysis by RP-HPLC

Samples for sinigrin analysis were filter-sterilized (0.22 μm, Millipore, Cork, Ireland) in sterile HPLC vials. HPLC solvents used were 0.02 M tetrabutylammonium hydrogen sulphate (pH 5.5) and acetonitrile. Solvents initially were sterilized using 0.45 mm Millipore nylon filters (Fisher Scientific, Nepean, ON, Canada), and degassed for 30 min in a Bransonic bath (Branson, 5510, Grass Valley, CA, USA). Sinigrin degradation was analyzed based on the method used by

Lara-Lledó et al. (2012) using an RP-HPLC system (Waters 2695, Waters Corporation, Milford, MA, USA). Sinigrin was quantified using a calibration curve established from 100-1500 ppm using a sinigrin standard (Sigma-Aldrich, St Louis MO, USA).

5.3.6 Statistical analysis

All tests were performed in two experiments with duplicate analyses (n = 4) and results were represented by means ± SD. Data were subject to an analysis of variance (ANOVA) using JMP 10.0 software (SAS Inst. Inc., Cary, N.C., U.S.A.) and significant differences between strains or treatments were estimated by student's t test ($P < 0.05$).

5.4 Results and Discussion

5.4.1 Effect of temperature on myrosinase-like activity of *Salmonella* and *L. monocytogenes*

The degradation of sinigrin by bacterial myrosinase increased at higher temperatures (21 ºC > 10 ºC > 4 ºC). By 21d, the *Salmonella* cocktail hydrolyzed 13.0, 22.0 or 59.1% of sinigrin at 4, 10 or 21 ºC, respectively, while the *L. monocytogenes* cocktail hydrolyzed 9.1, 26.3 or 53.2% of sinigrin at the same temperatures, respectively (Table 5.1). These results are similar to those of Tani et al. (1974) who found that the optimum temperature for bacterial myrosinase from *Enterobacter cloacae* ranged from 25 ºC to 40 ºC, with higher stability between 25-30 ºC. Similarly, a thioglucoside hydrolase capable of sinigrin hydrolysis in cell-free extracts of *Sphingobacterium* sp. strain OTG1 had higher stability between 20-30 ºC (Meulenbeld and Hartmans, 2001). Although *L. monocytogenes* showed the ability to grow at 4 ºC, significant sinigrin degradation (10%) was observed only at day 3, beyond which no significant changes occurred up to day 21. At 4 ºC it was likely that myrosinase synthesis by *Salmonella* and *L.*

monocytogenes was delayed by the lower temperature. In the case of plant myrosinase, Al-Turki and Dick (2003) and Ohtsuru and Kawatani (1979) found that the optimum temperature for myrosinase activity following extraction from *Sinapis alba* and *Wasabia japonica* was 37 ºC. In addition, Li and Kushad (2005) found that myrosinase extracted from horseradish (*Armoracia rusticana*) roots exhibited high activity at 37 and 45 ºC. However, in other studies it was found that plant myrosinase had an optimum temperature range of 50 to 75 ºC (Bones and Slupphaug, 1989; Hochkoeppler and Palmieri, 1992; Sharma and Garg, 1996; Springettand Adams, 1989).

5.4.2 Effect of iron compounds on myrosinase-like activity of *Salmonella* and *L. monocytogenes*

Interestingly, both ferric and ferrous ions at 10 mM significantly enhanced sinigrin degradation by *Salmonella* and *L. monocytogenes* cocktails. Ferrous ion was initially more effective with both organisms, enabling *Salmonella* to hydrolyze 93.5% and *L. monocytogenes* to degrade 94.8% of sinigrin by 3 d (Table 5.2), while sinigrin was stable in the absence of bacteria (reduced by $\leq$ 6.9%) and when bacteria were heat-killed. At day 7, sinigrin was not detected when the *Salmonella* cocktail was cultured in MH broth containing ferrous iron or when the *L. monocytogenes* cocktail was cultured in MH broth containing ferric iron. By day 21 with both pathogens sinigrin was not detected in MH broth with either ferric or ferrous iron (Table 5.2). In contrast, 10 mM ferric or ferrous iron inhibited the activity of 0.002 U/ml myrosinase from white mustard by 63% and 35% at day 1 under the same incubation conditions (Table 5.3). In the present work, it was not clear whether the presence of ferric or ferrous iron enhanced the expression and/or activity of bacterial myrosinases. However, with purified myrosinase from *E. cloacae*, Tani et al. (1974) found that 1 mM ferric and ferrous chloride reduced its activity by 18% and 68%, respectively, at 37 ºC compared to a control without added iron. Further, Uda et

al. (1986) found 2.5 mM ferrous ion had no inhibitory effect on sinigrin degradation by myrosinase extracted from takana (*Brassica juncea* L.). Myrosinase from cauliflower was completely inhibited in the presence of 1 mM ferrous ion, while 1 mM ferric ion reduced myrosinase activity by 50% compared to a negative control. This inhibition was attributed to iron blocking the substrate binding site or its causing instability of the enzyme-substrate complex (Prakash et al., 2013). Since two sulfate salts of iron were initially used in the current work, ferric and ferrous chloride were used in separate tests at 1-10 mM to confirm the role of iron. These compounds were also found to enhance the degradation of sinigrin in the presence of *Salmonella* or *L. monocytogenes* at a rate similar to the sulfate salts (sinigrin was not detected after 7 d), but they inhibited sinigrin degradation by white mustard myrosinase. It was evident that the effect of iron compounds on myrosinase activity was influenced by the source of the enzyme. It is recognized that the comparison here between unpurified and purified enzymes is less than ideal, but is necessitated by the small amount of information available on purified bacterial myrosinases. In the current study, iron compounds appeared to improve the catalytic activity of bacterial myrosinase-like enzymes.

5.4.3 Effect of glucose concentration on myrosinase-like activity of *Salmonella* and *L. monocytogenes*

The degradation of sinigrin by myrosinase-like activity decreased with higher glucose concentrations. *Salmonella* and *L. monocytogenes* cocktails were able to degrade > 80% of sinigrin at 0.05 and 0.1% glucose, but 0.25 to 1.0% glucose significantly reduced its hydrolysis. Glucose at 1.0% reduced sinigrin degradation by 2.4 to 4.0-fold at 21 d; only 21.1% and 38.2% of sinigrin was hydrolyzed by *Salmonella* and *L. monocytogenes*, respectively, compared to 81.3% and 82.3% sinigrin at 0.1% glucose (Table 5.4). Sinigrin hydrolysis by the myrosinase-

like activity of *Pediococcus pentosaceus* was also enhanced by glucose at 0.1% or 0.25% and 25 ºC after 21 d (unpublished data, this lab). As was observed here at higher glucose levels, Tani et al. (1974) found that 1.8% glucose reduced *E. cloacae* myrosinase activity by 68% at pH 6.8 and 37 ºC. The activity of myrosinase from cauliflower was also decreased in the presence of 0.09-0.9% glucose and the higher concentration (0.9%) reduced myrosinase activity by >50% (Prakash et al., 2013). Glucose can cause repression of enzyme synthesis/action, termed feedback inhibition, when the catalytic products of the targeted enzyme include glucose (Muñoz et al., 2011). It is likely that in the presence of glucose and sinigrin, *Salmonella* and *L. monocytogenes* preferentially used glucose for biosynthesis and energy production because of its ease of assimilation. Following glucose depletion from the medium, repression of myrosinase synthesis would be reversed, leading to glucose release following sinigrin hydrolysis by that enzyme.

5.4.4 Effect of sinigrin degradation on viability of *Salmonella* or *L. monocytogenes*

Although *Salmonella* and *L. monocytogenes* cocktails degraded significantly more sinigrin to form AITC in the presence of ferric and ferrous ions after 3 d, the growth of both pathogens was not prevented (Table 5.5). The inhibitory effect of AITC against the tested cultures was studied by Olaimat and Holley (2013 [Chapter 3]) and it was found that the minimum inhibitory concentration (MIC) of AITC against *Salmonella* and *L. monocytogenes* cocktails was 10 ppm and 40 ppm, respectively, at 21 ºC. Kawakishi and Muramatsu (1966) found that the amount of AITC formed during degradation of glucosinolates by plant myrosinase could reach 90%. If the action of bacterial myrosinase-like enzyme(s) were similar here, the sinigrin hydrolyzed (355-997 ppm) would have been sufficient to form AITC at levels (319-898 ppm) substantially higher than the MIC against either pathogen. However, it has been found that iron compounds can affect the hydrolysis products generated by plant myrosinase by shifting their proportion such

that more allyl nitrile than AITC is produced from sinigrin at pH 4.0 or 6.0 when 10 mM ferric or ferrous ions was present (Borek et al., 1994). In the current study, AITC formed was measured by RP-HPLC and reached a maximum level of only 6.2 ppm at day 3 which would not have been inhibitory to either pathogen even though ferric or ferrous ions had caused enhanced sinigrin hydrolysis.

However, the hydrolysis of sinigrin to form AITC by bacterial myrosinase at 21 ºC in the presence of < 0.25% glucose without added iron did not prevent growth of *Salmonella* or *L. monocytogenes* cocktails either (Table 5.6). It is probable that the gradual hydrolysis of sinigrin to form AITC did not produce an inhibitory level of AITC and/or the AITC formed was unstable in the aqueous medium and rapidly decomposed to new compounds which were less bactericidal against the pathogens. The instability of AITC in sterile MH broth at neutral pH was observed by Olaimat and Holley (2013 [Chapter 3]). Under these conditions AITC was not recoverable after 18 h at 37 ºC or 6d at 21 ºC. It was evident that the growth of *Salmonella* and *L. monocytogenes* was unaffected by sinigrin degradation products under the experimental conditions used. Similar results were obtained by Lara-Lledó et al. (2012) who found that deodorized mustard and pure sinigrin were not inhibitory to *L. monocytogenes* in broth, however, the pathogen was not detected at day 52 in inoculated bologna packed in an edible film containing Oriental mustard extract (5% sinigrin) stored at 4 ºC.

5.5 Conclusions

The degradation of sinigrin by the myrosinase-like enzyme(s) of *Salmonella* and *L. monocytogenes* was clearly affected by incubation temperature, the presence of iron compounds and the glucose concentration, where its activity was increased with higher temperature (21 ºC > 10 ºC > 4 ºC), and the presence of 10 mM ferric or ferrous ions. However glucose at ≥ 0.25%

significantly reduced the activity/synthesis of bacterial myrosinase. Both pathogens degraded significantly more sinigrin at 21 ºC, with 10 mM ferric or ferrous iron, or with < 0.25% glucose at 25 ºC, but in spite of the potential for greater AITC formation, the growth of *Salmonella* or *L. monocytogenes* cocktails was not prevented. It is probable that AITC was not the major compound formed during the hydrolysis of sinigrin in the presence of ferric or ferrous ions, or that the AITC formed was unstable in the aqueous medium and rapidly decomposed to new compounds which were less bactericidal against the pathogens.

Table 5.1: Effect of temperature on degradation of sinigrin by *Salmonella* or *L. monocytogenes* cocktails.

Bacteria	Temperature (ºC)	Percent of sinigrin hydrolyzed during days storage [A, B, C]			
		3	7	14	21
Salmonella	4	10.2±0.4 [c]	9.5±0.1 [bc]	15.8±7.9 [bc]	13.0±1.3 [d]
	10	11.9±0.8 [bc]	11.7±0.4 [bc]	19.9±2.4 [b]	22.0±3.4 [c]
	21	20.5±1.5 [a]	26.1±3.1 [a]	41.0±0.7 [a]	59.9±2.9 [a]
L. monocytogenes	4	10.9±0.1 [c]	7.8±1.6 [c]	8.1±2.9 [c]	9.1±0.9 [d]
	10	12.4±4.8 [bc]	13.2±0.4 [b]	14.4±6.5 [bc]	26.3±0.3 [c]
	21	16.3±0.9 [ab]	26.2±3.2 [a]	47.6±0.5 [a]	53.2±1.9 [b]

[A]Values are the means of two experiments ± standard deviation.

[B]Means of sinigrin hydrolyzed (%) at each sampling time in the same column with the same letters are not significantly different ($p > 0.05$).

[C] 100% = 1285 ppm.

Table 5.2: Effect of 10 mM iron on degradation of sinigrin by *Salmonella* or *L. monocytogenes* cocktails at 25 ºC.

Bacteria	Iron	Percent of sinigrin hydrolyzed during days storage [A, B, C]			
		3	7	14	21
Salmonella	Ferric	33.7±0.4 [b]	65.2±0.1 [b]	95.0±0.6 [b]	>99.3 [a*]
	Ferrous	93.5±0.6 [a]	>99.3 [a]	>99.3 [a]	>99.3 [a]
L. monocytogenes	Ferric	84.0±6.7 [a]	>99.3 [a]	>99.3 [a]	>99.3 [a]
	Ferrous	94.8±6.0 [a]	98.8±2.5 [a]	>99.3 [a]	>99.3 [a]

[A] Values are the means of two experiments ± standard deviation.

[B] Means of sinigrin hydrolyzed (%) at each sampling time in the same column with the same letters are not significantly different ($p > 0.05$)

[C] 100% = 1052 ppm.

[*] sinigrin was not detected, 100%-detection level (10 ppm = 0.7%)

Table 5.3: Sinigrin hydrolysis (%) by white mustard myrosinase in MH broth without or with 10 mM ferric or ferrous iron at 25 ºC for 24 h.

Control (without iron)	Ferric	Ferrous
96.65±1.76	35.75±1.19	62.61±5.98

Table 5.4: Effect of glucose concentration on degradation of sinigrin *Salmonella* or *L. monocytogenes* cocktails at 25 ºC.

Bacteria	Glucose %	Percent of sinigrin hydrolyzed during days storage [A, B, C]		
		7	14	21
Salmonella	0	48.8±1.2 [a]	67.9±7.5 [abc]	86.2±0.1 [a]
	0.05	34.4±0.9 [bcd]	54.6±8.0 [abc]	80.3±1.6 [a]
	0.1	29.5±0.1 [cd]	53.1±6.6 [abc]	81.3±1.0 [a]
	0.25	25.4±0.6 [cd]	43.8±2.2 [cd]	53.7±3.7 [c]
	0.5	20.9±1.2 [d]	43.9±2.0 [bcd]	52.9±1.0 [c]
	1	4.3±3.5 [e]	15.3±4.2 [e]	21.1±7.2 [d]
L. monocytogenes	0	52.3±2.1 [a]	78.6±0.3 [a]	90.0±1.0 [a]
	0.05	45.6±2.0 [ab]	73.4±4.6 [a]	85.2±1.9 [a]
	0.1	45.4±0.1 [ab]	71.9±1.8 [ab]	82.3±0.8 [a]
	0.25	34.8±0.2 [bcd]	58.0±1.0 [abc]	74.6±1.7 [ab]
	0.5	24.3±1.2 [cd]	44.5±2.0 [bcd]	55.8±1.5 [bc]
	1	6.5±2.8 [e]	17.9±4.8 [de]	38.2±2.6 [cd]

[A] Values are the means of two experiments ± standard deviation.

[B] Means of sinigrin hydrolyzed (%) at each sampling time in the same column with the same letters are not significantly different ($p > 0.05$)

[C] 100% = 922.2 ppm for *Salmonella* strains or 1133.4 ppm for *L. monocytogenes* strains

Table 5.5: Viability of *Salmonella* or *L. monocytogenes* cocktails (log CFU/ml) in the presence of 0.1% sinigrin and 10 mM ferric or ferrous iron in buffered MH broth (pH 7.0) at 25 ºC.

Bacteria	Iron	Treatment	Viability of *Salmonella* or *L. monocytogenes* (Log_{10} CFU/ml) during days storage [A, B]				
			0	3	7	14	21
Salmonella	Ferric	Control	6.12±0.07 [b]	8.60±0.51 [ab]	8.91±0.04 [a]	8.65±0.18 [a]	8.58±0.49 [a]
		Singrin	6.12±0.07 [b]	8.80±0.20 [a]	8.35±0.13 [bc]	7.77±0.07 [d]	7.78±0.13 [cd]
	Ferrous	Control	6.12±0.07 [b]	8.97±0.16 [a]	8.89±0.12 [a]	8.69±0.19 [a]	8.31±0.33 [ab]
		Singrin	6.12±0.07 [b]	8.75±0.06 [a]	8.11±0.08 [cd]	7.92±0.07 [cd]	7.97±0.07 [bc]
L.	Ferric	Control	7.14±0.22 [a]	8.16±0.49 [c]	8.07±0.37 [d]	8.07±0.27 [bc]	7.90±0.33 [c]
monocytogenes		Singrin	7.14±0.22 [a]	8.26±0.12 [bc]	8.22±0.10 [bcd]	7.91±0.08 [cd]	7.63±0.08 [cd]
	Ferrous	Control	7.14±0.22 [a]	8.12±0.12 [c]	8.23±0.14 [bcd]	8.02±0.15 [bcd]	7.53±0.04 [d]
		Singrin	7.14±0.22 [a]	8.69±0.22 [a]	8.38±0.28 [b]	8.26±0.25 [b]	7.49±0.01 [d]

[A] Values are the means of two experiments ± standard deviation.

[B] Means of bacterial growth at each sampling time in the same column with the same letters are not significantly different ($p > 0.05$).

Table 5.6: Effect of temperature on degradation of sinigrin by *Salmonella* or *L. monocytogenes* cocktails.

Bacteria	Temperature (ºC)	Sinigrin hydrolyzed (%) during days storage [A, B, C]			
		3	7	14	21
Salmonella	4	10.2 (0.4) [c]	9.5 (0.1) [bc]	15.8 (7.9) [bc]	13.0 (1.3) [d]
	10	11.9 (0.8) [bc]	11.7 (0.4) [bc]	19.9 (2.4) [b]	22.0 (3.4) [c]
	21	20.5 (1.5) [a]	26.1 (3.1) [a]	41.0 (0.7) [a]	59.9 (2.9) [a]
L. monocytogenes	4	10.9 (0.1) [c]	7.8 (1.6) [c]	8.1 (2.9) [c]	9.1 (0.9) [d]
	10	12.4 (4.8) [bc]	13.2 (0.4) [b]	14.4 (6.5) [bc]	26.3 (0.3) [c]
	21	16.3 (0.9) [ab]	26.2 (3.2) [a]	47.6 (0.5) [a]	53.2 (1.9) [b]

[A] Values are the means of two replicates (standard deviations).
[B] Means of sinigrin hydrolyzed (%) at each sampling time in the same column with the same letters are not significantly different ($p > 0.05$)
[C] 100%=1285 ppm.

Chapter 6

Inhibition of *Listeria moncytogenes* on Cooked, Cured Chicken Breasts by Malic or Acetic Acid-κ-Carrageenan/Chitosan-Based Coatings Containing Allyl Isothiocyanate or Deodorized Oriental Mustard Extract

6.1 Abstract

Ready-to-eat meats are considered foods at high risk to cause life-threatening *L. monocytogenes* infections. This study aimed to screen 5 *L. monocytogenes* strains for their ability to hydrolyze sinigrin (a glucosinolate in Oriental mustard) and form allyl isothiocyanate (AITC) by monitoring the resulting reduction of *L. monocytogenes* viability on inoculated vacuum-packed, cooked, cured roast chicken slices at 4 ºC. This involved incorporation of 25 to 50 µl/g AITC directly or 100 to 250 mg/g Oriental mustard extract in 0.5% (w/v) κ-carrageenan/ 2% (w/v) chitosan-based coatings prepared using 1.5% (w/v) malic acid or 1.5% (v/v) acetic acid. *L. monocytogenes* strains degraded 33.6% to 48.4% of pure sinigrin in MH broth tests by 21 d at 25 ºC. Acetic or malic acid-κ-carrageenan/chitosan coatings containing 25 to 50 µl/g AITC or 100 to 250 mg/g mustard reduced viable numbers of *L. monocytogenes* and aerobic bacteria on cooked, cured roast chicken slices by 4.1 to > 7.0 $\log_{10}$ CFU/g compared to uncoated chicken stored at 4 ºC by 70 d. However, coatings containing malic acid were significantly more antimicrobial than acetic acid-based coatings. During storage, acetic or malic acid-κ-carrageenan/chitosan coatings containing 25 to 50 µl/g AITC or 250 mg/g mustard extract reduced LAB numbers 3.8 to 5.4 $\log_{10}$ CFU/g on chicken slices by 70 d compared to uncoated samples. Malic or acetic acid-κ-

carrageenan/chitosan-based solutions containing 25 to 50 µl/g AITC or 100 to 250 mg/g Oriental mustard extract were shown to have the ability to control *L. monocytogenes* viability and delay growth by potential spoilage bacteria on refrigerated, vacuum-packed cured roast chicken.

6.2 Introduction

Listeria monocytogenes is a Gram-positive, nonspore-forming, motile, facultatively anaerobic, rod-shaped bacterium. The organism is commonly found in many environments including soil, water, manure, dust, silage as well as in animals and it can grow over a wide range of temperatures (-0.4–45 °C), pH values (4.3–9.6), salt concentrations (10%), and water activity < 0.93 (McClure et al., 1997; Pellicer et al., 2011). These characteristics enable the pathogen to survive and multiply in the food processing environment and in/on fresh and processed foods which make *L. monocytogenes* a significant problem for the food industry. The heat treatment used for the manufacture of ready-to-eat (RTE) meat and poultry products is sufficient to eliminate *L. monocytogenes*; however, these products can be re-contaminated with the organism during subsequent processing steps (Food Safety and Inspection Service, FSIS, 2014). Todd and Notermans (2010) reported that the prevalence rates of *L. monocytogenes* in RTE meat products in the US and Canada ranged from 0.4 to 71% and 0.0 to 21%, respectively.

L. monocytogenes is an opportunistic foodborne pathogen that can cause severe forms of infection in humans. In healthy people, listeriosis causes flu-like symptoms. However, severe complications like meningitis, septicemia, spontaneous abortion or infection of the newborn may also occur in susceptible groups or individuals (FSIS, 2014). Listeriosis outbreaks are rare, but the mortality rate can be high (20-30%), and may reach 75% in pregnant women, neonates, and immunocompromised adults (Jalali and Abedi, 2008). In the US, listeriosis accounts for about

1600 illnesses annually (Scallan et al., 2011). In 2008, a significant listeriosis outbreak occurred in 7 provinces in Canada which was linked to delicatessen meat and caused 57 illnesses with 23 deaths (Farber et al., 2011).

Plant-derived natural antimicrobials have been effectively used as food preservatives (Holley and Patel, 2005). Mustard, including the black cultivar (*Brassica nigra* L.), Oriental (brown, *B. juncea* L.), and white or yellow types (*Sinapis alba* or *B. hirta*), belong to the family *Brassicaceae* which also includes cabbage, broccoli, horseradish, wasabi and cauliflower. These plants contain secondary metabolites, glucosinolates, which are organic compounds containing nitrogen and sulfur. The glucosinolate, sinigrin, present in Oriental mustard, is hydrolyzed by endogenous plant myrosinase upon physical injury in the presence of moisture to yield allyl isothiocyanate (AITC) which is lethal toward major foodborne pathogens such as *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* and *Campylobacter jejuni* (Luciano and Holley, 2009; Olaimat and Holley, 2013[Chapter 3]; Olaimat et al., 2014a [Chapter 8]). In deodorized mustard (devoid of myrosinase), sinigrin is converted into AITC by the action of bacterial myrosinase-like enzyme(s) present in *E. coli* O157:H7, other VTEC *E. coli, Salmonella*, *L. monocytogenes* and *C. jejuni* (Herzallah et al., 2011; Luciano and Holley, 2011; Olaimat and Holley, 2014b [Chapter 4]; Olaimat et al., 2014a, b [Chapters 5, 8]). Therefore, it is probable that hydrolysis of sinigrin from Oriental mustard could be used to cause the inhibition of *L. monocytogenes* on RTE poultry meats.

Since incorporation of AITC or mustard powder may affect the physicochemical and organoleptic properties of poultry meat, an antimicrobial film or coating approach appeared to be a more compelling application. In other work reported later in the thesis, κ-carrageenan/chitosan-based coatings containing AITC or Oriental mustard extract were effective against *C. jejuni* and

Salmonella on fresh chicken breasts (Olaimat and Holley, 2014a [Chapter 7]; Olaimat et al., 2014a [Chapter 8]). The objectives of the current study were i) to determine the minimum inhibitory (MIC) and bactericidal (MBC) concentrations of AITC against 5 *L. monocytogenes* strains at 4 ºC, ii) to screen the 5 *L. monocytogenes* strains for their ability to degrade pure sinigrin by bacterial myrosinase, iii) to reduce *L. monocytogenes* viability on cooked, cured, sliced chicken breasts using edible κ-carrageenan/chitosan-based coatings (prepared with malic or acetic acid) containing AITC or Oriental mustard extract.

6.3 Materials and Methods

6.3.1 Chemicals

AITC (94%), chitosan (molecular weight: 100,000-300,000 Da; deacetylation degree: 75-85%), and HPLC grade tetrabutylammonium hydrogen sulphate (TBA), were from Acros Organics (Morris Plains, NJ, USA). Sinigrin from horseradish, glycerol (>99%) and κ-carrageenan from *Eucheuma cottonii* were from Sigma-Aldrich Co. (St. Louis, MO, USA). Acetonitrile HPLC grade, malic acid, acetic acid and hydrochloric acid (HCl) were from Fisher Scientific Co. (Fair Lawn, NJ, USA).

6.3.2 Bacterial strains and culture preparation

Five *L. monocytogenes* strains (2-138, 2-243, GLM-1, GLM-3, GLM-5) from the culture collection of the Food Science Department, University of Manitoba were stored in Brain Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, England) containing 25% (v/v) glycerol at -80 ºC. One loopful from each strain was streaked on *Listeria* Selective Agar Base with *Listeria* Selective Supplement (LSA, Oxoid) and incubated at 37 ºC for 48 h. A single colony of each *L.*

monocytogenes strain was transferred to BHI broth and incubated overnight at 37 ºC. Then, 0.1 % (v/v) of this culture was transferred to fresh BHI broth and incubated as before. Freshly prepared single cultures of *L. monocytogenes* were used in a sinigrin degradation test. A mixture of *L. monocytogenes* cultures was prepared by combining 8 ml of each the 5 freshly cultured strains in a sterile container and used for inoculation of cooked, cured chicken in experiments to evaluate the antimicrobial activity of coatings.

6.3.3 MIC and MBC of AITC against *L. monocytogenes* strains at 4 ºC

The MIC and MBC of AITC against *L. monocytogenes* strains were determined in 9 ml screw-capped tubes containing 7.92 ml Mueller-Hinton (MH) broth (pH 7.2) (Oxoid) with 1.25 to 640 ppm AITC and then 0.08 ml of each strain of *L. monocytogenes* (8 $\log_{10}$ CFU/ml) was added to yield 6 $\log_{10}$ CFU/ml in tubes which were mixed and incubated with shaking at 200 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA) for 10 d at 4 ºC. The lowest concentration at which no visible growth occurred was identified as the MIC, while the MBC was the lowest concentration which reduced the initial numbers by ≥ 3 $\log_{10}$ CFU/ml.

6.3.4 Screening of *L. monocytogenes* strains for sinigrin degradation

Mueller Hinton (MH, Oxoid) broth containing approximately 1000 ppm sinigrin was prepared by mixing 10 ml of a filter-sterilized (0.22 μm, Millipore, Cork, Ireland) sinigrin stock solution (50,000 ppm) with 490 ml sterile MH broth (pH 7.2). Then, 9.9 ml of MH broth with sinigrin was transferred to sterile 12 ml screw-capped tubes containing 0.1 ml of 8 $\log_{10}$ CFU/ml of individual stationary phase cultures of *L. monocytogenes* strains. Tubes containing sinigrin and cultures were incubated with shaking at 200 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA) for 21 d at 25 ºC. A negative control without *L. monocytogenes*

was prepared by mixing 0.1 ml sterile distilled water with 9.9 ml MH broth containing sinigrin. Samples of 1 ml were taken and filter-sterilized (0.22 µm, Millipore, Cork, Ireland) in sterile HPLC vials at 0, 1, 2, 3, 7, 14 and 21 d to measure the remaining sinigrin using a reversed phase-liquid chromatograph (RP-HPLC, model 2695, Waters Corporation, Milford, MA, USA) based on the method described by Lara-Lledó et al. (2012). Briefly, TBA, acetonitrile and double-distilled water were used as HPLC solvents which were sterilized using 0.45 mm Millipore nylon filters (Fisher Scientific, Nepean, ON, Canada) and degassed for 30 min in a Bransonic bath (Branson, 5510, Grass Valley, CA, USA). The sample injection volume was 10 µl. Sinigrin was quantitatively determined using a calibration curve (100-1500 ppm) established with a sinigrin standard under the same analytical conditions.

6.3.5 Preparation of antimicrobial coatings

An aqueous mustard extract was prepared as described by Olaimat et al. (2014a [Chapter 8]). κ-Carrageenan/chitosan coatings were prepared by adding 0.5% (w/v) κ-carrageenan to sterile distilled water with stirring for 1 h at 60 ºC. After cooling to room temperature, the mixture was divided in half and acetic acid (1.5%, v/v) or malic acid (1.5%, w/v) were added separately to each part. Chitosan (2% w/v) and glycerol (5% v/v) were added and mixed with a magnetic stirring bar overnight, and then homogenized at 8000 rpm using a homogenizer (L2R, Silverson Machines, Ltd., Waterside, England). Mustard extract to a final concentration of 100 and 250 mg/ml or AITC to concentrations of 25 and 50 µl/ml were added separately to the acetic or malic acid-κ-carrageenan/chitosan solutions. Control acetic or malic acid-κ-carrageenan/chitosan coatings without AITC or mustard extract were also prepared. Coatings were homogenized as described above and then the pH was adjusted to 3.5 using 0.2 M HCl.

6.3.6 Antimicrobial activity of coatings on cooked, cured chicken breast

Fresh cooked, cured chicken roast (chicken breast, water, salt, dextrose, sodium phosphate, spice extractives, carrageenan, sodium erythorbate, sodium nitrite, caramel) was obtained from Piller's Fine Foods, Waterloo, ON, Canada, the day after manufacture. The roast chicken was sliced into 20 ± 1 g pieces (8.5 cm diameter and 4 mm thick) using a model 410 meat slicer (Hobart Manufacturing Co., Troy, OH, USA). Inoculation culture was prepared by combining 8 ml from each of 5 *L. monocytogenes* strains and the mixture was centrifuged at 1100 xg for 20 min at 4 ºC. The pellets were washed twice using 40 ml sterile 0.1 M potassium phosphate buffer (pH 7.2, Sigma-Aldrich Co.) and re-centrifuged using the same conditions. The pellets were resuspended in 400 ml 0.1% (w/v) buffered peptone water to yield 8.0 $\log_{10}$ CFU/ml.

The cooked, cured roast chicken slices were inoculated by dipping in 400 ml of the *L. monocytogenes* mixture for 20 sec. Inoculated slices were allowed to dry in a laminar airflow hood for 30 min and then dipped in 100 ml κ-carrageenan/chitosan solutions for 20 sec. The samples were allowed to dry in a laminar-flow hood under ventilation for 45 min on each side. Inoculated samples without coating were also prepared. Single roast chicken slices were placed in commercial multi-layer high oxygen barrier plastic bags (Deli*1, 17.8 cm × 22.9 cm, Winpak Ltd., Winnipeg, MB, Canada) and sealed under vacuum using a model GM 2002 vacuum packaging machine (Bizerba Canada Inc., Mississauga, ON, Canada).

The vacuum-packaged slices were stored at 4 ºC for 70 d and samples were analyzed for viability of *L. monocytogenes* on LSA, lactic acid bacteria (LAB) on de Man, Rogosa and Sharpe agar (Oxoid), and aerobic bacteria on Plate Count Agar (Oxoid) at 25, 50, 75 and 100% of the shelf-life of the cooked, cured chicken (17, 35, 52 and 70 d, respectively). The PCA and LSA plates

were incubated aerobically at 37 ºC for 48 h while the MRS plates were incubated aerobically at 25 ºC for 48 h. The pH of the chicken slices was measured using an Accumet model 910 digital pH meter (Fisher Scientific, Pittsburgh, PA, USA).

6.3.7 Statistical analysis

All tests were performed in two experiments with duplicate analyses and results were expressed as means ± SD. Statistical differences among treatments were compared by Tukey's test using JMP 10.0 software (SAS Inst. Inc., Cary, N.C., USA) and differences were considered significant when $P < 0.05$.

6.4 Results and Discussion

6.4.1 Screening of *L. monocytogenes* strains for myrosinase-like activity

L. monocytogenes strains varied in their ability to hydrolyze sinigrin with the lowest degradation (33.6%) by strain GLM-3 and the highest (48.4%) by strain 2-243, although this variation was not significantly different (Table 6.1). Similarly, other pathogens including *E. coli* O157:H7 (Graumann and Holley, 2008), *Salmonella* (Herzallah et al., 2011, Olaimat and Holley 2014b [Chapter 4]), and *C. jejuni* (Olaimat et al., 2014a [Chapter 8]) were able to cause significant sinigrin hydrolysis. In contrast, *Pseudomonas fluorescens* showed little ability to degrade sinigrin (Herzallah et al., 2011). However, the latter authors found that *L. monocytogenes* GLM-4 was only able to hydrolyze 9.6% of the sinigrin present during 12 d at 30 ºC. It is apparent that sinigrin degradation takes place slowly in the presence of available glucose but is accelerated when glucose is exhausted (< 0.25%) from the growth medium (Olaimat et al. 2014b [Chapter 5]).

6.4.2 Antimicrobial activity of coatings against *L. monocytogenes* on cooked, cured chicken

L. monocytogenes is a psychrotrophic pathogen which is able to grow at refrigerator temperatures. In the current study, the numbers of *L. monocytogenes* on cooked, cured chicken increased 1.9 $\log_{10}$ CFU/g at 17 d and then remained constant until 70 d. However, the κ-carrageenan/chitosan control coating (without AITC or mustard extract) prepared with 1.5% (w/v) malic acid was significantly more inhibitory toward *L. monocytogenes* (3.2 $\log_{10}$ CFU/g reduction compared to uncoated chicken meat at 70 d) than coating prepared using 1.5% (v/v) acetic acid where there was a 2.3 $\log_{10}$ CFU/g reduction at 70 d) (Table 6.2). Similarly, Park et al. (2011) found higher reductions in numbers of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on fresh apple and lettuce which were dipped in a 1 to 2% malic acid solution than on those dipped in the same concentrations of acetic acid solution for 10 min at 22 ºC. In contrast, Li et al. (2013) found that chitosan coatings containing 2% (v/v) acetic acid were slightly more inhibitory at 4 ºC than those containing 2% (w/v) malic acid toward *L. monocytogenes* on cooked shrimp at 16 d. Antimicrobial action of organic acids depends on pH reduction, carbon chain length, degree of branching, and the ratio of undissociated forms of the acid (Eswaranandam et al., 2004). Although malic acid is an organic acid with low lipid solubility, it was effective against *L. monocytogenes* since the cell wall of Gram-positive bacteria contains only a thick peptidoglycan layer covering a lipid bilayer cytoplasmic membrane. In addition, malic acid is of a small molecular weight and this may facilitate its entry into cells and change the internal pH (Eswaranandam et al., 2004). The undissociated form of the acid is responsible for its antimicrobial activity because organic acids at pH values close to their pKa(s) become very weakly ionized and are more inhibitory (Doores, 2005). In the current study, κ-carrageenan/chitosan solutions were adjusted to pH 3.5 which is close to the first pKa value of

malic acid (3.46).

Acetic acid-κ-carrageenan/chitosan coatings containing 25 to 50 µl/g AITC or 100 to 250 mg/g Oriental mustard extract reduced the number of *L. monocytogenes* on cooked, cured chicken 4.2 to 6.0 $\log_{10}$ CFU/g at 70 d compared to uncoated chicken but only 2.0 to 3.7 $\log_{10}$ CFU/g compared to the acetic acid control coating. In addition, malic acid-κ-carrageenan/chitosan coatings containing 25 to 50 µl/g AITC or 100 to 250 mg/g mustard extract reduced *L. monocytogenes* numbers 4.8 to > 7.0 $\log_{10}$ CFU/g at 70 d compared to the uncoated chicken. It should be noted that *L. monocytogenes* cells were not detected at 70 d on cooked, cured chicken coated with malic acid-κ-carrageenan/chitosan coating containing 50 µl/g AITC (Table 6.2). Similar results were found by Olaimat and Holley (2014a [Chapter 7]) and Olaimat et al. (2014a [Chapter 8]) when κ-carrageenan/chitosan coatings were prepared with 1% (v/v) acetic acid and 50 µl/g AITC or 250 to 300 mg/g Oriental mustard extract and applied to fresh raw chicken breasts inoculated with *Salmonella* and *C. jejuni*. In another study, Lara-Lledó et al. (2012) found that a polymeric film containing Oriental mustard extract with 5% (w/w) sinigrin reduced *L. monocytogenes* cells on bologna slices to an undetectable level (≤ 1.6 log10 CFU/g) by 52 d at 4 ºC. In the current study, coatings with 100 or 250 mg/g mustard extract contained 21.5 and 53.8 mg/g sinigrin which was available for hydrolysis by the myrosinase-like enzyme(s) in *L. monocytogenes.* This can theoretically form 19.4 and 48.4 µl/g AITC, respectively, if it is assumed that 90% of the sinigrin present was degraded by the bacterial myrosinase and converted to AITC (Kawakishi and Muramatsu, 1966). However, in an earlier study, it was found that the same *L. monocytogenes* cocktail at 4 ºC degraded only 10.0% of the sinigrin present by 21 d (Olaimat et al., 2014b [Chapter 5]). Therefore, hydrolysis of sinigrin in 100 or 250 mg/g mustard extract may form 1.9 to 4.8 µl/g (1900 to 4800 ppm, respectively)

which are bactericidal since the MIC and MBC of AITC against the *L. monocytogenes* strains tested at 4 ºC ranged from 5 to 10 ppm and from 160 to 320 ppm, respectively, after 10 d storage (Table 6.3). However, it should be mentioned that AITC was more inhibitory toward *L. monocytogenes* at neutral than acidic pH where 200 ppm AITC in MH broth at pH 7.0 reduced viability of the same *L. monocytogenes* cocktail used in this study 4.1 $\log_{10}$ CFU/ml by 10 d at 4 ºC, but the reduction was only 1.2 $\log_{10}$ CFU/g when the pH was adjusted to 5.0 (Olaimat and Holley, 2013 [Chapter 3]). In the current study, chicken samples coated with solutions containing mustard extract had a lower pH (4.2 to 4.5) than those coated with other solutions (4.8 to 5.2, Table 6.4). The differences in pH values were attributed to the presence of phenolic acids which may also contribute in part to the antimicrobial activity of Oriental mustard extract (Olaimat et al., 2014a [Chapter 8]). Furthermore, it should be noted that the pH values of cooked, cured chicken samples coated with malic acid-κ-carrageenan/chitosan solutions were slightly lower than those coated with acetic acid-based coatings when products coated with the same antimicrobial concentration of AITC or mustard extract were compared (Table 6.4).

It is evident that the combination of malic or acetic acid with AITC or Oriental mustard extract in edible κ-carrageenan/chitosan coatings significantly reduced *L. monocytogenes* viability on cooked, cured roast chicken slices during its commercial shelf-life, and when coatings were prepared with malic acid they had greater antimicrobial activity. In a previous study, it was found that 0.5 % (w/v) mustard extract combined with 0.2% (w/v) malic acid reduced numbers of *L. monocytogenes* at 21 ºC to undetectable levels by 10 d, but their combination was not more inhibitory at 10 ºC than the individual agents (Olaimat and Holley, 2014b [Chapter 4]). Rhee et al. (2003) reported that no synergistic effect occurred when 10 or 20% (w/v) mustard flour was combined with 0.5% (v/v) acetic acid against *E. coli* O157:H7, *S.*

Typhimurium, and *L. monocytogenes* at 5 or 22 ºC. However, the combination of 10 or 20% (w/v) mustard with 1% (v/w) acetic acid had greater antimicrobial activity than mustard alone.

6.4.3 Antimicrobial activity of coatings against aerobic and lactic acid bacteria on cooked, cured chicken

Numbers of aerobic bacteria on uncoated, cooked, cured chicken increased by 1.6 $\log_{10}$ CFU/g at 17 d and then remained roughly constant until 70 d. The antimicrobial activity of edible coatings against aerobic bacteria followed the same pattern as with *L. monocytogenes*, where the malic acid-κ-carrageenan/chitosan coatings were more inhibitory. Coatings containing 25 to 50 µl/g AITC or 100 to 250 mg/g mustard extract reduced aerobic bacteria 4.1 to > 7.1 $\log_{10}$ CFU/g compared to uncoated chicken (Table 6.5). However, with LAB, there were no significant differences between malic acid-κ-carrageenan/chitosan coatings and acetic acid-κ-carrageenan/chitosan coatings at the same concentration of each antimicrobial. Coatings containing 25 to 50 µl/g AITC or 250 mg/g mustard extract reduced LAB numbers 3.8 to 5.4 $\log_{10}$ CFU/g compared to uncoated samples, while the activity of coatings containing 100 mg/g mustard extract was similar in activity to the control coating which reduced the numbers of LAB at 70 d by < 0.8 $\log_{10}$ CFU/g (Table 6.6). It is likely that the numbers of aerobic bacteria (Table 6.5) and *L. monocytogenes* (Table 6.2) were similar since the latter were able to grow on PCA and MRS agars. However, bacterial numbers on MRS (Table 6.6) were generally higher than *L. monocytogenes* numbers on LSA agar (Table 6.2) which may have occurred because both *L. monocytogenes* and LAB were able to grow on MRS agar, but LAB are resistant to AITC (Holley, 1997; Graumann and Holley, 2009; Luciano and Holley, 2011). In treatments where AITC reduced the large numbers of initially inoculated *L. monocytogenes* (Table 6.2), the more AITC resistant LAB become detectable on MRS agar (70 d, Table 6.6).

6.5 Conclusions

The 5 *L. monocytogenes* strains tested degraded 33.6% to 48.4% of the sinigrin at 25 ºC by 21 d. In general, κ-carrageenan/chitosan coatings prepared using 1.5% (w/v) malic acid were more inhibitory than those prepared using 1.5% (v/v) acetic acid. Acetic acid-κ-carrageenan/chitosan coatings containing 25 to 50 µl/g AITC or 100 to 250 mg/g Oriental mustard extract reduced numbers of *L. monocytogenes* on cooked, cured chicken slices by 5.9 to 6.0 $\log_{10}$ CFU/g and 4.2 to 4.6 $\log_{10}$ CFU/g, respectively. Malic acid coatings containing 25 to 50 µl/g AITC or 100 to 250 mg/g Oriental mustard extract reduced *L. monocytogenes* by 6.6 to > 7.0 $\log_{10}$ CFU/g (ie. less than the detection limit) and 4.8 to 5.5 $\log_{10}$ CFU/g, respectively, compared to uncoated chicken by 70 d at 4 ºC. Incorporation of 25 to 50 µl/g AITC or 100 to 250 mg/g Oriental mustard extract into κ-carrageenan/chitosan-based coatings prepared using malic or acetic acids was able to control *L. monocytogenes* on vacuum-packed, cooked cured roast chicken slices.

Table 6.1: Degradation of sinigrin by 5 *L. monocytogenes* strains in MH broth (pH 7.2) at 25 ºC.

Strain	Sinigrin hydrolyzed (%) during days storage			
	3	7	14	21
Control (without bacteria)	0.9 ± 0.4^{aA}	1.2 ± 0.3^{aB}	2.4 ± 0.7^{aB}	2.1 ± 0.4^{aB}
L. monocytogenes GLM-1	5.1 ± 4.3^{cA}	17.5 ± 1.1^{bcA}	36.5 ± 8.3^{abA}	42.3 ± 3.1^{aA}
L. monocytogenes GLM-3	10.5 ± 2.2^{cA}	15.1 ± 1.2^{bcA}	26.3 ± 0.3^{abA}	33.6 ± 5.7^{aA}
L. monocytogenes GLM-5	5.0 ± 4.9^{bA}	12.4 ± 4.2^{bA}	28.2 ± 3.0^{abA}	39.1 ± 10.6^{aA}
L. monocytogenes 2-138	7.9 ± 0.1^{cA}	14.7 ± 4.1^{bcA}	27.6 ± 2.2^{abA}	39.0 ± 6.7^{aA}
L. monocytogenes 2-243	9.6 ± 2.3^{cA}	19.5 ± 3.0^{bcA}	30.9 ± 6.0^{abA}	48.4 ± 4.1^{aA}

Means of sinigrin hydrolyzed (%) by each strain in the same row with different letters are significantly different ($p < 0.05$)
Means of sinigrin hydrolyzed (%) by strains at each time in the same column with different letters are significantly different ($p < 0.05$)
100%=1051 ppm

Table 6.2: Antimicrobial activity of acetic or malic acid-κ-carrageenan/chitosan-based coatings containing AITC or Oriental mustard extract against a 5 strain *L. monocytogenes* cocktail (log_{10} CFU/g) on vacuum-packed cooked, cured roast chicken held at 4 °C for 70 d.

Day	Viability of *L. monocytogenes* (log_{10} CFU/g) on vacuum-packed cooked, cured roast chicken slices coated with acetic or malic acid-κ-carrageenan/chitosan solutions containing AITC or Oriental mustard extract										
		1.5% (v/v) Acetic Acid					1.5% (w/v) Malic Acid				
	Uncoated control	Control coating	AITC (25 µl/g)	AITC (50 µl/g)	Mustard (100 mg/g)	Mustard (250 mg/g)	Control coating	AITC (25 µl/g)	AITC (50 µl/g)	Mustard (100 mg/g)	Mustard (250 mg/g)
0	6.32±0.26^{a}	6.32±0.26^{a}	6.32±0.26^{a}	6.32±0.26^{a}	6.32±0.26^{a}	6.32±0.26^{a}	6.32±0.26^{a}	6.32±0.26^{a}	6.32±0.26^{a}	6.32±0.26^{a}	6.32±0.26^{a}
17	8.23±0.14^{a}	6.10±0.13^{b}	4.34±0.18^{fg}	3.82±0.29^{gh}	5.35±0.08^{cd}	4.79±0.23^{ef}	5.64±0.13^{bc}	3.50±0.15^{h}	3.36±0.38^{h}	4.93±0.26^{de}	4.86±0.33^{def}
35	8.18±0.18^{a}	5.43±0.22^{b}	3.71±0.06^{d}	3.60±0.19^{d}	5.23±0.07^{b}	4.41±0.37^{c}	5.14±0.12^{b}	2.30±0.22^{e}	2.02±0.38^{e}	4.58±0.09^{c}	3.81±0.05^{d}
52	8.06±0.09^{a}	5.73±0.23^{b}	2.62±0.11^{f}	2.28±0.06^{f}	4.42±0.14^{d}	3.54±0.13^{e}	5.19±0.18^{c}	1.75±0.28^{g}	1.17±0.14^{h}	3.86±0.35^{e}	3.43±0.05^{e}
70	8.01±0.15^{a}	5.75±0.29^{b}	2.10±0.20^{fg}	2.03±0.25^{g}	3.78±0.17^{d}	3.39±0.23^{de}	4.80±0.09^{c}	1.43±0.19^{h}	ND (+)	3.23±0.06^{e}	2.54±0.13^{f}

Values within the same row with different letters are significantly different ($p < 0.05$). Bacteria were enumerated on LSA.
ND: *L. monocytogenes* cells were not detected (the detection limit was 1.0 log_{10} CFU/g).
(+): *L. monocytogenes* cells were detected after overnight enrichment in MH broth.

Table 6.3: MIC and MBC (ppm) of AITC against five individual *L. monocytogenes* strains after 10 d of storage at 4 ºC.

Strain	MIC [a]	MBC [b]
L. monocytogenes GLM1	10	320
L. monocytogenes GLM3	5	320
L. monocytogenes GLM5	10	160
L. monocytogenes 2-138	10	320
L. monocytogenes 2-243	10	320

[a] The lowest concentration at which no visible growth occurred
[b] The lowest concentration which reduced the initial inoculated number by ≥ 3 $\log_{10}$ CFU/ml.

Table 6.4: Effect of acetic or malic acid-κ-carrageenan/chitosan-based coatings containing AITC or Oriental mustard on the pH values of vacuum-packed cooked cured roast chicken held at 4 °C for 70 d.

Day	pH of vacuum-packed cooked, cured roast chicken slices coated with acetic or malic acid-κ-carrageenan/chitosan solutions containing AITC or Oriental mustard extract										
		1.5% (v/v) Acetic Acid					1.5% (w/v) Malic Acid				
	Uncoated control	Control coating	AITC (25 µl/g)	AITC (50 µl/g)	Mustard (100 mg/g)	Mustard (250 mg/g)	Control coating	AITC (25 µl/g)	AITC (50 µl/g)	Mustard (100 mg/g)	Mustard (250 mg/g)
17	5.45±0.06^{a}	4.95±0.01^{cd}	5.15±0.05^{b}	5.06±0.08^{bc}	4.45±0.04^{e}	4.56±0.06^{e}	4.78±0.04^{d}	4.95±0.04^{cd}	4.89±0.02^{cd}	4.43±0.06^{e}	4.56±0.03^{e}
35	5.32±0.04^{a}	5.13±0.02^{ab}	5.38±0.11^{a}	5.29±0.01^{a}	4.60±0.08^{cd}	4.61±0.15^{cd}	4.84±0.02^{bc}	5.10±0.01^{ab}	4.98±0.11^{b}	4.38±0.08^{d}	4.54±0.05^{d}
52	4.94±0.04^{b}	4.93±0.01^{b}	5.20±0.04^{a}	5.16±0.08^{a}	4.45±0.04^{d}	4.50±0.04^{cd}	4.64±0.02^{c}	4.90±0.01^{b}	4.93±0.03^{b}	4.26±0.02^{e}	4.27±0.05^{e}
70	4.82±0.04^{bcd}	4.97±0.11^{cde}	5.21±0.04^{a}	5.14±0.06^{ab}	4.49±0.08^{def}	4.51±0.11^{def}	4.80±0.09^{c}	4.96±0.05^{abc}	4.92±0.05^{abc}	4.24±0.18^{f}	4.21±0.13^{f}

Values within the same row with different letters are significantly different ($p < 0.05$).

Table 6.5: Antimicrobial activity of acetic or malic acid-κ-carrageenan/chitosan-based coatings containing AITC or Oriental mustard extract against aerobic bacteria (log_{10} CFU/g) on vacuum-packed cooked, cured roast chicken held at 4 °C for 70 d.

Day	Viability of aerobic bacteria (log_{10} CFU/g) on vacuum-packed cooked, cured roast chicken slices coated with acetic or malic acid-κ-carrageenan/chitosan solutions containing AITC or Oriental mustard extract										
		1.5% (v/v) Acetic Acid					1.5% (w/v) Malic Acid				
	Uncoated control	Control coating	AITC (25 µl/g)	AITC (50 µl/g)	Mustard (100 mg/g)	Mustard (250 mg/g)	Control coating	AITC (25 µl/g)	AITC (50 µl/g)	Mustard (100 mg/g)	Mustard (250 mg/g)
0	6.64±0.42^{a}	6.64±0.42^{a}	6.64±0.42^{a}	6.64±0.42^{a}	6.64±0.42^{a}	6.64±0.42^{a}	6.64±0.42^{a}	6.64±0.42^{a}	6.64±0.42^{a}	6.64±0.42^{a}	6.64±0.42^{a}
17	8.22±0.19^{a}	6.27±0.26^{b}	4.42±0.29^{ef}	3.49±0.16^{g}	5.39±0.09^{cd}	4.94±0.08^{de}	5.79±0.46^{bc}	4.14±0.15^{f}	3.25±0.23^{g}	4.92±0.10^{de}	4.98±0.10^{de}
35	8.28±0.20^{a}	5.35±0.35^{b}	3.62±0.11^{d}	2.59±0.27^{e}	5.32±0.11^{b}	4.52±0.34^{c}	5.53±0.42^{b}	2.82±0.17^{e}	2.51±0.04^{e}	4.71±0.16^{c}	3.87±0.09^{d}
52	8.09±0.05^{a}	5.76±0.12^{b}	2.91±0.23^{e}	1.63±0.50^{fg}	4.69±0.09^{c}	3.68±0.09^{d}	5.48±0.39^{b}	2.21±0.10^{f}	1.29±0.35^{g}	3.97±0.06^{d}	3.59±0.08^{d}
70	8.07±0.13^{a}	5.90±0.11^{b}	1.95±0.20^{g}	1.19±0.23^{h}	3.94±0.06^{d}	3.59±0.19^{de}	5.16±0.02^{c}	1.62±0.17^{g}	ND (+)	3.44±0.19^{e}	2.51±0.15^{f}

Values within the same row with different letters are significantly different ($p < 0.05$).
ND: aerobic bacteria were not detected (the detection limit was 1.0 log_{10} CFU/g).
(+): aerobic bacteria were detected after overnight enrichment in MH broth. Bacteria were enumerated on PCA.

Table 6.6: Antimicrobial activity of acetic or malic acid-κ-carrageenan/chitosan-based coatings containing AITC or Oriental mustard extract against LAB (log_{10} CFU/g) on vacuum-packed cooked, cured roast chicken held at 4 °C for 70 d.

Day	Viability of LAB (log_{10} CFU/g) on vacuum-packed cooked, cured roast chicken slices coated with acetic or malic acid-κ-carrageenan/chitosan solutions containing AITC or Oriental mustard extract										
		1.5% (v/v) Acetic Acid					1.5% (w/v) Malic Acid				
	Uncoated control	Control coating	AITC (25 µl/g)	AITC (50 µl/g)	Mustard (100 mg/g)	Mustard (250 mg/g)	Control coating	AITC (25 µl/g)	AITC (50 µl/g)	Mustard (100 mg/g)	Mustard (250 mg/g)
0	6.30±0.17^{a}	6.30±0.17^{a}	6.30±0.17^{a}	6.30±0.17^{a}	6.30±0.17^{a}	6.30±0.17^{a}	6.30±0.17^{a}	6.30±0.17^{a}	6.30±0.17^{a}	6.30±0.17^{a}	6.30±0.17^{a}
17	8.16±0.18^{a}	6.25±0.20^{b}	5.29±0.09^{d}	4.41±0.44^{e}	6.31±0.18^{b}	5.52±0.09^{cd}	5.73±0.20^{cd}	5.25±0.22^{d}	4.62±0.22^{e}	5.96±0.10^{bc}	5.35±0.24^{d}
35	8.11±0.12^{a}	5.15±0.12^{cd}	4.68±0.07^{e}	3.93±0.07^{f}	5.63±0.13^{b}	5.15±0.09^{cde}	5.47±0.22^{bc}	4.76±0.15^{de}	3.81±0.16^{f}	5.69±0.05^{b}	5.23±0.49^{bcd}
52	7.90±0.07^{a}	5.82±0.09^{b}	3.48±0.24^{d}	3.61±0.08^{d}	5.37±0.20^{b}	4.64±0.39^{c}	5.46±0.26^{b}	3.92±0.40^{d}	3.43±0.31^{d}	5.40±0.38^{b}	4.81±033^{c}
70	7.93±0.16^{a}	5.86±0.06^{b}	3.10±0.16^{de}	2.57±0.11^{f}	5.47±0.19^{b}	4.09±0.13^{c}	5.65±0.04^{b}	3.40±0.34^{d}	2.94±0.08^{ef}	5.59±0.15^{b}	4.18±0.17^{c}

Values within the same row with different letters are significantly different ($p < 0.05$).
Bacteria were enumerated on MRS agar.

Chapter 7

Control of *Salmonella* on Fresh Chicken Breasts by κ-Carrageenan/Chitosan-Based Coatings Containing Allyl Isothiocyanate or Deodorized Oriental Mustard Extract plus EDTA

7.1 Abstract

Control of *Salmonella* in poultry is a public health concern as salmonellosis is one of the most common foodborne diseases worldwide. This study aimed to screen the ability of 5 *Salmonella* serovars to degrade the mustard glucosinolate, sinigrin (by bacterial myrosinase) in Mueller-Hinton broth at 25 ºC for 21 d and to reduce *Salmonella* on fresh chicken breasts by developing an edible 0.2% (w/v) κ-carrageenan/2% (w/v) chitosan-based coating containing Oriental mustard extract, allyl isothiocyanate (AITC), EDTA or their combinations. Individual *Salmonella* serovars degraded 50.2% to 55.9% of the sinigrin present in 21 d. κ-Carrageenan/chitosan-based coatings containing 250 mg Oriental mustard extract/g or 50 µl AITC/g reduced the numbers of *Salmonella* on chicken breasts 2.3 $\log_{10}$ CFU/g by 21 d at 4 ºC. However, when either mustard extract or AITC was combined with 15 mg/g EDTA in κ-carrageenan/chitosan-based coatings, *Salmonella* numbers were reduced 2.3 $\log_{10}$ CFU/g at 5 d and 3.0 $\log_{10}$ CFU/g at 21 d. Moreover, these treatments reduced numbers of lactic acid bacteria and aerobic bacteria by 2.5 to 3.3 $\log_{10}$ CFU/g at 21 d. κ-Carrageenan/chitosan coatings containing either 50 µl AITC/g or 250 mg Oriental mustard extract/g plus 15 mg EDTA/g have the potential to reduce *Salmonella* on raw chicken.

7.2 Introduction

Foodborne illnesses, resulting from consumption of contaminated food, represent a substantial public health threat. *Salmonella,* STEC *E. coli* and *Listeria monocytogenes* are significant pathogens present in food and the agricultural environment which account for large numbers of illness outbreaks, cases and deaths (Scharff, 2010). In the US, it was estimated that 82 million cases of foodborne illness occur every year, costing $152 billion. Cost associated with *Salmonella* was estimated to be $15 billion (Scharff, 2010). In recent years, the incidence of salmonellosis has increased worldwide and *Salmonella* is considered to be one of the most common causes of foodborne illness internationally. Scallan et al. (2011) reported that more than 1.2 million cases of salmonellosis with 452 deaths occur in the US annually. In another study, Scharff (2010) estimated that 1.6 million salmonellosis cases occur in the US every year. Greig and Ravel (2009) found there were 1918 salmonellosis outbreaks (47% of total outbreaks) reported internationally from 1988 to 2007. In Canada, *Salmonella* is the third leading bacterial pathogen and accounts for 87,510 foodborne illnesses every year (Thomas et al., 2013). The consumption of undercooked meat products has been recognized as a major risk factor for infection by *Salmonella*; however, poultry is the most common reservoir of *Salmonella* (Humphrey and Jorgensen, 2006).

The use of natural compounds as antimicrobials during processing can extend the shelf-life of food as well as reduce health hazards and economic loss due to foodborne pathogens (Oussalah et al., 2007). Spices, herbs and their essential oils have been found to have antimicrobial activity against a variety of food pathogens (Holley and Patel, 2005). Mustard and other cruciferous vegetables of the *Brassica* family (i.e. broccoli, horseradish and wasabi) contain high levels of glucosinolates which have antioxidant and antimicrobial properties when hydrolyzed by endogenous plant myrosinase (thioglucoside glucohydrolase, EC.3.2.1.147) in the

presence of water to yield isothiocyanates, nitriles, D-glucose and sulphate. In the past few years, glucosinolate metabolites (especially the isothiocyanates) have not only been shown to be inhibitory toward pathogenic bacteria (Fahey et al., 2001; Vig et al., 2009), but they are also potent anticancer agents (Zhang, 2010).

Luciano and Holley (2010, 2011) found that *E. coli* O157:H7, *Staphylococcus carnosus* and *Pediococcus pentosaceus* were able to degrade glucosinolates present in Oriental and yellow mustards (sinigrin and sinalbin) and form isothiocyanates which killed *E. coli* O157:H7. This showed that the tested microorganisms had myrosinase-like activity and raised the question of how widespread the distribution of this activity might be among other pathogens like *Salmonella*. At the point in time when the present work was done, only one study had tested the ability of *Salmonella* to convert sinigrin into allyl isothiocyanate (AITC) by myrosinase-like activity and this was limited to testing a single strain of *S.* Typhimurium (Herzallah et al., 2011). AITC from natural sources is permitted as a food preservative in Japan, and is regarded as a "generally recognized as safe" (GRAS) flavoring agent in the US (Delaquis and Mazza, 1995; Kim et al., 2002). AITC has been shown to be effective as an antimicrobial against various pathogens including *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* and *Campylobacter jejuni* (Luciano and Holley, 2009; Olaimat and Holley, 2013 [Chapter 3]; Olaimat et al., 2014a [Chapter 8])

Edible films and coatings containing antimicrobials have been demonstrated to be useful tools to control foodborne pathogens on food. κ-Carrageenan/chitosan-based coatings containing AITC or Oriental mustard extract were found to be effective against *C. jejuni* on chicken breasts (Olaimat et al, 2014a [Chapter 8]). In another study, Olaimat and Holley (2014 [Chapter 4]) found that mustard extract at 0.5% (w/v) significantly reduced numbers of *Salmonella* in Brain Heart Infusion (BHI) broth at 4 ºC at 21d, but the additive inhibitory effects of 0.5% mustard

extract with 0.2% ethylenediamine tetraacetic acid (EDTA) eliminated *S.* Typhimurium by 7 d and 10 d at 21 ºC and 10 ºC, respectively. Incorporation of mustard extract, AITC and EDTA in an edible coating may reduce *Salmonella* on fresh chicken. Therefore, the objectives of the present study were i) to screen for the ability of 5 *S. enterica* serovars to degrade pure sinigrin by bacterial myrosinase, ii) to reduce *Salmonella* viability on fresh chicken breasts by an edible antimicrobial coating containing mustard extract, AITC and EDTA or their combinations.

7.3 Materials and Methods

7.3.1 Bacterial strains and culture preparation

Salmonella strains (*S.* Heidelberg 271, *S.* Typhimurium 02:8423, *S.* Copenhagen PT 99, *S.* Enteritidis CRIFS 1016, and *S.* Kentucky 64701) were from the culture collection of the Food Science Department, University of Manitoba. *Salmonella* serovars were stored in BHI broth (Oxoid Ltd., Basingstoke, England) containing 25% (v/v) glycerol at -80 ºC. To activate the organisms, one loopful from each strain was streaked on BHI agar (Oxoid) and incubated at 37 ºC for 24 h to 48 h. One colony of each *Salmonella* serovar was streaked on Xylose Lysine Deoxycholate (XLD) agar (Oxoid), and incubated at 37 ºC for 24 h. A single colony from XLD agar was transferred to BHI broth and incubated overnight at 37 ºC. *Salmonella* serovars were suspended again in BHI broth and incubated as before. Then, 0.1 % (v/v) of this culture was transferred to fresh BHI broth and incubated overnight at 37 ºC. Either freshly prepared single cultures or mixtures of *Salmonella* cultures, prepared by combining equal volumes of each the 5 freshly cultured organisms in a sterile container, were used in the experiments.

7.3.2 Screening of *Salmonella* for sinigrin hydrolysis

A 50 g/L sinigrin (Sigma-Aldrich Co., St Louis, MO, USA) stock solution was prepared in 10 ml Mueller Hinton (MH, Oxoid) broth and filter-sterilized (0.22 μm, Millipore, Cork, Ireland). Sinigrin stock solution was added to 490 ml MH broth (pH 7.2), mixed to achieve a concentration of 1000 mg/l sinigrin and 9.9 ml was transferred to sterile 12 ml screw-capped tubes. To screen *Salmonella* serovars for myrosinase-like activity, 0.1 ml of 8 $\log_{10}$ CFU/ml of individual stationary phase *Salmonella* cultures was added to tubes containing MH broth. Tubes containing sinigrin and cultures were mixed to yield 6 $\log_{10}$ CFU/ml *Salmonella* cultures which were incubated with shaking at 200 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA) for 21 d at 25 ºC. Samples of 1 ml were taken at 0, 3, 7, 14 and 21 d, filter-sterilized (0.22 μm, Millipore, Cork, Ireland) and transferred to sterile high-performance liquid chromatography (HPLC) vials. Samples were stored at 4 ºC until HPLC analysis.

7.3.3 Sinigrin analysis by reversed-phase high-performance liquid chromatography (RP-HPLC)

HPLC solvents used were tetrabutylammonium hydrogen sulfate (TBA, J. T. Baker, NJ, USA), acetonitrile (Fisher Scientific Co., NJ, USA) and double-distilled water. Solvents initially were sterilized using 0.45 mm Millipore nylon filters (Fisher Scientific, Nepean, ON, Canada), and then degassed for 30 min in a Bransonic bath (Branson, 5510, Grass Valley, CA, USA). Sinigrin degradation was monitored based on the method used by Luciano and Holley (2011). Quantification of sinigrin during storage at 0, 3, 7, 14 and 21 d was performed using an RP-HPLC (Waters 2695, Waters Corporation, Milford, MA, USA) equipped with a C18 column (Gemini-NX, 150×4.60 mm, 5 μm; Phenomenex, Torrance, CA, USA) and a security guard

cartridge (Gemini C18, 4×3 mm; Phenomenex). A Waters model 486 detector (wavelength at 227 nm), and a Waters 600E system controller were used. Waters LC-module 1Millennium software (version 32) was used to process the data. Sinigrin was quantitatively determined using a calibration curve (100-1500 mg/l) established with a sinigrin standard under the same analytical conditions.

7.3.4 Fresh chicken and antimicrobial coatings

Fresh chicken breast fillets were purchased from a local grocery store in Winnipeg, MB, Canada. Oriental mustard powder (Sakai spice, Calgary, AB, Canada) was autoclaved at 121 ºC for 20 min and the mustard extract was prepared as described by Lara-Lledó et al. (2012). Antimicrobial coatings were prepared as described by Olaimat et al. (2014a [Chapter 8]). Briefly, 0.2% (w/v) κ-carrageenan from *Eucheuma cottonii* (Sigma) and 2% (w/v) chitosan (molecular weight of 100-300 kDa; deacetylation degree of 75-85%, Acros Organics, Morris Plains, NJ, USA) were mixed in a 1% acetic solution and stirred for 1 h at 60 ºC. Glycerol (Sigma) at 4% (v/v) was added to the mixture as a stabilizer, mixed with a magnetic stirring bar overnight and homogenized at maximum speed using a homogenizer (L2R, Silverson Machines, Ltd., Waterside, England). Before use 5 ml AITC (Acros Organics) was dissolved in 1 ml of Tween 80 (Sigma). Mustard extract or AITC was added separately or in combination with EDTA to yield 6 coating treatments including: a control without antimicrobials; 250 mg/g mustard extract; 50 µl/g AITC; 15 mg/g EDTA; 250 mg/g mustard extract with 15 mg/g EDTA, or 50 µl/g AITC with 15 mg/g EDTA. All coating solutions were homogenized as described above and then the pH was adjusted to 3.5 using 0.2 M acetic acid.

7.3.5 Antimicrobial activity of coatings on chicken meat

A mixture of 20 ml *Salmonella* (4 ml from each of 5 strains) was centrifuged at 1100 *xg* for 20 min at 4 ºC. The pellets were washed twice using 20 ml sterile 0.1 M potassium phosphate buffer (pH 7.2, Sigma-Aldrich) and re-centrifuged using the same conditions. The pellets were resuspended in 200 ml 0.1% (w/v) buffered peptone water to yield 8.0 $\log_{10}$ CFU/ml.

The chicken breast fillets were cut into 20 ± 1 g pieces (5 cm x 4 cm) and dipped in 100 ml of the 5 strain *Salmonella* cocktail mixture containing 10^8 CFU/ml for 20 sec and drained for 10 sec. Inoculated samples were dried in a laminar airflow hood for 30 min. The chicken pieces were dipped in 100 ml κ-carrageenan/chitosan solution for 20 sec and the excess coating was drained for 10 sec. Samples were allowed to dry in a laminar-flow hood under ventilation for 30 min on each side. Inoculated samples without coating were also prepared. Control and treated inoculated chicken pieces were placed into commercial multi-layer high oxygen barrier plastic bags (Deli*1, 17.8 cm × 22.9 cm, Winpak Ltd., Winnipeg, MB, Canada) and sealed under vacuum using a model GM 2002 vacuum packaging machine (Bizerba Canada Inc., Mississauga, ON, Canada). The packed chicken breasts were stored at 4 ºC for 21 d. Samples were taken at 5, 11, 16 and 21 d, and homogenized with 180 ml 0.1% (w/v) sterile buffered peptone water in stomacher bags (filtra-bags, VWR Scientific, Edmonton, AB, Canada) for 30 sec using a stomacher (Bagmixer®400CC, Interscience Inc., Markham, ON, Canada). Serial dilutions were made in 0.1% buffered peptone water, and plated in duplicate on plate count agar (PCA, Oxoid) for aerobic bacteria, XLD agar for *Salmonella* and de Man, Rogosa and Sharpe agar (MRS, Oxoid) for lactic acid bacteria (LAB). The PCA and XLD plates were incubated at 37 ºC for 24-48 h, while MRS plates were incubated at 25 ºC for 48 h. The pH of the chicken pieces was measured using an Accumet model 910 digital pH meter (Fisher Scientific, Pittsburgh, PA,

USA).

7.3.6 Statistical analysis

Sinigrin degradation tests were performed in two experiments with duplicate analyses (n = 4) and the antimicrobial coating tests were performed in two experiments with 3 replicates of each experiment (n = 6). Results were expressed as means ± SD and statistical differences among treatments were compared by Tukey's test using JMP 10.0 software (SAS Inst. Inc., Cary, N.C., U.S.A.). Differences between the treatments were considered significant when $p < 0.05$.

7.4 Results and Discussion

7.4.1 Screening of *Salmonella* strains for myrosinase-like activity

Salmonella serovars were able to degrade 50.2% to 55.9% of sinigrin by 21 d incubation at 25 ˚C (Table 7.1). These results agree with findings of other studies which showed that glucosinolates can be degraded by different pathogens like *E. coli* O157:H7 (Luciano and Holley, 2010; 2011), *Enterococcus faecalis*, *L. monocytogenes* (Herzallah et al., 2011) and *C. jejuni* (Olaimat et al., 2014a [Chapter 8]). Herzallah et al. (2011) also found that a different strain of *S.* Typhimurium (02:8421) reduced sinigrin 28.0% at 12 d. However, the sinigrin degradation rate by *Salmonella* strains in the present study was higher (2.4-2.7%/d or 26.2-29.5 mg/l/d) compared to 1.3%/d (4.0 mg/l/d) in the study by Herzallah et al. (2011). This could have been because of differences among the tested strains, the growth medium (MH broth here vs Tryptone Soy broth), the incubation temperature (25 ºC here vs 35 ºC), or the initial concentration of sinigrin (>1000 mg/l here vs 307 mg/l).

7.4.2 Antimicrobial activity of coatings against *Salmonella* on chicken meat

Vacuum packaging can be used successfully for preserving fresh poultry carcass parts because the normal spoilage microorganisms active at 4 ºC are mainly aerobic. However, *Salmonella* is a facultatively anaerobic bacterium unable to grow at < 7 ºC and in the present study was able to survive on uncoated chicken breasts without significant change in numbers over 21 d storage (Table 7.2). Similarly, Wen and Dickson (2012) reported that *S.* Typhimurium numbers were not significantly changed on vacuum-packed pork slices after 28 d at 4 or 10 ºC.

Edible coatings enhance food product quality and safety and extend their shelf-life by protecting them from physical, chemical, and biological deterioration. Chitosan has excellent potential to be used in the food industry because of its particular physicochemical properties including biodegradability, lack of allergenicity, non-toxicity and its antimicrobial activity (Aider, 2010). In the current study, an edible coating containing 2% chitosan and 0.2% κ-carrageenan significantly reduced numbers of *Salmonella* on chicken breast 0.8 $\log_{10}$ CFU/g compared to a 0.2. $\log_{10}$ CFU/g reduction on uncoated pieces (Table 7.2). Chen et al. (2012) also found that a 2% chitosan coating reduced *Salmonella* numbers on cantaloupe 1.5 $\log_{10}$ CFU/cm^2 after 24 h at 22 °C. Chitosan may change cell permeability by its interaction with electronegative charges on the bacterial cell (Martínez-Camacho et al., 2010). Chitosan with low molecular weight also interacts with DNA and interferes with messenger RNA synthesis (Rabea et al., 2003). Moreover, chitosan can act as a chelating agent by binding essential metals and nutrients which inhibits microbial growth (Tripathi et al., 2010).

κ-Carrageenan/chitosan coating containing 15 mg/g EDTA showed similar antimicrobial activity to the control coating (Table 7.2). It was evident that EDTA had no additive inhibitory

effect with the coating against *Salmonella*. However, coating containing 50 µl AITC/g significantly reduced *Salmonella* numbers on chicken samples by 2.3 $\log_{10}$ CFU/g at 21 d (Table 7.2). Few studies have tested the antimicrobial activity of AITC incorporated in an edible coating. Chen et al. (2012) found that AITC at 60 µl/ml in chitosan coatings reduced *Salmonella* on cantaloupe surfaces > 5 $\log_{10}$ CFU/cm^2 by 24 h at 22 ºC. Jin et al. (2013) reported that a polylactic acid polymer coating or a chitosan coating with 60 µl/ml AITC reduced the numbers of *Salmonella* in a cocktail mixture on egg shells 1.2 and 1.7 $\log_{10}$ CFU/cm^2, respectively at 22 ºC. An AITC vapor release rate of 1.2 µg/h reduced *S.* Typhimurium in modified atmosphere-packaged fresh chicken by 1.3 $\log_{10}$ CFU/g after 21 d storage at 4 ºC (Shin et al., 2010). The mode of AITC action has been attributed to three probable mechanisms. First, AITC may attack the cell membrane and increase its permeability which leads to leakage of intracellular components and subsequent loss of cell viability (Zou et al., 2013). Second, AITC may disrupt bacterial metabolism and energy production (Chan et al., 2013). Third, AITC may alter protein structure and inactivate enzymes via reaction with free amino groups and cause oxidative cleavage of disulphide bonds (Luciano and Holley, 2009).

In the current study, coating containing 250 mg mustard extract/g (53.8 mg/g sinigrin) also reduced *Salmonella* numbers on chicken breasts 2.3 $\log_{10}$ CFU/g at 21 d (Table 7.2). Sinigrin represented 21.5% of the mustard extract by weight; therefore, the κ-carrageenan/chitosan solution with 250 mg/g Oriental mustard extract contained 53.8 mg/g sinigrin. The amount of AITC formed during degradation of glucosinolates by plant myrosinase was found to reach 90% (Kawakishi and Muramatsu, 1966). If the conversion by bacterial myrosinase followed the same pattern, the degradation of 53.8 mg/g sinigrin would yield 48.4 µl/ml AITC. However, *Salmonella* strains only degraded up to 56% sinigrin by 21 d at 25 ˚C (Table 7.1). It should be

noted that although the AITC formed was more stable at 4 °C (Olaimat and Holley, 2013 [Chapter 3]), the rate of sinigrin hydrolysis by *Salmonella* at 4 °C was 5 times lower than at 25 °C (Olaimat and Holley, 2014b [Chapter 4]; Olaimat et al., 2014b [Chapter 5]), and this was believed due to reduced synthesis or activity of myrosinase at 4 ºC (Olaimat et al., 2014b [Chapter 5]). Oriental mustard also has a high phenolic content (> 2300 ug/g gallic acid equivalents) which may be partially responsible for the antimicrobial activity of mustard against *Salmonella* (Wu, 2013). In addition, coating containing mustard extract significantly reduced the pH of chicken breasts which reached pH 5.0 at 21 d compared to the control coating which had a pH value of 5.9 (Table 7.3).

κ-Carrageenan/chitosan coatings containing a combination of 15 mg/g EDTA with either 250 mg/g mustard extract or 50 µl/ml AITC had improved antimicrobial activity against *Salmonella* where numbers were reduced 2.3 $\log_{10}$ CFU/g at 5 d and 3.0 $\log_{10}$ CFU/g at 21 d (Table 7.2). Olaimat and Holley (2014b [Chapter 4]) also found that addition of 0.2% EDTA enhanced the activity of mustard extract at 0.5% against *S.* Typhimurium in MH broth at 21 or 10 ºC. Zhou et al. (2007) found that the combination of EDTA with either thymol or carvacrol caused significantly greater reductions in *S.* Typhimurium numbers than each compound alone. Chelating agents like EDTA interfere with Ca^{2+} and Mg^{2+} cations in the lipopolysaccharide layer of Gram-negative bacteria which disrupt the outer membrane structure, alter its permeability, and consequently allow other antimicrobials to reach the cytoplasmic membrane (Alakomi et al., 2000; 2003).

7.4.3 Antimicrobial activity of coatings against aerobic and lactic acid bacteria

Numbers of aerobic bacteria and LAB gradually grew on the uncoated chicken breasts to reach 7.8 and 8.3 $\log_{10}$ CFU/g at 21 d. However, the κ-carrageenan/chitosan control coating or coating with 15 mg/g EDTA reduced numbers of both organisms < 0.6 $\log_{10}$ CFU/g at 21 d. The coating containing mustard extract was more inhibitory and reduced the numbers of aerobic bacteria and LAB 1.1 $\log_{10}$ CFU/g and 1.4 $\log_{10}$ CFU/g, respectively, at 21 d. When mustard extract was combined with EDTA in κ-carrageenan/chitosan coatings, greater reductions in numbers of aerobic bacteria (2.5 $\log_{10}$ CFU/g) and LAB (3.1 $\log_{10}$ CFU/g) were observed at 21d. κ-Carrageenan/chitosan coatings containing 50 µl/ml AITC or 50 µl/ml AITC plus 15 mg/g EDTA reduced the numbers of aerobic bacteria 1.8 $\log_{10}$ CFU/g and 2.7 $\log_{10}$ CFU/g, respectively, and the numbers of LAB 3.0 $\log_{10}$ CFU/g and 3.3 $\log_{10}$ CFU/g, respectively, at 21 d (Tables 7.4 and 7.5). It has been reported that LAB starter cultures and adventitious LAB in meat were more resistant to AITC than *E. coli* O157:H7 (Graumann and Holley, 2009; Luciano and Holley, 2011). In the current study, AITC in the coating was more inhibitory to naturally occurring LAB than 20 µl/l AITC in the headspace of cooked roast beef against *Lactobacillus sakei* stored 7 d at 12 °C (Ward et al., 1998). In other work, hot yellow mustard powder containing natural AITC reduced aerobic surface bacteria 5 $\log_{10}$ CFU/g on dry-cured ham after 80 d maturation at 14 ^{o}C, but 400 µg/kg microencapsulated AITC caused ≤ 2 $\log_{10}$ CFU/g reductions of aerobic bacteria and LAB (Graumann and Holley, 2009). From the present work it is evident that the control coating prevented growth of LAB and aerobic bacteria; however, coatings containing either AITC or mustard extract combined with EDTA significantly reduced the numbers of both groups of organisms and this may enhance the quality of chicken and extend its shelf-life.

7.5 Conclusions

The individual 5 *Salmonella* strains degraded > 50% of sinigrin by 21 d at 25 ºC. κ-Carrageenan/chitosan coating containing 250 mg mustard extract/g or 50 µl AITC/g reduced numbers of *Salmonella* on vacuum-packed chicken breasts 2.3 $\log_{10}$ CFU/g by 21 d at 4 ºC. However, the combination of 15 mg/g EDTA with either 250 mg mustard extract/g or 50 µl AITC/g improved the antimicrobial activity and reduced *Salmonella* numbers 2.3 and 3.0 $\log_{10}$ CFU/g by 5 and 21 d storage at 4 ºC, respectively. Moreover, numbers of LAB and aerobic bacteria were significantly reduced by these treatments. Application of κ-carrageenan/chitosan coatings containing either AITC or mustard extract combined with EDTA on fresh, refrigerated, vacuum-packed chicken breasts was shown to have the potential to reduce *Salmonella,* LAB and aerobic bacteria, and consequently, to enhance the safety of chicken and extend its shelf-life.

Table 7.1: Degradation of sinigrin by 5 *Salmonella* strains in MH broth (pH 7.2) at 25 ºC.

Strain	Sinigrin hydrolyzed (%) during days storage [A, B, C]			
	3	7	14	21
Control (without bacteria)	1.4±0.2[a]	1.1±0.1[a]	1.9±0.3[a]	2.3±0.5[a]
S. Typhimurium 02:8423	16.4±1.7[b]	27.1±4.1[b]	36.0±10.3[ab]	54.6±7.5[a]
S. Copenhagen PT 99	12.5±3.1[b]	27.6±6.5[b]	44.6±2.2[a]	51.1±0.2[a]
S. Enteritidis CRIFS 1016	15.3±1.8[c]	23.8±0.5[c]	43.2±1.5[b]	55.2±4.2[a]
S. Kentucky 64701	8.3±0.1[c]	26.3±1.9[bc]	40.4±6.3[ab]	55.9±5.9[a]
S. Heidelberg 271	12.4±0.2[c]	21.5±2.8[bc]	35.7±2.9[ab]	50.2±6.4[a]

[A] Values are the means of two experiments ± standard deviations.
[B] Means of sinigrin hydrolyzed (%) by each strain in the same row with different letters are significantly different ($p < 0.05$)
[C] 100%=1093 mg/l

Table 7.2: Antimicrobial activity of κ-carrageenan/chitosan-based coatings containing AITC, Oriental mustard extract, EDTA or their combinations against a 5 strain *Salmonella* cocktail (log_{10} CFU/g) on vacuum-packed chicken breasts held at 4 °C for 21 d.

Day	Viability of *Salmonella* (log_{10} CFU/g) on vacuum-packed chicken breasts coated with different solutions						
	Uncoated control	Coating control	AITC (50 µl/g)	Mustard (250 mg/g)	EDTA (15 mg/g)	AITC (50 µl/g) + EDTA (15 mg/g)	Mustard (250 mg/g) + EDTA (15 mg/g)
0	6.05±0.06^{a}	6.05±0.06^{a}	6.05±0.06^{a}	6.05±0.06^{a}	6.05±0.06^{a}	6.05±0.06^{a}	6.05±0.06^{a}
5	5.91±0.23^{a}	5.37±0.13^{ab}	4.55±0.08^{cd}	4.79±0.50^{bc}	5.38±0.05^{ab}	3.81±0.33^{de}	3.73±0.56^{e}
11	5.97±0.01^{a}	5.27±0.25^{ab}	4.26±0.21^{cd}	4.69±0.90^{bc}	5.35±0.07^{ab}	3.44±0.38^{d}	3.82±0.40^{cd}
16	5.73±0.02^{a}	5.22±0.23^{a}	4.17±0.37^{b}	4.02±0.84^{b}	5.22±0.04^{a}	3.01±0.13^{c}	3.53±0.37^{bc}
21	5.84±0.40^{a}	5.30±0.13^{b}	3.74±0.01^{c}	3.80±0.23^{c}	5.20±0.06^{b}	3.07±0.33^{d}	3.01±0.01^{d}

Values are the means of two experiments ± standard deviations.
Values within the same row with different letters are significantly different ($p < 0.05$).

Table 7.3: Effect of κ-carrageenan/chitosan-based coatings containing AITC, Oriental mustard extract, EDTA or their combinations on the pH values of vacuum-packed chicken breasts held at 4 °C for 21 d.

Day	pH values of vacuum-packed chicken breasts coated with different solutions						
	Uncoated control	Coating control	AITC (50 µl/g)	Mustard (250 mg/g)	EDTA (15 mg/g)	AITC (50 µl/g) + EDTA (15 mg/g)	Mustard (250 mg/g) + EDTA (15 mg/g)
0	6.28±0.04^{a}	5.95±0.06^{abc}	5.90±0.13^{abc}	5.55±0.07^{c}	6.02±0.02^{ab}	5.98±0.18^{ab}	5.60±0.14^{bc}
5	6.37±0.06^{a}	5.88±0.15^{ab}	5.77±0.01^{ab}	5.11±0.33^{bc}	6.07±0.10^{a}	6.04±0.06^{a}	4.98±0.34^{c}
11	6.23±0.05^{a}	5.75±0.13^{a}	5.88±0.08^{a}	4.84±0.23^{b}	6.00±0.16^{a}	5.86±0.10^{a}	5.01±0.32^{b}
16	6.35±0.07^{a}	5.87±0.05^{ab}	5.80±0.11^{ab}	4.99±0.44^{b}	6.02±0.08^{a}	5.97±0.10^{a}	4.99±0.34^{b}
21	6.19±0.09^{a}	5.87±0.24^{a}	5.85±0.04^{a}	5.03±0.24^{b}	5.95±0.03^{a}	6.01±0.29^{a}	4.95±0.09^{b}

Values are the means of two experiments ± standard deviations.
Values within the same row with different letters are significantly different ($p < 0.05$).

Table 7.4: Antimicrobial activity of κ-carrageenan/chitosan-based coatings containing AITC, Oriental mustard extract, EDTA or their combinations against aerobic bacteria (log_{10} CFU/g) on vacuum-packed chicken breasts held at 4 °C for 21 d.

Day	Viability of aerobic bacteria (log_{10} CFU/g) on vacuum-packed chicken breasts coated with different solutions						
	Uncoated control	Coating control	AITC (50 µl/g)	Mustard (250 mg/g)	EDTA (15 mg/g)	AITC (50 µl/g) + EDTA (15 mg/g)	Mustard (250 mg/g) + EDTA (15 mg/g)
0	6.01±0.13^{a}	6.01±0.13^{a}	6.01±0.13^{a}	6.01±0.13^{a}	6.01±0.13^{a}	6.01±0.13^{a}	6.01±0.13^{a}
5	6.06±0.28^{a}	5.43±0.35^{ab}	4.70±0.14^{ab}	5.48±0.27^{ab}	5.46±0.08^{ab}	3.97±0.26^{b}	4.02±0.81^{b}
11	6.26±0.11^{a}	5.33±0.23^{b}	4.48±0.30^{c}	4.34±0.19^{c}	5.50±0.05^{ab}	3.66±0.16^{c}	3.97±0.30^{c}
16	7.25±0.26^{a}	5.35±0.11^{b}	4.62±0.60^{bc}	4.91±0.59^{b}	5.42±0.06^{b}	3.06±0.56^{c}	4.14±0.39^{bc}
21	7.81±0.30^{a}	5.47±0.33^{b}	4.21±0.31^{bcd}	4.88±0.74^{bc}	5.34±0.04^{b}	3.36±0.42^{d}	3.48±0.07^{cd}

Values are the means of two experiments ± standard deviations.
Values within the same row with different letters are significantly different ($p < 0.05$).

Table 7.5: Antimicrobial activity of κ-carrageenan/chitosan-based coatings containing AITC, Oriental mustard extract, EDTA or their combinations against LAB (log_{10} CFU/g) on vacuum-packed chicken breasts held at 4 °C for 21 d.

Day	Viability of aerobic bacteria (log_{10} CFU/g) on vacuum-packed chicken breasts coated with different solutions						
	Uncoated control	Coating control	AITC (50 µl/g)	Mustard (250 mg/g)	EDTA (15 mg/g)	AITC (50 µl/g) + EDTA (15 mg/g)	Mustard (250 mg/g) + EDTA (15 mg/g)
0	5.94±0.16^{a}	5.94±0.16^{a}	5.94±0.16^{a}	5.94±0.16^{a}	5.94±0.16^{a}	5.94±0.16^{a}	5.94±0.16^{a}
5	6.23±0.53^{a}	5.25±0.24^{abc}	4.65±0.35^{abc}	5.21±0.36^{abc}	5.43±0.03^{ab}	3.59±0.17^{c}	3.78±0.80^{bc}
11	6.74±0.58^{a}	5.22±0.34^{b}	3.66±0.05^{cd}	4.63±0.45^{bc}	5.29±0.07^{b}	2.86±0.20^{d}	3.59±0.32^{cd}
16	7.39±0.25^{a}	5.34±0.23^{b}	3.13±0.60^{c}	4.78±0.65^{b}	5.29±0.08^{b}	1.88±0.39^{c}	3.07±0.16^{c}
21	8.26±0.17^{a}	5.37±0.12^{b}	2.96±0.21^{c}	4.58±0.42^{b}	5.48±0.21^{b}	2.67±0.87^{c}	2.86±0.04^{c}

Values are the means of two experiments ± standard deviations.
Values within the same row with different letters are significantly different ($p < 0.05$).

Chapter 8

Inhibition of *Campylobacter jejuni* on Fresh Chicken Breasts by κ-Carrageenan/Chitosan-Based Coatings Containing Allyl Isothiocyanate or Deodorized Oriental Mustard Extract

8.1 Abstract

Campylobacter species are common bacterial pathogens associated with human gastroenteritis worldwide. The objectives of this study were to determine the minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of allyl isothiocyanate (AITC) against 4 *Campylobacter jejuni* strains in Mueller-Hinton (MH) broth at 4, 21, 37 and 42 °C, and screen the *C. jejuni* strains for their ability to degrade sinigrin (which forms AITC) in pH 7.0 MH broth at 35 °C for 21 d. Also evaluated was the antimicrobial activity of an edible 0.2% κ-carrageenan/2% chitosan-based coating containing AITC or deodorized Oriental mustard extract against a 4 strain *C. jejuni* cocktail (6.2 $\log_{10}$ CFU/g) on vacuum-packaged fresh chicken breasts during 4 °C storage. MIC values of AITC were 0.63 to 1.25 ppm and 2.5 to 5 ppm against tested strains at 37 and 42 °C, respectively. However, the MBC was 2.5 and 5 ppm at 37 and 42 °C, respectively, and increased to a range of 40 to 160 ppm at 4 °C. κ-Carrageenan/chitosan-based coatings containing 50 or 100 µl/g AITC reduced viable *C. jejuni* to undetectable levels on chicken breast after 5 d at 4 °C, while 25 µl/g AITC or 200 to 300 mg/g mustard extract in coatings reduced *C. jejuni* numbers by 1.75 to 2.78 $\log_{10}$ CFU/g more than control coatings without antimicrobial. Both Oriental mustard extract (50 to 300 mg/g) and AITC (≥ 25 µl/g) reduced aerobic bacteria by 1.72 to 2.75 $\log_{10}$ CFU/g and lactic acid bacteria (LAB) by 0.94 to 3.36 $\log_{10}$ CFU/g by 21 d compared to the control coating. κ-Carrageenan/chitosan coatings

containing ≥ 50 ul/g AITC or ≥ 300 mg/g Oriental mustard showed excellent potential to control *C. jejuni* viability on raw chicken.

8.2 Introduction

Campylobacteriosis is an acute zoonotic bacterial infection, primarily of the gastrointestinal tract, that can become invasive. It is caused by *Campylobacter* spp. which include more than 90 biotypes and serotypes (Adedayo and Kirkpatrick, 2008). Among the organisms in this genus, *Campylobacter jejuni* is the most frequent cause of foodborne illness in humans (Adedayo and Kirkpatrick, 2008). *C. jejuni* is a Gram-negative, slender, spirally-curved and highly motile bacterium (Adedayo and Kirkpatrick, 2008; Keener et al., 2004). *C. jejuni* can survive in a wide range of conditions, but especially in moist environments at lower temperatures (Keener et al., 2004). However, it grows over a restricted range of temperatures (32-45 °C), at pH values of 4.9–9.0, and at water activities (a_w) > 0.987. In addition, the organism is microaerophilic and requires a reduced oxygen atmosphere (5%) for growth (Keener et al., 2004). *Campylobacter* may cause severe clinical symptoms including acute enteritis with diarrhea ranging from massive watery to grossly bloody stools, malaise, fever, and abdominal pain sometimes leading to partial paralysis (Keener et al., 2004; Nachamkin et al., 2002). It has been reported in Canada and the US that *Campylobacter* is the second and the third most frequent bacterial cause of foodborne illnesses and is responsible for 145,350 and 845,024 cases/year in those countries, respectively (Scallan et al., 2011; Thomas et al., 2013).

The reservoir for *Campylobacter* is the intestinal tract of animals, most commonly poultry (Adedayo and Kirkpatrick, 2008; Suzuki and Yamamoto, 2009). Although many sources of *Campylobacter* are recognized, campylobacteriosis outbreaks are mainly associated with the

consumption of poultry meat (Suzuki and Yamamoto, 2009). Suzuki and Yamamoto (2009) summarized research describing the prevalence of *Campylobacter* in retail poultry and by-products in various countries and found that the pathogen was present in 72% and 58% of samples in the US and Canada, respectively.

Raw poultry is often contaminated by feces during slaughter and it is frequently identified as the source of human infection (Keener et al., 2004). Washing poultry carcasses with chlorine, organic acids, chlorine dioxide, trisodium phosphate or acidified sodium chlorite reduced *Campylobacter* numbers by 0.5 to 1.5 $\log_{10}$ CFU/g; however, this is a negligible reduction compared to the 5.0 to 8.0 $\log_{10}$ CFU/g of poultry feces that may enter the processing area (Keener et al., 2004).

Application of natural antimicrobials to reduce *Campylobacter* in poultry products may contribute to a significant reduction of campylobacteriosis. Oriental and yellow mustards which contain high levels of glucosinolates (sinigrin and sinalbin, respectively) were inhibitory to *Escherichia coli* O157:H7 and *Listeria monocytogenes* in meat products (Lara-Lledó et al., 2012; Luciano et al., 2011). The glucosinolate sinigrin in Oriental mustard is the precursor of allyl isothiocyanate (AITC) which is a potent antimicrobial with activity against major foodborne pathogens including *E. coli* O157:H7, *Campylobacter* spp., *Salmonella* and *L. monocytogenes* (Dufour et al., 2012; Luciano and Holley, 2009; Olaimat and Holley, 2013 [Chapter 3]). However, AITC formed by plant myrosinase contributes to the pungent flavor of Oriental mustard which has limited its use in food systems (Luciano et al., 2011). To address this issue, heat treatment at >100 °C is used to inactivate the endogenous myrosinase and produce aroma-neutral deodorized mustard, which is still inhibitory to spoilage and pathogenic organisms that express myrosinase-like activity by virtue of its conversion of sinigrin to AITC in the absence of

plant myrosinase (Luciano and Holley, 2011; Olaimat and Holley 2014b [Chapter 4]).

Edible films or coatings provide a novel option to improve the safety and shelf-life of food by serving as selective barriers to moisture transfer, oxygen uptake, lipid oxidation, and prevent loss of volatile aromas and flavors (Kester and Fennema, 1986). The films and coatings can have antimicrobials incorporated which are gradually released at the surface of food packaged with these materials. Therefore, lower concentrations are required to inhibit foodborne pathogens (Sangsuwan et al., 2009). Chitosan is a derivative of chitin and has good-film forming, antimicrobial and biocompatibility properties when combined with other polar compounds due to its high densities of amino and hydroxyl groups (Chen et al., 2012). κ-Carrageenan, a sulfated polysaccharide extracted from edible red seaweeds, has excellent functional properties that facilitate gelling, thickening, emulsifying and stabilizing characteristics, and it is widely used in the food industry. For example, it is used as a binder and stabilizer in the meat industry for the manufacture of sausages, patties and low-fat hamburgers (Li et al., 2014b). Since chitosan and κ-carrageenan are oppositely charged polysaccharides, a mixture of the two compounds has good coating properties, improved action as a gas barrier and can facilitate delayed release of incorporated bioactive compounds (Pinheiro et al., 2012a; b).

Polyvinyl polyethylene glycol film containing deodorized mustard extract with 5% w/w sinigrin was effective in elimination of *L. monocytogenes* from bologna after 52 d at 4 ºC (Lara-Lledó et al., 2012). In addition, chitosan coating containing AITC inhibited *Salmonella* on the surface of egg shells and cantaloupe (Chen et al., 2012; Jin et al., 2013). However, little information is available on the antimicrobial activity of AITC against *C. jejuni* (Dufour et al., 2012). No studies have investigated the activity of AITC or deodorized Oriental mustard extract incorporated in an edible coating against *C. jejuni* on chicken. Therefore, the objectives of the

current study were to: i) determine the minimum inhibitory (MIC) and bactericidal concentrations (MBC) of AITC against 4 *C. jejuni* strains at different temperatures; ii) screen *C. jejuni* strains for their ability to degrade sinigrin which forms AITC, and iii) develop an edible κ-carrageenan/chitosan-based coating containing AITC or deodorized Oriental mustard extract to inhibit *C. jejuni* on vacuum-packaged fresh chicken breasts at 4 ºC.

8.3 Material and Methods

8.3.1 Materials

Fresh chicken breast fillets were purchased from a local grocery store in Winnipeg, MB, Canada. Hot Oriental mustard was from Sakai spice (Calgary, AB, Canada). AITC (94%), chitosan ((1→4)-2-amino-2-deoxy-β-D-glucan, molecular weight: 100,000-300,000 Da; deacetylation degree: 75-85%), and HPLC grade tetrabutylammonium hydrogen sulphate (TBA), were from Acros Organics (Morris Plains, NJ, USA). Sinigrin (potassium [Є-1-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]sulfanylbut-3-enylideneamino] sulfate) from horseradish, glycerol (>99%) and κ-carrageenan [(1→4)-3,6-anhydro-2-*O*-sulfonato-α-D-galactopyranosyl-(1→3)-4-*O*-sulfonato-β-D-galactopyranan] from *Eucheuma cottonii* were from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Other chemicals used were from Fisher Scientific Co. (Fair Lawn, NJ, USA).

8.3.2 Bacterial strains and inoculum preparation

A human clinical *C. jejuni* strain (NCTC1168) and 3 *C. jejuni* strains (*C. jejuni* 206, *C. jejuni* 217 and *C. jejuni* 230) isolated from chicken feces in Saskatchewan, Canada, were provided by Dr. B. J. Allan, University of Saskatchewan. All strains were stored at – 80 ºC in

Mueller-Hinton (MH) broth (Oxoid Ltd., Basingstoke, England) containing 25% glycerol. Frozen cultures were activated by streaking a loopful on MH agar (Oxoid Ltd.) and incubated microaerobically using *Campylobacter* atmosphere-generating sachets (5% oxygen, 10% carbon dioxide, and 85% nitrogen, CampyGen, Oxoid Ltd.) at 42 ºC for 48 h in 3.5 L anaerobic jars (BBL gaspak jars, Becton Dickinson and Company, Franklin Lakes, NJ, USA). A single colony was streaked on *Campylobacter* agar base (Karmali, Oxoid Ltd.) containing *Campylobacter* selective supplement (Oxoid Ltd.) and incubated as described above. Colonies of each strain on plates were harvested using 10 ml MH broth and transferred into a sterile test tube. One hundred µl was transferred to a MH agar plate covered with 5 ml MH broth and incubated microaerobically (CampyGen, Oxoid Ltd.) at 42 ºC for 20 to 24 h. The liquid on culture plates was transferred and mixed with 5 ml MH broth in a sterile test tube for use in experiments.

8.3.3 MIC and MBC of AITC against *C. jejuni* strains

The MIC and MBC of AITC against *C. jejuni* strains were determined by a macro-dilution method. MH broth (pH 7.2) containing 0.31 to 160 ppm AITC was prepared in 9 ml screw-capped tubes using stock solutions of 100 ppm and 4000 ppm. The AITC (0.4 ml) was first dissolved in 2 ml 95% ethanol. Separate tubes containing MH broth and AITC were inoculated with 6.0 $\log_{10}$ CFU/ml of each *C. jejuni* strain and incubated microaerobically (CampyGen, Oxoid Ltd.) in an anaerobic jar with shaking at 100 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA) at 4, 21, 37 and 42 ºC for 24 h. Since *C. jejuni* strains were not able to grow below 32 ºC, the MIC was determined at 37 and 42 ºC. To better define the inhibitory endpoint of AITC, to each MIC test 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium violet (Sigma-Aldrich) dissolved in sterile water was added and incubated for 2 h at room temperature to allow *C. jejuni* cells to reduce the tetrazolium dye and form a red color. The MIC was the

lowest AITC concentration at which no red color appeared because sensitive *C. jejuni* cells lost their ability to reduce the dye (Palaniappan and Holley, 2010). The MBC values were determined at tested temperatures by transferring 100 µl of incubated mixtures to Karmali selective agar plates which were incubated microaerobically at 42 ºC for 48 h. The MBC was the lowest AITC concentration that caused ≥ 3 $\log_{10}$ reduction (99.9% mortality) of the initial *C. jejuni* numbers.

8.3.4 Sinigrin degradation by *C. jejuni* strains

A stock solution of sinigrin was prepared by dissolving 560 mg sinigrin in 10 ml buffered MH broth (pH 7.0). The solution was filter-sterilized (0.22 µm, Millipore, Cork, Ireland) and added to 490 ml sterile buffered MH broth. A 100 µl sample containing 8.0 $\log_{10}$ CFU/ml of individual *C. jejuni* strains was added to 9.9 ml buffered MH broth containing a final concentration of 1120 ppm sinigrin in sterile screw-capped tubes. These were incubated microaerobically with shaking at 200 rpm at 35 ºC for 21 d. One ml from the samples at 0, 3, 7, 14 and 21 d was filter-sterilized in sterile HPLC vials and analyzed for the presence of sinigrin using reversed phase-liquid chromatography (RP-HPLC, model 2695, Waters Corporation, Milford, MA, USA) based on the method described by Lara-Lledó et al. (2012). HPLC solvents used were TBA, acetonitrile (Fisher Scientific) and double-distilled water.

8.3.5 Preparation of κ-carrageenan/chitosan-based coating containing AITC or deodorized Oriental mustard extract

Oriental mustard was autoclaved at 121 ºC for 20 min and the extract was prepared as described by Lara-Lledó et al. (2012). Briefly, the autoclaved (deodorized) mustard was mixed with sterile distilled water (10% w/v) and stirred for 1 h at room temperature. The mixture was centrifuged for 20 min at 4200 *xg* at 4 ºC and filtered using a Whatman no. 4 filter. After boiling

for 30 min at 100 ºC, the mixture was centrifuged and filtered as described above. The filtrate was frozen overnight at −20 ºC and freeze-dried for 72 h.

Coating solutions were prepared by mixing 0.2% (w/v) κ-carrageenan in sterile distilled water and stirring for 1 h at 60 ºC. The steps followed in coating preparation were done under aseptic conditions and utensils used were treated with 70% ethanol. The solution was held for 1 h to cool to room temperature and then 2% (w/v) chitosan was added and mixed with a mechanical stirrer for 30 min. Acetic acid at 1% (v/v) was added to dissolve the chitosan and form a polymeric solution. Glycerol at 4% (v/v) was added as a stabilizer to improve the strength and increase the viscosity of the coating solution. The mixture was mechanically mixed with a magnetic stirring bar overnight until the polymer was completely dissolved. The solution was homogenized at approximately 8000 rpm for 2 min using a homogenizer (L2R, Silverson Machines, Ltd., Waterside, England). The homogenized mixture was divided into 9 parts and mustard extract or AITC (previously dissolved in 1 ml of Tween 80, Sigma) was added separately to 8 parts at 50, 100, 200 and 300 mg/g or 10, 25, 50 and 100 µl/g, respectively. The ninth part was used as a control coating without antimicrobial. All coating solutions were homogenized as described above and then the pH was adjusted to 4.5 (similar to the pH of the control coating) using 0.2 M acetic acid.

8.3.6 Inoculation and coating of chicken meat

The *C. jejuni* cocktail containing 32 ml of a mixture of equal numbers of each of the 4 strains was centrifuged at 1100 *x g* for 20 min at 4 ºC. The culture pellets were washed twice using 32 ml sterile 0.1 M potassium phosphate buffer (pH 7.2) and re-centrifuged using the same conditions. The pellets were diluted with 320 ml 0.1% (w/v) buffered peptone water to yield 8.0

$\log_{10}$ CFU/ml.

Chicken breasts were tested for the presence of aerobic bacteria on Brain Heart Infusion (BHI) agar (Oxoid Ltd.) and for lactic acid bacteria (LAB) on de Man, Rogosa and Sharpe agar (MRS, Oxoid Ltd.) before experiments. The breast fillets were cut into 20 ± 1 g pieces (5 cm x 4 cm) using a sterile knife and scissors. Chicken pieces were dip-inoculated in 100 ml of the *C. jejuni* cocktail mixture for 20 sec and drained for 10 sec. Inoculated chicken breasts were placed on a sterile aluminum tray for 30 min in a laminar airflow hood (Forma 1286, Thermo Electron Corporation, Marietta, OH, USA) to dry and allow *C. jejuni* to attach to the surface of chicken pieces. The inoculated chicken pieces were dipped in 100 ml κ-carrageenan/chitosan solution for 20 sec. The excess coating on samples was drained for 10 sec and treated samples were dried on a sterile aluminum tray for 1 h (30 min/side) in a laminar airflow hood. All samples were packaged in commercial multi-layer high oxygen barrier plastic bags (Deli*1, 17.8 cm × 22.9 cm, Winpak Ltd., Winnipeg, MB, Canada), vacuum-packed and heat-sealed using a vacuum packaging machine (GM 2002, Bizerba Canada Inc, Mississauga, ON, Canada).

8.3.7 Chicken shelf-life study

Vacuum-packed raw chicken breasts slices were stored at 4 ºC and analyzed at 5, 11, 16 and 21 d. Samples were aseptically opened and homogenized with 180 ml 0.1% (w/v) sterile buffered peptone water in stomacher bags (filtra-bags, VWR Scientific, Edmonton, AB, Canada) for 30 sec using a stomacher (Bagmixer®400CC, Interscience Inc., Markham, ON, Canada). Appropriate serial dilutions of each sample were prepared in 0.1% (w/v) buffered peptone water and plated in duplicate on BHI agar (incubated aerobically at 37 ºC for 24 h) for aerobic bacteria, Karmali selective agar (incubated microaerobically at 42 ºC for 48 h) for *C. jejuni* and on MRS

agar (incubated aerobically at 25 ºC for 72 h) for LAB. For treatments stored ≥ 5 d, after the homogenate had been plated on agar, 180 ml of double-strength MH broth was added to the remaining homogenized samples and incubated microaerobically at 42 ºC for 24 h. After incubation, 100 µl was plated in duplicate on Karmali selective agar, incubated at 42 ºC for 48 h and colonies developed were assessed. The pH of chicken pieces was measured using a pH meter (Accumet digital meter 910, Fisher Scientific, Pittsburgh, PA, USA).

8.3.8 Statistical analysis

All data reported are the average values of two experiments with 3 replicates of each experiment (n = 6) and are represented by means ± standard deviation (SD). The results were analyzed by one-way analysis of variance (ANOVA) and statistical differences among treatments were compared by Tukey's test using JMP 10.0.0 software from SAS (SAS Institute Inc., Cary, NC, USA). Significant differences between treatments were denoted if $p \leq 0.05$.

8.4 Results and Discussion

8.4.1 MIC and MBC of AITC against *C. jejuni*

The MIC of AITC against *C. jejuni* strains ranged from 0.63 to 1.25 ppm or 2.5 to 5.0 ppm at 37 and 42 °C, respectively (Table 8.1). The MIC values at 37 °C were slightly lower than those reported by Dufour et al. (2012) who found that the MIC of AITC ranged from 5.0 to 10 ppm against *C. jejuni* strains at that temperature. AITC inhibitory activity is affected by several factors including its stability, the physiological state of cells, temperature and pH. Since AITC is more stable at lower temperatures, it was more inhibitory at 37 °C than at 42 °C (Olaimat and Holley, 2013 [Chapter 3]).

MBC values of AITC against *C. jejuni* strains were also affected by temperature (Table 8.1). AITC was most bactericidal at 37 °C and least active at 4 °C with MBC values of 2.5 to 160 ppm against tested strains. Dufour et al. (2012) found similar MBC values of AITC against *C. jejuni* strains (from 5 to 10 ppm at 37 °C). As observed here, Olaimat and Holley (2013 [Chapter 3]) found that MBC values were higher when AITC was tested at lower temperature against *Salmonella* and *L. monocytogenes*. It has been reported that *C. jejuni* lacks cold-shock proteins, but it is able to survive and resist antimicrobial action at lower temperatures (Hazeleger et al., 1995). In the current study, the higher MBC values of AITC at 4 °C compared to other temperatures may have been due to the presence of cyclic fatty acids in the cell membrane which maintained its fluidity and integrity. It is notable that at 4 °C, *C. jejuni* was still able to generate ATP and catalase activity (Mild et al., 2011). Even though *C. jejuni* NCTC1168 and *C. jejuni* 206 were more resistant to AITC than the other strains at 4 °C, an equal mixture of the 4 strains (Table 8.1) was used in the chicken shelf-life experiments.

8.4.2 Viability of *C. jejuni* on chicken breasts

Although *C. jejuni* is not able to grow at < 32 ºC, it can survive for long periods at lower temperatures (Adedayo and Kirkpatrick, 2008). *C. jejuni* on fresh chicken without coating or antimicrobial was reduced by 1.55 $\log_{10}$ CFU/g at 21 d and 4 ºC (Table 8.2). These results are similar to the findings of Wen and Dickson (2012) who reported that *C. jejuni* numbers were reduced by 0.9 $\log_{10}$ CFU/g on vacuum-packed pork slices after 28 d at 4 or 10 ºC. However, Kudra et al. (2012) found that *C. jejuni* survived up to 42 d without any reduction on vacuum-packed fresh chicken breasts at 4 ºC. Several factors may have influenced the *C. jejuni* reduction in the current study, including strain differences and competition with the natural chicken microflora (aerobic and LAB). Slightly more reduction (1.86 $\log_{10}$ CFU/g) was observed on

chicken breasts coated with unmodified κ-carrageenan/chitosan than on uncoated chicken at 21d. The positive charge on the amino groups in chitosan can interact with the negatively-charged bacterial cell membrane at pH < 6.3, leading to leakage of intracellular constituents. The chitosan used here was of high molecular weight (100,000-300,000 Da) and would be unable to cross the outer membrane of Gram-negative bacteria including that of *C. jejuni* (Helander et al., 2001). Although the antibacterial activity of chitosan increased as its molecular weight was decreased below 91,600 Da and as the degree of deacetylation was increased (Chung et al., 2004; Liu et al., 2001), treatment of the 75-85 % deacetylated chitosan with 1% (v/v) acetic acid during preparation of the coatings in the present work would have had both positive effects on the antimicrobial activity of chitosan by solubilizing chitosan in the reaction mixture and negative effects by re-acetylating chitosan and reducing its antimicrobial activity. However, these changes were not monitored. Choice of the type of chitosan used here was based on its ability to form a suitable coating. Reductions of *C. jejuni* numbers on the uncoated samples were smaller than on those coated with unmodified κ-carrageenan/chitosan, but differences were not significant.

C. jejuni numbers on chicken samples treated with 50 mg/g mustard extract (10.8 mg sinigrin/g coating) were not significantly different from those treated with the control coating up to 21 d. However, κ-carrageenan/chitosan solutions containing mustard extract at 100, 200 or 300 mg/g (coatings contained 21.5, 43 and 64.5 mg/g sinigrin, respectively) reduced *C. jejuni* numbers by 0.89, 1.75 and 2.78 $\log_{10}$ CFU/g more than the control coating by 21 d (Table 8.2). Similarly, Lara-Lledó et al. (2012) found that Oriental mustard extract with 5% (w/w) sinigrin incorporated into a polymeric film reduced *L. monocytogenes* on bologna to ≤ 1.6 $\log_{10}$ CFU/g by 52 d at 4 ºC. Deodorized yellow mustard as an ingredient in dry-fermented sausages also reduced *E. coli* O157:H7 numbers by > 5.0 $\log_{10}$ CFU/g in 18 to 38 d (Graumann and Holley,

2008; Luciano et al., 2011). The antimicrobial activity of Oriental mustard may be attributed to two factors. First, it is probable that the major chemical species formed by bacterial hydrolysis of sinigrin in the mustard extract was AITC (Luciano and Holley, 2011). Since AITC is difficult to measure accurately in aqueous media because it is volatile and easily decomposes to new products under these circumstances (Liu and Yang, 2010), sinigrin degradation was used as a more dependable measure of its formation. Further, it was evident that *C. jejuni* strains possess myrosinase-like enzyme(s) as has been observed with many other foodborne bacteria (Herzallah et al., 2011) and were able to degrade 87% to 91% (975 to 1020 ppm) of the sinigrin present in buffered MH broth (pH 7.0) by 21 d at 35 °C (Table 8.3). Although sinigrin hydrolysis by *C. jejuni* at 35 °C was greater than at 4 °C, the AITC formed is more stable at 4 °C (Olaimat and Holley, 2013 [Chapter 3]). The second factor is the phenolic compounds present in Oriental mustard. It has been found that the MIC and MBC values of Oriental mustard containing phenolic compounds but no sinigrin (sinigrin was depleted by not inactivating endogenous plant myrosinase) were 10 to 20 mg/ml and 20 mg/ml, respectively, against tested *C. jejuni* strains at 37 °C.

κ-Carrageenan/chitosan solutions containing 50 or 100 µl/g AITC reduced *C. jejuni* to an undetectable level (< 1.0 $\log_{10}$ CFU/g) after 5 d storage. When the samples were incubated microaerobically in MH broth at 42 °C for 24 h, the organism was not detected in chicken breasts coated with 100 µl/g AITC, but was recovered from breasts coated with 50 µl/g AITC at all storage intervals (Table 8.2). From additional tests it was found that at 2 and 4 d, the numbers of *C. jejuni* on coated chicken samples treated with 50 µl/g AITC were decreased by 2.8 and 5.2 $\log_{10}$ CFU/g, respectively. κ-Carrageenan/chitosan coatings containing 10 or 25 µl/g AITC reduced *C. jejuni* by 0.65 and 1.88 $\log_{10}$ CFU/g more than the control coating by 21 d (Table

8.2). Similar results were obtained by Jin et al. (2013) and Chen et al. (2012) who found that chitosan coating containing 60 µl/ml AITC reduced *Salmonella* on egg shells or cantaloupe surfaces by 1.7 and > 5.0 $\log_{10}$ CFU/cm^2, respectively, after 24 h at 22 ºC. The antimicrobial activity of AITC against foodborne pathogens is related to its ability to cause leakage of intracellular components (Lin et al. 2000; Zou et al., 2013), inhibit intracellular enzyme activity (Luciano and Holley, 2009) and disrupt cellular metabolism and energy production (Ahn et al., 2001; Chan et al., 2013).

Informal sensory analysis showed that the use of AITC in coatings at > 50 µl/g could be detected by a slight odor and the mustard extract at 300 mg/g gave a slight yellowish color to the coating. Whether these changes would be regarded negatively by consumers has not been determined.

8.4.3 Viability of aerobic and lactic acid bacteria on chicken

On the non-coated chicken, aerobic bacterial numbers increased from 4.14 $\log_{10}$ CFU/g to 6.71 $\log_{10}$ CFU/g by 21 d, while in samples coated with only κ-carrageenan/chitosan, their numbers increased to 5.41 $\log_{10}$ CFU/g. On chicken breasts treated with coatings containing 50 to 300 mg/g mustard or 25 to 100 µl/g AITC, aerobic bacterial numbers were reduced by 1.72 to 3.75 $\log_{10}$ CFU/g more than the control coating at 21 d (Table 8.4). These results are similar to previous findings where AITC significantly inhibited aerobic bacteria on acidified chicken meat at 22 ºC (Lemay et al., 2002), in modified atmosphere-packaged chicken breast at 4 ºC (Shin et al., 2010), or cooked rice at 10, 25 and 60 ºC (Kim et al., 2002).

Numbers of LAB increased from 2.71 to 6.39 and 5.35 $\log_{10}$ CFU/g on non-coated or κ-carrageenan/chitosan-coated chicken breasts, respectively (Table 8.5). Chicken breasts coated

with 100 to 300 mg/g mustard or 50 to 100 µl/g had LAB reduced by 1.96 to 3.36 $\log_{10}$ CFU/g more than the control coating at 21 d. However, only 0.94 and 0.66 $\log_{10}$ CFU/g reductions were caused when the coating contained 50 mg/g mustard extract or 25 µl/g AITC, respectively, while 10 µl/g AITC was not inhibitory to the LAB (Table 8.5). Lara-Lledó et al. (2012) also found that Oriental mustard extract incorporated in an edible film significantly inhibited LAB on bologna at 4 °C. Although LAB may play a role in sinigrin degradation, it has been found that many LAB are resistant to AITC (Holley, 1997; Luciano and Holley, 2011). The results of the current study showed that at equivalent AITC levels (300 mg/g mustard extract would contain 58 µl/g AITC), Oriental mustard was slightly more inhibitory to LAB than pure AITC which suggests that phenolic compounds may contribute to the antimicrobial activity of Oriental mustard against this group of organisms. Phenolic compounds can damage the cytoplasmic membrane, cause leakage of intracellular constituents and also disrupt the cell wall peptidoglycan which can lead to loss of cellular structural integrity (Holley and Patel, 2005). Phenolic acids may also have been responsible in part for the pH reduction of chicken samples coated with mustard extract compared to those coated with AITC. It was noted that the pH of chicken breasts was reduced when the level of Oriental mustard extract increased in the coating and reached 5.20 in breasts with 300 mg/g mustard extract (Table 8.6).

8.5 Conclusions

The MIC and MBC of AITC ranged from 0.63 to 2.5 ppm against *C. jejuni* strains tested at 37 ºC, but *C. jejuni* was less sensitive at 4 ºC where MBCs increased to 40-160 ppm. *C. jejuni* strains hydrolyzed ≥ 87% of sinigrin in buffered MH broth (pH 7.0) by 21 d at 35 °C which would lead to AITC formation. κ-Carrageenan/chitosan coatings containing 50 or 100 µl/g AITC reduced numbers of *C. jejuni* on chicken breasts to an undetectable level (< 1.0 $\log_{10}$ CFU/g)

after 5 d storage at 4 ºC. However, coatings containing 200 to 300 mg/g Oriental mustard extract or 25 µl/g AITC reduced *C. jejuni* on chicken breasts by 1.75 to 2.78 $\log_{10}$ CFU/g compared to those coated with only κ-carrageenan/chitosan. These concentrations also significantly reduced the aerobic bacteria and LAB on chicken breasts and could be expected to extend vacuum-packaged shelf-life at 4 ºC. The results indicated that incorporation of ≥ 50 µl/g AITC or 300 mg/g deodorized Oriental mustard extract in κ-carrageenan/chitosan solutions as an edible coating significantly reduced viable numbers of *C. jejuni* on vacuum-packed chicken breasts and thus enhanced its safety.

Table 8.1: MIC and MBC (ppm) of AITC against 4 individual *C. jejuni* strains at different temperatures.

Strain	MBC [b] (ppm)				MIC [a] (ppm)	
	4 ºC	21 ºC	37 ºC	42 ºC	37 ºC	42 ºC
C. jejuni NCTC1168	160	10	2.5	5	1.25	5
C. jejuni 206	160	10	2.5	5	1.25	5
C. jejuni 217	80	5	2.5	5	1.25	2.5
C. jejuni 230	40	10	2.5	5	0.63	2.5

[a] The lowest concentration at which no visible growth occurred
[b] The lowest concentration which reduced the initial inoculated number by ≥ 3 $\log_{10}$ CFU/ml.

Table 8.2: Antimicrobial activity of κ-carrageenan/chitosan-based coatings containing AITC or Oriental mustard extracts against a 4 strain *C. jejuni* cocktail (log_{10} CFU/g) on vacuum-packed chicken breasts at 4 °C for 21 d.

Day	Control		Mustard extract (mg/g)				AITC (µl/g)			
	Uncoated	Coated	50	100	200	300	10	25	50	100
0	6.18±0.69 [a]	6.18±0.69 [a]	6.18±0.69 [a]	6.18±0.69 [a]	6.18±0.69 [a]	6.18±0.69 [a]	6.18±0.69 [a]	6.18±0.69 [a]	6.18±0.69 [a]	6.18±0.69 [a]
5	5.14±0.30 [a]	4.37±0.84 [ab]	4.41±0.33 [ab]	3.97±0.43 [ab]	3.87±0.21 [ab]	3.53±0.10 [ab]	4.08±0.91 [ab]	2.89±0.29 [b]	ND	ND*
11	5.38±0.16 [a]	4.52±0.44 [ab]	4.45±0.23 [ab]	3.85±0.23 [ab]	3.82±0.51 [ab]	3.14±0.18 [b]	3.54±0.82 [ab]	2.71±0.96 [b]	ND	ND*
16	4.99±0.29 [a]	4.41±0.13 [ab]	4.12±0.38 [abc]	3.76±0.39 [bc]	3.32±0.26 [cd]	2.67±0.08 [de]	3.16±0.09 [cde]	2.15±0.47 [e]	ND	ND*
21	4.63±0.77 [a]	4.32±0.62 [ab]	4.15±0.43 [ab]	3.43±0.25 [abc]	2.57±0.81 [abc]	1.54±0.47 [cd]	3.67±0.25 [ab]	2.44±0.85 [bc]	ND	ND*

Values within the same row with the same letters are not significantly different ($p > 0.05$).

ND: *C. jejuni* cells were not detected (the detection limit was 1.0 log_{10} CFU/g).

* *C. jejuni* cells were not detected after overnight enrichment in MH broth.

Table 8.3: Degradation of sinigrin by 4 individual *C. jejuni* strains in buffered MH broth (pH 7.0) at 35 ºC.

Strain	Sinigrin remaining (ppm) during days storage				
	0	3	7	14	21
Control (without *C. jejuni*)	1122.2±33.2 [a]	1118.2±3.4 [a]	1120.7±8.8 [a]	1116 ±1.3 [a]	1112.4 ±2.7 [a]
C. jejuni NCTC1168	1122.2±33.2 [a]	814.8±6.1 [b]	517.2.8±4.3 [b]	254.1±20.0 [b]	119.1±3.1 [bc]
C. jejuni 206	1122.2±33.2 [a]	908.4±87.9 [b]	479.5±17.1 [b]	204.1±15.0 [b]	119.8±17.9 [bc]
C. jejuni 217	1122.2±33.2 [a]	957.2±21.6 [b]	438.8±16.1 [b]	188.2±31.8 [b]	102.2±2.6 [c]
C. jejuni 230	1122.2±33.2 [a]	870.2±33.4 [b]	455.1±52.0 [b]	177.5±18.7 [b]	147.6±0.1 [b]

Values within the same column with the same letters are not significantly different ($p > 0.05$).

Table 8.4: Antimicrobial activity of κ-carrageenan/chitosan-based coatings containing AITC or Oriental mustard extracts against aerobic bacteria (log_{10} CFU/g) on vacuum-packed chicken breasts at 4 °C for 21 d.

Day	Control		Mustard extracts (mg/g)				AITC (μl/g)			
	Uncoated	Coated	50	100	200	300	10	25	50	100
0	4.14±0.78 [a]	4.14±0.78 [a]	4.14±0.78 [a]	4.14±0.78 [a]	4.14±0.78 [a]	4.14±0.78 [a]	4.14±0.78 [a]	4.14±0.78 [a]	4.14±0.78 [a]	4.14±0.78 [a]
5	5.36±0.65 [a]	4.65±0.06 [ab]	4.11±0.72 [ab]	3.55±0.07 [ab]	3.56±0.54 [ab]	3.39±0.84 [ab]	3.31±0.45 [ab]	3.46±0.50 [ab]	3.14±0.47 [ab]	2.56±0.73 [b]
11	4.82±0.27 [ab]	5.46±0.28 [a]	3.39±0.43 [bc]	3.31±0.22 [bc]	3.35±0.66 [bc]	3.26±0.55 [bc]	4.14±0.90 [abc]	3.43±0.30 [abc]	2.54±0.71 [c]	2.30±0.42 [c]
16	5.77±0.71 [a]	5.56±0.43 [ab]	3.27±0.47 [bc]	3.18±0.54 [bc]	3.20±0.84 [bc]	3.05±0.91 [c]	4.46±0.72 [abc]	3.75±0.21 [abc]	2.20±0.42 [c]	2.38±0.52 [c]
21	6.71±0.15 [a]	5.41±0.16 [ab]	3.29±0.61 [cde]	2.74±0.90 [de]	2.16±0.30 [de]	1.68±0.11 [e]	4.71±0.66 [bc]	3.69±0.16 [bcd]	2.27±0.47 [de]	1.66±0.08 [e]

Values within the same row with the same letters are not significantly different ($p > 0.05$).

Table 8.5: Antimicrobial activity of κ-carrageenan/chitosan-based coatings containing AITC or Oriental mustard extracts against LAB on vacuum-packed chicken breasts at 4 °C for 21 d.

Day	Control		Mustard extracts (mg/g)				AITC (µl/g)			
	Uncoated	Coated	50	100	200	300	10	25	50	100
0	2.71±0.34^{a}	2.71±0.34^{a}	2.71±0.34^{a}	2.71±0.34^{a}	2.71±0.34^{a}	2.71±0.34^{a}	2.71±0.34^{a}	2.71±0.34^{a}	2.71±0.34^{a}	2.71±0.34^{a}
5	5.50±0.02^{a}	4.89±0.28^{ab}	3.34±0.09^{ef}	3.50±0.04^{def}	3.79±0.15^{bcde}	3.69±0.23^{cde}	4.73±0.04^{abc}	4.48±0.38^{abcd}	3.96±0.66^{bcde}	2.41±0.22^{f}
11	5.49±0.36^{a}	5.24±0.40^{ab}	3.56±0.56^{bcd}	3.62±0.11^{abcd}	2.90±0.20^{cd}	2.78±0.25^{d}	4.74±0.16^{abc}	3.29±0.25^{cd}	3.05±0.77^{cd}	2.25±0.92^{d}
16	5.64±0.22^{a}	5.18±0.08^{ab}	4.11±0.18^{bc}	3.69±0.21^{cd}	2.53±0.10^{d}	2.65±0.34^{d}	4.10±0.19^{bc}	3.99±0.06^{bc}	3.20±0.42^{cd}	2.53±0.71^{d}
21	6.39±0.12^{a}	5.35±0.44^{ab}	4.41±0.69^{abc}	3.39±0.69^{bcd}	2.16±0.27^{d}	1.99±0.4^{d}	5.80±0.40^{a}	4.69±0.71^{ab}	3.46±0.33^{bcd}	2.44±0.62^{cd}

Values within the same row with the same letters are not significantly different ($p > 0.05$).

Table 8.6: Effect of κ-carrageenan/chitosan-based coatings containing AITC or Oriental mustard extracts on the pH values of vacuum-packed chicken breasts at 4 °C for 21 d.

Day	Control		Mustard extracts (mg/g)				AITC (μl/g)			
	Uncoated	Coated	50	100	200	300	10	25	50	100
0	6.10±0.07^{a}	6.10±0.09^{a}	5.88±0.04^{ab}	5.68±0.07^{b}	5.65±0.06^{b}	5.28±0.09^{c}	6.00±0.01^{a}	5.99±0.03^{a}	6.09±0.08^{a}	5.95±0.02^{a}
5	6.25±0.33^{a}	6.08±0.17^{a}	5.94±0.28^{a}	5.67±0.01^{a}	5.54±0.40^{a}	5.17±0.01^{a}	6.16±0.08^{a}	6.17±0.25^{a}	6.14±0.38^{a}	6.21±0.29^{a}
11	6.19±0.36^{a}	6.10±0.51^{a}	5.87±0.40^{a}	5.61±0.36^{a}	5.41±0.16^{a}	5.14±0.01^{a}	6.08±0.37^{a}	6.03±0.38^{a}	6.12±0.39^{a}	6.04±0.29^{a}
16	6.07±0.35^{a}	6.01±0.42^{a}	5.92±0.39^{a}	5.59±0.18^{a}	5.34±0.09^{a}	4.98±0.04^{a}	5.98±0.44^{a}	5.99±0.49^{a}	5.98±0.49^{a}	6.03±0.36^{a}
21	6.01±0.09^{a}	5.99±0.14^{a}	5.77±0.10^{ab}	5.57±0.01^{ab}	5.39±0.33^{ab}	5.20±0.04^{b}	6.09±0.32^{a}	6.07±0.26^{a}	6.09±0.21^{a}	6.03±0.18^{a}

Values within the same row with the same letters are not significantly different ($p > 0.05$).

Chapter 9

Conclusions and Further Studies

9.1 Conclusions

Temperature and pH are the major factors influencing the stability and antimicrobial activity of AITC. AITC was more inhibitory against *Salmonella* or *L. monocytogenes* at refrigerator and room temperatures because its stability is higher than at 37 ºC. However, AITC was not inhibitory toward these pathogens when temperatures of 4 or 10 ºC were combined with alkaline pH because AITC became unstable and decomposed to new compounds that were less bactericidal (Tsao et al., 2000; Ohta et al., 1995). In contrast, AITC was more stable at acidic pH, and at pH 5 from 4 to 21 ºC its antimicrobial activity was enhanced against *Salmonella.* Although AITC was effective against *L. monocytogenes* at 4 to 21 ºC with neutral pH, it was less stable than at acidic pH and decomposed to new compounds which had interactive inhibitory effects with the residual AITC against *L. monocytogenes* (Chapter 3).

Temperature, iron compounds and glucose concentration affected the activity of myrosinase-like enzymes in *Salmonella* and *L. monocytogenes*. The degradation of sinigrin by *Salmonella* and *L. monocytogenes* cocktails was significantly enhanced by higher incubation temperature (21 ºC > 10 ºC > 4 ºC) and the presence of iron (10 mM ferric or ferrous ions), but it also was significantly reduced in the presence of $\geq$ 0.25% glucose. It seems that the presence of glucose and low temperature (4 ºC) delayed the synthesis and/or activity of myrosinase-like enzymes in *Salmonella* and *L. monocytogenes,* while ferrous or ferric iron improved the catalytic activity of the enzymes. In contrast, ferric and ferrous iron inhibited the activity of myrosinase

from white mustard by 63% and 35% at 1 d, which showed that the plant and bacterial enzymes had at least one substantial difference (Chapter 5).

The *in vitro* antimicrobial activity of Oriental mustard against *Salmonella* and *L. monocytogenes* was studied at different temperatures and it was found that 0.5% (w/v) Oriental mustard extract reduced numbers of a 5 strain *L. monocytogenes* cocktail 2.3 $\log_{10}$ CFU/ml at 4 ºC by 21 d and 2.4 $\log_{10}$ CFU/ml by 7 d at 10 ºC compared to controls, but it was only slightly inhibitory toward *Salmonella* at 4 ºC and reduced its viability 0.7 $\log_{10}$ CFU/ml at 21 d. Although *Salmonella* and *L. monocytogenes* at 21 ºC degraded 5 times more sinigrin than at 4 ºC, which enabled the formation of inhibitory AITC concentrations, Oriental mustard extract was unable to prevent growth of *Salmonella* and *L. monocytogenes* up to 21 d because of the instability of AITC in aqueous solution (Chapter 4).

To improve its activity at low concentration against *Salmonella* and *L. monocytogenes*, Oriental mustard was combined with EDTA and malic acid. *S*. Typhimurium was not detected at 7 d and 10 d when 0.5% Oriental mustard extract was combined with 0.2% EDTA at 21 and 10 ºC, respectively, while *L. monocytogenes* was not detected at 10 d when 0.5% mustard extract was combined with 0.2% malic acid at 21 ºC. However, the combination was not more inhibitory to *L. monocytogenes* than each of the agents alone at 10 ºC (Chapter 4).

These data suggested that AITC or material from which it can be generated like Oriental mustard alone or when combined with EDTA and malic acid, may have promise as antimicrobial agents in foods stored in the refrigerator such as fresh and cooked poultry meat. To minimize changes in the physicochemical and organoleptic properties of poultry meat, AITC and Oriental mustard were combined with EDTA and malic acid, incorporated in edible antimicrobial

chitosan-based coatings and tested against *L. monocytogenes* on cooked, cured, roast chicken or against *Salmonella* and *C. jejuni* on fresh raw chicken meat (Chapter 6-8) because these specific combinations of types of poultry meat and pathogens are of significant food safety concern.

Against *L. monocytogenes* on cooked, cured, vacuum-packed roast chicken slices, the antimicrobial coatings were prepared by dissolving 0.5% κ-carrageenan and 2% chitosan in a 1.5% malic or acetic acid solution. It was found that acetic or malic acid-κ-carrageenan/chitosan coatings containing 25 to 50 µl/g AITC or 100 to 250 mg/g Oriental mustard extract reduced numbers of *L. monocytogenes* on cooked, cured chicken slices 4.2 to > 7.0 $\log_{10}$ CFU/g by 70 d at 4 ºC compared to numbers on uncoated samples. It was found that malic acid-κ-carrageenan/chitosan coatings were more inhibitory than those prepared using acetic acid (Chapter 6). For tests against *Salmonella* on fresh, refrigerated, vacuum-packed chicken breasts, antimicrobial coatings were prepared by dissolving 0.2% κ-carrageenan and 2% chitosan in a 1% acetic acid solution. Coating containing 250 mg/g mustard extract or 50 µl/g AITC reduced numbers of *Salmonella* on vacuum-packed chicken breasts 2.3 $\log_{10}$ CFU/g by 21 d at 4 ºC, but this reduction was achieved in 5 d when 15 mg/g EDTA was added to the coatings (Chapter 7). It is likely that the ability of EDTA and chitosan to interfere with divalent cation stabilization of the *Salmonella* outer membrane (Alakomi et al., 2000; 2003; Sánchez-Ortega et al., 2014), and the ability of chitosan, malic acid and acetic acids to cause damage in the cytoplasm of *L. monocytogenes* (Eswaranandam et al., 2004; Sánchez-Ortega et al., 2014), enhanced the susceptibility of both pathogens to AITC and Oriental mustard extract in edible coatings (Chapters 4, 6, 7).

C. jejuni strains were more susceptible to AITC than *Salmonella* or *L. monocytogenes* (Chapter 8). This was attributed to the limited number or lack of key regulators of the stress

response systems in *Campylobacter* compared to other bacteria such as *Salmonella* and *E. coli* (Murphy et al., 2006; Park, 2002; Parkhill et al., 2000). Antimicrobial coatings which were prepared using 0.2% κ-carrageenan and 2% chitosan dissolved in a 1% acetic acid solution containing 50 or 100 µl/g AITC reduced numbers of *C. jejuni* on chicken breasts to an undetectable level (< 1.0 $\log_{10}$ CFU/g) after 5 d storage at 4 ºC, while coatings containing 200 to 300 mg/g Oriental mustard extract or 25 µl/g AITC reduced *C. jejuni* numbers on chicken breasts by 3.6 to 4.6 $\log_{10}$ CFU/g (Chapter 8).

Numbers of LAB and aerobic bacteria on fresh, refrigerated, vacuum-packed chicken breasts and cooked, cured, vacuum-packed chicken slices, were significantly reduced by the prepared antimicrobial coatings (Chapters 6-8). Therefore, coatings containing either AITC or mustard extract combined with EDTA, malic or acetic acid would be expected to extend the shelf-life of both fresh, refrigerated, vacuum-packed chicken breasts and cooked, cured, vacuum-packed chicken slices.

It was found that the individual strains of *Salmonella* (5), *C. jejuni* (4) and *L. monocytogenes* (5) tested possessed myrosinase-like enzyme(s) and were able to cause significant sinigrin hydrolysis in MH broth at 25 to 35 ºC (Chapters 6-8). The antimicrobial coatings with 100 or 300 mg/g mustard extract applied to chicken samples at 4 ºC contained 21.5 and 64.5 mg/g sinigrin that could have been hydrolyzed by *Salmonella*, *C. jejuni* and *L. monocytogenes* to form AITC with bactericidal potential. Although sinigrin hydrolysis was 5 times lower at 4 ºC (Chapters 4, 5), the AITC formed was more stable at this temperature (Chapter 3). In addition, the natural chicken microflora (aerobic and LAB) may play a role in sinigrin hydrolysis and raise the concentrations of AITC formed. In understanding mechanisms of antimicrobial action, it must be considered that Oriental mustard has a high phenolic content

(> 2300 ug/g gallic acid equivalents) which may enhance the antimicrobial activity of AITC formed (Wu, 2013).

The results of the current project indicated that AITC or deodorized Oriental mustard extract alone or when combined with EDTA, malic acid or acetic acid in edible κ-carrageenan/chitosan coatings effectively inhibited *L. monocytogenes* on vacuum-packed, cooked, cured roast chicken as well as *Salmonella* and *C. jejuni* on fresh, refrigerated, chicken breasts during their commercial shelf-life in vacuum-packaging at 4 ºC. These results will be of interest to: consumers by providing them with poultry meats having enhanced safety; to food regulatory agencies by creating new processing tools which improve compliance with requirements for reduced product contamination, and to the meat industry by reducing the potential for recalls and increasing the safety and shelf-life of poultry meat products, which will reduce economic loss.

9.2 Further Studies

Salmonella was more likely to tolerate AITC and mustard extract exposures than *L. monocytogenes*. Incorporation of more than three antimicrobials in edible coatings may further enhance the inhibitory effectiveness reported against *Salmonella* on fresh chicken. Gadang et al. (2008) found that whey protein isolate coatings containing a combination of grape seed extract, nisin, malic acid, and EDTA reduced *S.* Typhimurium numbers on turkey frankfurters by 5 $\log_{10}$ CFU/g after 28 d at 4 ºC. Further study should be done to investigate combinations of AITC or Oriental mustard with other natural antimicrobials like grape seed extract or bacteriocins with EDTA plus malic acid in κ-carrageenan/chitosan coatings.

When natural antimicrobials are used in food, the resulting organoleptic properties should be taken into consideration (Davidson et al., 2013; Holley and Patel, 2005). Further study should be done to examine the consumer acceptability of poultry meat coated with κ-carrageenan/chitosan solutions containing AITC or mustard extract.

Yellow mustard contains 4-fold greater glucosinolate levels (Zrybko et al. 1997) and > 2-fold greater phenolic content (Wu, 2013) than Oriental mustard. Further work should be done to investigate the antimicrobial activity of yellow mustard alone or combined with EDTA and organic acids in edible coatings against *L. monocytogenes* on cooked, cured chicken meat and against *Salmonella* and *Campylobacter* on fresh, refrigerated, packaged chicken. It was found that addition of 0.2% hot mustard (containing plant myrosinase) accelerated the antimicrobial activity of 2% yellow or Oriental mustard against *E. coli* O157:H7 in dry sausage (Cordeiro et al., 2014b). Therefore, further work is needed to examine whether the addition of low concentrations of hot mustard to deodorized mustard will improve antimicrobial action against *L. monocytogenes* on cooked, cured chicken meat and against *Salmonella* and *Campylobacter* on fresh, refrigerated, packaged chicken.

Myrosinase was isolated and purified from *E. cloacae* (Tani et al., 1974). In the current project, factors affecting the activity of myrosinase-like enzyme(s) were examined using bacterial cultures (Chapter 5). Additional work should be done to isolate and purify the myrosinase enzyme(s) from the foodborne pathogens examined and study factors that enhance enzyme activity.

Nanotechnology techniques can be used to prepare antimicrobial films containing encapsulated nanoparticles for improving the safety of meat products. Nanoparticles increase the

stability of incorporated antimicrobials, control their release and have the potential to enhance meat quality and extend product shelf-life. Moreover, with the small discrete size of encapsulated antimicrobials within edible films, changes in the appearance and texture of foods is minimized or are absent at effective antimicrobial levels (Hettiarachchy and Ravichandran, 2012). The potential for the use of edible films containing nanoencapsulated mustard extract and AITC on meat products should be examined.

References

Adak, G.K., Meakins, S.M., Yip, H., Lopman, B.A., O'Brien, S.J., 2005. Disease risks from foods, England and Wales, 1996–2000. Emerging Infectious Diseases 11, 365–372.

Adedayo, O., Kirkpatrick, B.D., 2008. *Campylobacter jejuni* infections: update on presentation, diagnosis and management. Hospital Physician 44, 9-15.

Agriculture and Agri-Food Canada, 2013. Poultry and egg market information - Canadian industry. Available at: http://www5.agr.gc.ca/eng/industry-markets-and-trade/statistics-and-market-information/by-product-sector/poultry-and-eggs/poultry-and-egg-market-information-canadian-industry/?id=1384971854388 (Accessed on September 30, 2014).

Ahn, E.S., 2001. Observation of bactericidal effect of allyl isothiocyanate on *Listeria monocytogenes*. Food Science and Biotechnology 10, 31–35.

Aider, M., 2010. Chitosan application for active bio-based films production and potential in the food industry: review. LWT - Food Science and Technology 43, 837–842.

Alakomi, H.L., Skytta, E., Saarela, M., Mattila-Sanholm, T., Latvakala, K., Helander, M., 2000. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Applied and Environmental Microbiology 66, 2001-2005.

Alakomi, H., Saarela, M., Helander, I.M., 2003. Effect of EDTA on *Salmonella enterica* serovar Typhimurium involves a component not assignable to lipopolysaccharide release. Microbiology 149, 2015-2021.

Al-Mutairi, M.F., 2011. The incidence of *Enterobacteriaceae* causing food poisoning in some meat products. Advance Journal of Food Science and Technology 3, 116-121.

Al-Turki, A., Dick, W., 2003. Myrosinase activity in soil. Soil Science Society of America Journal 67, 139-145.

Bender, A., 1992. Meat and meat products in human nutrition in developing countries. Animal Production and Health Division and the Food Policy and Nutrition Division of Food and Agriculture Organization of the United Nations (FAO). Food and Nutrition Papers, vol. 53. Available at: http://www.fao.org/docrep/t0562e/t0562e00.HTM (Accessed on September 29, 2014).

Bertsch, D., Rau, J., Eugster, M.R., Haug, M.C., Lawson, P.A., Lacroix, C., Meile L., 2013. *Listeria fleischmannii* sp. nov., isolated from cheese. International Journal of Systematic and Evolutionary Microbiology 63, 526-532.

Beverlya, R.L., Janes, M.E., Prinyawiwatkula, W., No, H.K., 2008. Edible chitosan films on ready-to-eat roast beef for the control of *Listeria monocytogenes*. Food Microbiology 25, 534–537.

Bhunia, A.K., 2008. *Salmonella enterica*. In: Heldman, D.R. (Ed.). Foodborne Microbial Pathogens: Mechanisms and Pathogenesis. (pp. 201-216). Springer, New York, USA.

Blaser, M.J., Engberg, J., 2008. Clinical aspects of *Campylobacter jejuni* and *Campylobacter coli* infections. In: Nachamkin, I., Blaser, J.M. (Eds). *Campylobacter* (pp. 99–121). American Society for Microbiology (ASM) Press, Washington DC, USA.

Bohaychuk, V.M., Gensler, G.E., King, R.K., Manninen, K.I., Sorensen O., Wu, J.T., Stiles, M.E., Mcmullen, L.M., 2006. Occurrence of pathogens in raw and ready-to-eat meat and poultry products collected from the retail marketplace in Edmonton, Alberta, Canada. Journal of Food Protection 69, 2176–2182.

Bones, A.M., Slupphaug, G., 1989. Purification, characterization and partial amino acid sequencing of β-thioglucosidase from *Brassica napus* L. Journal of Plant Physiology 134, 722-729.

Borek, V., Morra, M.J., Brown, P.D., McCaffrey, J.P., 1994. Allelochemicals produced during sinigrin decomposition in soil. Journal of Agricultural and Food Chemistry 42, 1030-1034.

Borris, R.P., 1996. Natural products research perspective from a major pharmaceutical company. Journal of Ethnopharamacology 51, 29-38.

Brabban, A.D., Edwards, C., 1994. Isolation of glucosinolate degrading organisms and their potential for reducing the glucosinolate content of rapemeal. FEMS Microbiology Letters 119, 83-88.

Brougher, C., Greene, J.L., 2011. The USDA's authority to recall meat and poultry products, congressional research service report for congress, prepared for members and committees of congress, CRS 7-5700/L34313 Available at: http://www.nationalaglawcenter.org/assets/crs/RL34313.pdf (Accessed on October 18, 2014).

Brown, P.M., 2009. Spices, seasonings, and flavors. In: Tarte, R. (Ed.). Ingredients in Meat Products. (pp. 199-210). Springer, New York, USA.

Brusewitz, G., Cameron, B.D., Chasseaud, L.F., Gorler, K., Hawkins, D.R., Koch, H., Mennicke, W.H., 1977. The metabolism of benzyl isothiocyanate and its cysteine conjugate. Biochemistry Journal 162, 99–107.

Cappelier, J.M., Minet, J., Magras, C., Colwell, R.R., Federighi, M., 1999. Recovery in embryonated eggs of viable but nonculturable *Campylobacter jejuni* cells and maintenance of ability to adhere to HeLa cells after resuscitation. Applied and Environmental Microbiology 65, 5154-5157.

Centers for Disease Control and Prevention, CDC, 2010. Foodborne diseases active surveillance network (FoodNet): FoodNet surveillance report for 2008 (final report). Atlanta, GA: U.S. Department of Health and Human Services. Available at: http://www.cdc.gov/foodnet/ar2008_508compliant_v3_final.pdf (Accessed on October 11, 2014).

Centers for Disease Control and Prevention, CDC, 2013. Foodborne illness surveillance systems fact sheet. Available at: http://www.cdc.gov/foodborneburden/PDFs/FACTSHEET_G_SURVEILLANCE.pdf. (Accessed on September 20, 2014).

Centers for Disease Control and Prevention, CDC, 2014a. List of selected multistate foodborne outbreak investigations. Available at: http://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html (Accessed on October 01, 2014).

Centers for Disease Control and Prevention, CDC, 2014b. Trends in foodborne illness in the United States. CDC estimates of foodborne illness in the United States. Available at: http://www.cdc.gov/foodborneburden/trends-in-foodborne-illness.html (Accessed on October 24, 2014).

Center for Science in the Public Interest, CSPI, 2009. Outbreak alert! Analyzing foodborne outbreaks 1998 to 2007. Available at: http://www.cspinet.org/new/pdf/outbreakalertreport 09.pdf (Accessed on October 13, 2014).

Center for Science in the Public Interest, CSPI, 2013. Risky meat: A CSPI field guide to meat & poultry safety. Available at: http://cspinet.org/foodsafety/riskymeat.html (Accessed on October 25, 2014).

Center for Science in the Public Interest, CSPI, 2014. Outbreak alert! 2014. A review of foodborne illness in America from 2002-2011. Available at: http://cspinet.org/reports/outbreakalert2014.pdf (Accessed on October 12, 2014).

Chacon, P.A., Muthukumarasamy, P., Holley, R.A., 2006. Elimination of *Escherichia coli* O157:H7 from fermented dry sausages at an organoleptically acceptable level of microencapsulated allyl isothiocyanate. Applied and Environmental Microbiology 72, 3096–3102.

Chan, A.C., Ager, D., Thompson, I.P., 2013. Resolving the mechanism of bacterial inhibition by plant secondary metabolites employing a combination of whole-cell biosensors. Journal of Microbiological Methods 93, 209–217.

Chen, C.-W., Ho, C.-T., 1998. Thermal degradation of allyl isothiocyanate in aqueous solution. Journal of Agriculture and Food Chemistry 46, 220–223.

Chen, W., Jin, T.Z., Gurtler, J.B., Geveke, D.J., Fan, X., 2012. Inactivation of *Salmonella* on whole cantaloupe by application of an antimicrobial coating containing chitosan and allyl isothiocyanate. International Journal of Food Microbiology 155, 165-170.

Cheng, D.-L., Hashimoto, K., Uda, Y., 2004. *In vitro* digestion of sinigrin and glucotropaeolin by single strains of *Bifidobacterium* and identification of the digestive products. Food and Chemical Toxicology 42, 351–357.

Chimalizeni, Y., Kawaza, K., Molyneux, E., 2010. The epidemiology and management of non-typhoidal *Salmonella* infections. Advances in Experimental Medicine and Biology 659, 33-46.

Chung, Y.C., Su, Y.P., Chen, C.C., Jia, G., Wang, H.I., Wu, J.C.G., Lin, J.G., 2004. Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. Acta Pharmacologica Sinica 25, 932-936.

Clark, C.G., Farber, J., Pagotto, F., Ciampan, N., Doré, K., Nadon, C., Bernard, K., Ng, L.K., the Canadian Public Health Laboratory Network, 2010. Surveillance for *Listeria monocytogenes* and listeriosis, 1995–2004. Epidemiology and Infection 138, 559-572.

Cordeiro, R.P., Krause, D.O., Doria, J.H., Holley, R.A., 2014a. Role of BaeSR two-component regulatory system in resistance of *Escherichia coli* O157:H7 to allyl isothiocyanate. Food Microbiology 42, 136–141.

Cordeiro, R.P., Luciano, F.B., Holley, R.A., 2013. Evaluation of deodorized yellow mustard concentrations for control of *Escherichia coli* O157:H7 viability in dry fermented sausage. Food Control 33, 20–24.

Cordeiro, R.P., Wu, C., Holley, R.A., 2014b. Contribution of endogenous plant myrosinase to the antimicrobial activity of deodorized mustard against *Escherichia coli* O157:H7 in fermented dry sausage. International Journal of Food Microbiology 189, 132–138

Cork, S.C., 2010. Epidemiology of pathogens in the food supply. In Krause, D., Hendrick, S. (Eds). Zoonotic Pathogens in the Food Chain (pp. 21-58), CAB International, Wallingford, Oxfordshire, UK.

Cox, N.A., Richardson, L.J., Maurer, J.J., Berrang, M.E., Fedorka-Cray, P.J., Buhr, R.J., Byrd, J.A., Lee, M.D., Hofacre, C.L., O'Kane, P.M., Lammerding, A.M., Clark, A.G., Thayer, S.G., Doyle, M.P., 2012. Evidence for horizontal and vertical transmission in *Campylobacter* passage from hen to her progeny. Journal of Food Protection 75, 1896-1902.

D'Aoust, J.Y., Maurer, J., Bailey J.S., 2001. *Salmonella* species. In: Doyle, M.P., Beuchat, L.R. Montville, T.J., (Eds) Food Microbiology: Fundamentals and Frontiers. (pp. 141-179). ASM Press, Washington DC, USA.

Davidson, M.P., Critzer, F.J., Taylor, T.M., 2013. Naturally occurring antimicrobials for minimally processed foods. Annual Review of Food Science and Technology 4, 163-190.

Delaquis, P.J., Mazza, G., 1995. Antimicrobial properties of isothiocyanates in food preservation. Food Technology 49, 73–84.

Doores, S., 2005. Organic acids. In Davidson, P.M., Sofos, J.N., Branen A.L. (Eds.). Antimicrobials in Food. (pp. 91-142). CRC Press, Boca Raton, FL, USA.

Dortet, L., Veiga-Chacon, E., Cossart, P., 2009. *Listeria monocytogenes*. In: Schaechter M. (Ed.) Encyclopedia of Microbiology (pp. 182-198). Institut Pasteur, Paris, France.

Drevets, D.A., Bronze, M.S., 2008. *Listeria monocytogenes*: epidemiology, human disease, and mechanisms of brain invasion. FEMS Immunology Medical Microbiology 53, 151-165.

Dufour, V., Alazzam, B., Ermel, G., Thepaut, M., Rossero, A., Tresse, O., Baysse, C., 2012. Antimicrobial activities of isothiocyanates against *Campylobacter jejuni* isolates. Frontiers in Cellular and Infection Microbiology 2, 53 (13 pages).

Dussurget, O., 2008. New insights into determinants of *Listeria monocytogenes* virulence. International Review of Cell and Molecular Biology 270, 1-38.

Elfoul, L., Rabot, S., Khelifa, N., Quinsac, A., Duguay, A., Rimbault, A., 2001. Formation of allyl isothiocyanate from sinigrin in the digestive tract of rats monoassociated with a human colonic strain of *Bacteroides thetaiotaomicron*. FEMS Microbiology Letters 197, 99-103.

Eswaranandam, S., Hettiarachchy, N.S., Johnson, M.G., 2004. Antimicrobial activity of citric, lactic, malic, or tartaric acids and nisin-incorporated soy protein film against *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella gaminara*. Journal of Food Science 69, 79–84.

European Food Safety Authority, EFSA, European Centre for Disease Prevention and Control, ECDC, 2013. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011. EFSA Journal 11(4), 3129 (250 pages).

European Food Safety Authority, EFSA, European Centre for Disease Prevention and Control, ECDC, 2014. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. EFSA Journal 12(2), 3547 (312 pages).

Fahey, J.W., Zalcmann, A.T., Talalay, P., 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 56, 5–51.

Farber, J.M., Daley, E.M., Mackie, M.T., Limerick, B., 2000. A small outbreak of listeriosis potentially linked to the consumption of imitation crab meat. Letters in Applied Microbiology 31, 100-104.

Farber, J.M., Kozak, G.K., Duquette, S., 2011. Changing regulation: Canada's new thinking on *Listeria*. Food Control 22, 1506-1509.

Farber, J.M., Peterkin P.J., 1991. *Listeria monocytogenes*, a foodborne pathogen. Microbiological Reviews 55, 476-511.

Fenwick, G.R., Heaney, R.K., Mullin, W.J., VanEtten, C.H., 1982. Glucosinolates and their breakdown products in food and food plants. Critical Reviews in Food Science and Nutrition 18, 123-201.

Ferreira, M.A.S., Lund, B.M., 1987. The influence of pH and temperature on initiation of growth of *Salmonella* spp. Letters in Applied Microbiology 5, 67-70.

Finiguerra, M.G., Iori R., Palmieri, S., 2001. Soluble and total myrosinase activity in defatted *Crambe abyssinica* meal. Journal of Agricultural and Food Chemistry 49, 840-845.

Fleckenstein, J.M., Patel, S.K., 2005. Bioterrorism and threats to food safety: *Campylobacter*, *Salmonella*, *Shigella*, diarrheagenic *Escherichia coli*, and *Listeria.* In: Bronze, M.S., Greenfield, R.A. (Eds). Biodefense Principles and Pathogens. (pp. 521-586). Horizon Bioscience, Norfolk, England.

Foley, S., Nayak, R., Hanning, I.B., Johnson, T.L., Han, J., Ricke, S.C., 2011. Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. Applied and Environmental Microbiology 77, 4273–4279.

Food and Agriculture Organization and World Health Organization, FAO/WHO, 2002. Risk assessments of *Salmonella* in eggs and broiler chickens. Microbiological Risk Assessment Series 2. Available at: http://www.fao.org/docrep/005/y4392e/y4392e00.htm (Accessed on October 29, 2014).

Food and Agriculture Organization and World Health Organization, FAO/WHO, 2004. Risk assessment of *Listeria monocytogenes* in ready-to-eat foods. Interpretative Summary. Microbiological Risk Assessment Series. Available at: http://www.who.int/foodsafety/publications/ micro/en/mra4.pdf. (Accessed on October 09, 2014).

Food Safety and Inspection Service, FSIS, 2014. FSIS Compliance Guideline: Controlling *Listeria monocytogenes* in post-lethality exposed ready-to-eat meat and poultry products. January 2014. Available at: http://www.fsis.usda.gov/wps/wcm/connect/d3373299-50e6-47d6-a577-e74a1e549fde/Controlling_LM_RTE_Guideline_0912?MOD=AJPERES (Accessed on Sep 22, 2014).

Fox, E., Hunt K., O'Brien, M., Jordan, K., 2010. *Listeria monocytogenes* in Irish farmhouse cheese processing environments. International Journal of Food Microbiology 145,S39-S45.

Fratmico, P.M., Bhunia, A.K, Smith, J.L., 2005. Foodborne Pathogens in Microbiology and Molecular Biology, 273 pp. Caister Academic Press, Wymondham, Norfolk, UK.

Gadang, V.P., Hettiarachchy, N.S., Johnson, M.G., Owens, C.M., 2008. Evaluation of antibacterial activity of whey protein isolate coating incorporated with nisin, grape seed extract, malic acid, and EDTA on a turkey frankfurter system. Journal of Food Science 73, M389−M394.

Gandhi, M., Chikindas, M.L., 2007. *Listeria*: a foodborne pathogen that knows how to survive. International Journal of Food Microbiology 113, 1-15.

George, S.M., Lund, B.M., Brocklehur T.F., 1988. The effect of pH and temperature on initiation of growth of *Listeria monocytogenes*. Letters in Applied Microbiology 6, 153-156.

Glass, K.A., Doyle, M.P., 1989. Fate of *Listeria monocytogenes* in processed meat products during refrigerated storage. Applied and Environmental Microbiology 55, 1565-1569.

Gómez-Estaca, J., Montero, P., Gimenez, B., Gómez- Guillen, M.C., 2007. Effect of functional edible films and high pressure processing on microbial and oxidative spoilage in cold-smoked sardine (*Sardina pilchardus*). Food Chemistry 105, 511–520.

Gonçalves, M.P.J.C., Pires, A.C.D.S., Soares, N.D.F.F., Araújo, E.A., 2009. Use of allyl isothiocyanate sachet to preserve cottage cheese. Journal of Foodservice 20, 275-279.

Gonzalez-Fandos, E., Herrera, B., 2013. Efficacy of malic acid against *Listeria monocytogenes* attached in poultry skin during refrigerated storage. Poultry Science 92, 1936–1941.

Gonzalez-Fandos, E., Herrera, B., 2014. Efficacy of acetic acid against *Listeria monocytogenes* attached to poultry skin during refrigerated storage. Foods 3, 527-540.

Gottlieb, S.L., Newbern, E.C., Griffin, P.M., Graves, L.M., Hoekstra, R.M., Baker, N.L., Hunter, S.B., Holt, K.,G., Ramsey, F., Head, M., Levine, P., Johnson, G., Schoonmaker-Bopp, D., Reddy, V., Kornstein, L., Gerwel, M., Nsubuga, J., Edwards, L., Stonecipher, S., Hurd, S., Austin, D., Jefferson, M.A., Young, S.D., Hise, K., Chernak, E.D., Sobel, J., the Listeriosis Outbreak Working Group, 2006. Multistate outbreak of listeriosis linked to turkey deli meat and subsequent changes in US regulatory policy. Clinical Infectious Diseases 42, 29-36.

Goulet, V., King, L.A., Vaillant, V., de Valk, H., 2013. What is the incubation period for listeriosis?. BMC Infectious Diseases 13, 11 (7 pages).

Government of Canada, 2009. Listeriosis investigative review. Available at: http://www.listeriosis-listeriose.investigation-enquete.gc.ca/index_e.php?s1=rpt&page=tab (Accessed on September 22, 2014).

Goy, R.C., de Britto, D., Assis, O.B.G., 2009. A review of the antimicrobial activity of chitosan. Polímeros: Ciência e Tecnologia 19, 241-247.

Graumann, G.H., Holley, R.A., 2008. Inhibition of *Escherichia coli* O157:H7 in ripening dry fermented sausage by ground yellow mustard. Journal of Food Protection 71, 486–493.

Graumann, G.H., Holley, R.A., 2009. Survival of *E. coli* O157:H7 during manufacture of dry-cured Westphalian ham surface-treated with allyl isothiocyanate or hot mustard powder. Journal of the Science of Food and Agriculture 89, 617–624.

Graves, L.M., Helsel, L.O., Steigerwalt, A.G., Morey, R E., Daneshvar, M.I., Roof, S.E., Orsi, R.H., Fortes, E.D., Milillo, S.R., denBakker, H.C., Wiedmann, M., Swaminathan, B., Sauders, B.D., 2010. *Listeria marthii* sp. nov., isolated from the natural environment, Finger Lakes National Forest. International Journal of Systematic and Evolutionary Microbiology 60, 1280-1288.

Greig, J.D., Ravel, A., 2009. Analysis of foodborne outbreak data reported internationally for source attribution. International Journal of Food Microbiology 130, 77–87.

Günlü, A., Koyun, E., 2013. Effects of vacuum packaging and wrapping with chitosan-based edible film on the extension of the shelf-life of sea bass (*Dicentrarchus labrax*) fillets in cold storage (4 °C). Food and Bioprocess Technology 6, 1713–1719.

Guo, M., Jin, T.Z., Scullen, O.J., Sommers, C.H., 2013. Effects of antimicrobial coatings and cryogenic freezing on survival and growth of *Listeria innocua* on frozen ready-to-eat shrimp during thawing. Journal of Food Science 78, M1195–M1200.

Guo, M., Jin, T.Z., Wang, L., Scullen, O.J., Sommers, C.H., 2014. Antimicrobial films and coatings for inactivation of *Listeria innocua* on ready-to-eat deli turkey meat. Food Control, 40, 64–70.

Halter, E.L., Neuhaus, K., Scherer, S., 2013. *Listeria weihenstephanensis* sp. nov., isolated from the water plant *Lemna trisulca* taken from a freshwater pond. International Journal of Systematic and Evolutionary Microbiology 63, 641-647.

Han, J.H., Gennadios, A., 2005. Edible films and coatings: a review. In Han, J.H. (Ed.). Innovations in Food Packaging. (pp. 239–262). Elsevier Science, NY, USA.

Hasegawa, N., Matsumoto, Y., Hoshino, A., Iwashita, K., 1999. Comparison of effects of *Wasabica japonica* and allyl isothiocyanate on the growth of four strains of *Vibrio parahaemolyticus* in lean and fatty tuna meat suspensions. International Journal of Food Microbiology 49, 27-34.

Hazeleger, W.C., Janse, J.D., Koenraad, P.M., Beumer, R.R., Rombouts, F.M., Abee, T., 1995. Temperature-dependent membrane fatty acid and cell physiology changes in coccoid forms of *Campylobacter jejuni*. Journal of Applied and Environmental Microbiology 61, 2713-2719.

Health Canada, 2011a. Eating well with Canada's food guide – a resource for educators and communicators. Available at: http://www.hc-sc.gc.ca/fn-an/food-guide-aliment/index-eng.php (Accessed on October 10, 2014).

Health Canada, 2011b. Food-related illnesses. Available at: http://www.hc-sc.gc.ca/fn-an/securit/ill-intox/index-eng.php (Accessed on October 10, 2014).

Health Canada, 2011c. Policy on *Listeria monocytogenes* in ready-to eat foods. Available at http://www.hc-sc.gc.ca/fn-an/legislation/pol/policy_listeria_monocytogenes_2011-eng.php#comply (Accessed on October 8, 2014).

Heaney, R.K., Fenwick, G.R., 1980. Glucosinolates in *Brassica* vegetables. Analysis of 22 varieties of brussels sprout (*Brassica oleracea* var. *gemmifera*). Journal of the Science of Food and Agriculture 31, 785-793.

Helander, I.M., Nurmiaho-Lassila, E.-L., Ahvenainen, R., Rhoades, J., Roller, S., 2001. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. International Journal of Food Microbiology 71, 235–244.

Herzallah, S., Lara Lledó, M., Holley, R.A., 2011. Influence of NaCl and $NaNO_3$ on sinigrin hydrolysis by food-borne bacteria. Journal of Food Protection 74, 2162–2168.

Hettiarachchy, N., Ravichandran, M., 2012. Potential use of edible nanoscale coatings for meat. Food Safety Magazine June/July 2012.

Higueras, L., López-Carballo, G., Hernández-Muñoz, P., Gavara, R., Rollini, M., 2013. Development of a novel antimicrobial film based on chitosan with LAE (ethyl-Nα-dodecanoyl-*L*-arginate) and its application to fresh chicken. International Journal of Food Microbiology 165, 339–345.

Hochkoeppler, A., Palmieri, S., 1992. Kinetic properties of myrosinase in hydrated reverse micelles. Biotechnology Progress 8, 91-96.

Holley, R.A., 1997. Impact of slicing hygiene upon shelf-life and distribution of spoilage bacteria in vacuum packaged cured meats. Food Microbiology 14, 201–211.

Holley, R.A., Patel, D., 2005. Improvement of shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. Food Microbiology 22, 273–292.

Humphrey, T., Jorgensen, F., 2006. Pathogens on meat infection in animals – Establishing a relationship using *Campylobacter* and *Salmonella* as examples. Meat Science 74, 89-97.

Humphrey, T., O'Brien, S., Madsen, M., 2007. Campylobacters as zoonotic pathogens: a food production perspective. International Journal of Food Microbiology 117, 237–257.

Hwang, S., Jeon, B., Yun, J., Ryu, S., 2011. Roles of RpoN in the resistance of *Campylobacter jejuni* under various stress conditions. BMC Microbiology 11, 207 (8 pages).

Hyldgaard, M., Mygind, T., Meyer, R.L., 2012. Essential oils in food preservation: mode of action, synergies and interactions with food matrix components. Frontiers in Microbiology 3, 12 (24 pages).

Isshiki, K., Tokuoka, K., Mori, R., Chiba, S., 1992. Preliminary examination of allyl isothiocyanate vapor for food preservation. Bioscience, Biotechnology and Biochemistry 9, 1476–1477.

Jalali, M., Abedi, D., 2008. Prevalence of *Listeria* species in food products in Isfahan, Iran. International Journal of Food Microbiology 122, 336–340.

James, D., Rossiter J.T., 1991. Development and characteristics of myrosinase in *Brassica napus* during early seedling growth. Physiologia Plantarum 82, 163-170.

Jeyaletchumi, P., Tunung R., Margaret S.P., Son, R., Farinazleen M.G., Cheah, Y.K., 2010. Detection of *Listeria monocytogenes* in foods. International Food Research Journal 17, 1-11.

Jiang, Z., Neetoo, H., Chen, H., 2011. Efficacy of freezing, frozen storage and edible antimicrobial coatings used in combination for control of *Listeria monocytogenes* on roasted turkey stored at chiller temperatures. Food Microbiology 28, 1394–1401.

Jiménez-Colmenero, F., Carballo, J., Cofrades, S., 2001. Healthier meat and meat products: their role as functional foods. Meat Science 59, 5-13.

Jin, T.Z., Gurtler, J.B., 2011. Inactivation of *Salmonella* in liquid egg albumen by antimicrobial bottle coatings infused with allyl isothiocyanate, nisin and zinc oxide nanoparticles. Journal of Applied Microbiology 110, 704-712.

Jin, T.Z., Gurtler, J.B., Li, S-Q., 2013. Development of antimicrobial coatings for improving the microbiological safety and quality of shell eggs. Journal of Food Protection 76, 779–785.

Junttila, J.R., Niemela, S.I., Hirn, J., 1988. Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*. Journal of Applied Bacteriology 65, 321–327.

Kallipolitis, B.H., Ingmer, H., 2001. *Listeria monocytogenes* response regulators important for stress tolerance and pathogenesis. FEMS Microbiology Letters 204, 111-115.

Kawakishi, S., Muramatsu, K., 1966. Studies on the decomposition of sinalbin. Agricultural and Biological Chemistry 30, 688–692.

Keener, K.M., Bashor, M.P., Curtis, P.A., Sheldon, B.W., Kathariou, S., 2004. Comprehensive review of *Campylobacter* and poultry processing. Comprehensive Reviews in Food Science and Food Safety 3, 105-116.

Kelly, A.F., Park, S.F., Bovill, R., Mackey, B.M., 2001. Survival of *Campylobacter jejuni* during stationary phase: evidence for the absence of a phenotypic stationary-phase response. Applied and Environmental Microbiology 67, 2248–2254.

Kester, J.J., Fennema, O.R., 1986. Edible films and coatings: A review. Food Technology 40, 47-59.

Kim, W.T., Chung, H., Shin, I.S., Yam, K.L., Chung, D.H., 2008. Characterization of calcium alginate and chitosan-treated calcium alginate gel beads entrapping allyl isothiocyanate. Carbohydrate Polymers 71, 566–573.

Kim, Y.S., Ahn, E.S., Shin, D.H., 2002. Extension of shelf-life by treatment with allyl isothiocyanate in combination with acetic acid on cooked rice. Journal of Food Science 67, 274–279.

Ko, J.A., Kim, W.Y., Park H.J., 2012. Effects of microencapsulated allyl isothiocyanate (AITC) on the extension of the shelf-life of *Kimchi.* International Journal of Food Microbiology 153, 92-98.

Ko, K.Y,, Mendonca, A.F., Ismail, H., Ahn, D.U., 2009. Ethylenediaminetetraacetate and lysozyme improves antimicrobial activities of ovotransferrin against *Escherichia coli* O157:H7. Poultry Science 88, 406-414.

Krul, C., Humblot, C., Philippe, C., Vermeulen, M., van Nuenen, M., Havenaar, R., Rabot, S., 2002. Metabolism of sinigrin (2-propenyl glucosinolate) by the human colonic microflora in a dynamic *in vitro* large-intestinal model. Carcinogenesis 23, 1009–1016.

Kudra, L.L., Sebranek, J.G., Dickson, J.S., Mendonca, A.F., Zhang, Q., Jackson-Davis, A., Prusa, K.J., 2012. Control of *Campylobacter jejuni* in chicken breast meat by irradiation combined with modified atmosphere packaging including carbon monoxide. Journal of Food Protection 75, 1728–1733.

Kyung, K.H., Fleming, H.P., 1997. Antimicrobial activity of sulfur compounds derived from cabbage. Journal of Food Protection 60, 67-71.

Lado, B.H., Yousef, A.E., 2007. Characteristics of *Listeria monocytogenes* important to food processors. In Ryser E.T., Marth E.H. (Eds). *Listeria*, Listeriosis and Food Safety. (pp. 157-213). CRC Press, Boca Raton, FL, USA.

Lara-Lledó, M., Olaimat, A., Holley, R.A., 2012. Inhibition of *Listeria monocytogenes* on bologna sausages by an antimicrobial film containing mustard extract or sinigrin. International Journal of Food Microbiology 156, 25–31.

Leclercq, A., Clermont, D., Bizet, C., Grimont, P.A., Fleche-Mateos, A.L., Roche, S.M., Buchrieser, C., Cadet-Daniel, V., Monnier, A.L., Lecuit, M., Allerberger, F., 2009. *Listeria rocourtiae* sp. nov. International Journal of Systematic and Evolutionary Microbiology 60, 2210-2214.

Lemay, M-J., Choquette, J., Delaquis, P.J, Gariepy, C., Rodrigue, N., Saucier, L., 2002. Antimicrobial effect of natural preservatives in a cooked and acidified chicken meat model. International Journal of Food Microbiology 78, 217–226.

Li, L., Mendis, N., Trigui, H., Oliver, J.D., Faucher, S.P., 2014a. The importance of the viable but non-culturable state in human bacterial pathogens. Frontiers in Microbiology 5, 258 (20 pages).

Li, L., Ni, R., Shao, Y., Mao, S., 2014b. Carrageenan and its applications in drug delivery. Carbohydrate Polymers 103, 1– 11.

Li, M., Wang, W., Fang, W., Li, Y., 2013. Inhibitory effects of chitosan coating combined with organic acids on *Listeria monocytogenes* in refrigerated ready-to-eat shrimps. Journal of Food Protection 76, 1377–1383.

Li, X., Kushad, M.M., 2005. Purification and characterization of myrosinase from horseradish (*Armoracia rusticana*) roots. Plant Physiology and Biochemistry 43, 503-511.

Lin C.M., Preston J.F., III, Wei, C.I., 2000. Antibacterial mechanism of allyl isothiocyanate. Journal of Food Protection 63, 727–734.

Liu, T-T., Yang T-S., 2010. Stability and antimicrobial activity of allyl isothiocyanate during long-term storage in an oil-in-water emulsion. Journal of Food Science 75, C445-C451.

Liu, X.F., Guan, Y.L., Yang, D.Z., Li, Z., Yao, K.D., 2001. Antibacterial action of chitosan and carboxymethylated chitosan. Journal of Applied Polymer Science 79, 1324-1335.

Lönnerdal, B., Janson, J.-C., 1972. Studies on myrosinases. II. Purification and characterization of a myrosinase from rapeseed (*Brassica napus* L.). Biochimica et Biophysica Acta 315, 421-429.

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

Luciano, F.B., Holley, R.A., 2010. Bacterial degradation of glucosinolates and its influence on the growth of *E. coli* O157:H7 in a dry-fermented sausage model — Part 1. Fleischwirtschaft International 25, 67–70.

Luciano, F.B., Holley, R.A., 2011. Bacterial degradation of glucosinolates and its influence on the growth of *E. coli* O157:H7 in a dry-fermented sausage model —Part 2. Fleischwirtschaft International 26, 78-81.

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

Lupescu, M., 2014. Canada: poultry and products annual. USDA Foreign Agricultural Service, Global Agricultural Information Network. Available at: http://www.fas.usda.gov/data/canada-poultry-and-products-annual-0 (Accessed on October 17, 2014).

Mani-López, E., García, H.S., López-Malo, A., 2012. Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. Food Research International 45, 713-721.

Mari, M., Leoni, O., Bernardi, R., Neri, F., Palmieri, S., 2008. Control of brown rot on stone fruit by synthetic and glucosinolate-derived isothiocyanates. Postharvest Biology and Technology 47, 61-67.

Martínez-Camacho, A.P., Cortez-Rocha, M.O., Ezquerra-Brauer, J.M., Graciano-Verdugo, A.Z., Rodriguez-Félix, F., Castillo-Ortega, M.M., Yépiz-Gómez, M.S., Plascencia-Jatomea, M., 2010.

Chitosan composite films: Thermal, structural, mechanical and antifungal properties. Carbohydrate Polymers 82, 305-315.

Matan, N., Rimkeeree, H., Mawson, A. J., Chompreeda, P., Haruthaithanasan, V., Parker, M., 1982. Antimicrobial activity of cinnamon and clove oils under modified atmosphere conditions. International Journal of Food Microbiology 107, 180-185.

McClure, P.J., Beaumont, A.L., Sutherland, J.P., Roberts, T.A., 1997. Predictive modelling of growth of *Listeria monocytogenes*. The effects on growth of NaCl, pH, storage temperature and $NaNO_2$. International Journal of Food Microbiology 34, 221-232.

McDermid, A.S, McKee, A.S., Dowsett, A.B., Marsh, P.D., 1996. The effect of environmental pH on the physiology and surface structures of *Salmonella* serotype Enteritidis phage type 4. Journal of Medical Microbiology 45, 452-458.

Mead, P.S., Dunne, E.F., Graves, L., Wiedmann, M., Patrick, M., Hunter, S., Salehi, E., Mostashari, F., Craig, A., Mshar, P., Bannerman, T., Sauders, B.D., Hayes, P., Dewitt, W., Sparling, P., Griffin, P., Morse, D., Slutsker, L., Swaminathan, B., 2006. Nationwide outbreak of listeriosis due to contaminated meat. Epidemiology & Infection 134, 744–751.

Medeiros, D.T., Sattar, S.A., Farber, J.M., Carrillo, C.D., 2008. Occurrence of *Campylobacter* spp. in raw and ready-to-eat foods and in a Canadian food service operation. Journal of Food Protection 71, 2087–2093.

Meulenbeld, G.H., Hartmans, S., 2001. Thioglucosidase activity from *Sphingobacterium* sp. strain OTG1. Applied Microbiology and Biotechnology 56, 700–706.

Micha, R., Wallace, S.K., Mozaffarian, D., 2010. Red and processed meat consumption and risk of incident coronary heart disease, stroke, and diabetes mellitus. A systematic review and meta-analysis. Circulation 121, 2271–2283.

Mild, R.M., Joens, L.A., Friedman, M., Olsen, C.W., McHugh, T.H., Law, B., Ravishankar S., 2011. Antimicrobial edible apple films inactivate antibiotic resistant and susceptible *Campybobacter jejuni* strains on chicken breast. Journal of Food Science 76, 163-168.

Miller, A.J., Call, J.E., Bowles, B.L., 1996. Sporostatic, sporocidal and heat sensitizing action of malic acid against spores of proteolytic *Clostridium botulinum*. Journal of Food Protection 59, 115–20.

Minn-Dak Growers Ltd., 2002. Mustard. Available at: http://www.minndak.com/Mustard.htm (Accessed on September 26, 2014)

Mulder, R.W.A.W., Schlundt, J.,1999. The safety of poultry meat: from farm to table. International Consultative Group on Food Irradiation (FAO, IAEA, WHO). Available at: www-naweb.iaea.org/nafa/fep/public/poultrymeat.pdf. (Accessed on October 02, 2014).

Muñoz, A.J., Hernández-Chávez, G., de Anda, R., Martínez, A., Bolívar, F. Gosset, G., 2011. Metabolic engineering of *Escherichia coli* for improving L-3,4 dihydroxyphenylalanine (L-DOPA) synthesis from glucose. Journal of Industrial Microbiology & Biotechnology 38, 1845–1852.

Murphy, C., Carroll, C., Jordan, K.N., 2006. Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. Journal of Applied Microbiology 100, 623–632.

Nachamkin, I., Liu, J., Li, M., Ung, H., Moran, A.P., Prendergast, M.M., Sheikh K., 2002. *Campylobacter jejuni* from patients with Guillain–Barré syndrome preferentially expresses a GD_{1a}-like epitope. Infection and Immunity 70, 5299–5303.

Nadarajah, D., Han, J.H., and Holley, R.A., 2005. Use of mustard flour to inactivate *Escherichia coli* O157:H7 in ground beef under nitrogen flushed packaging. International Journal of Food Microbiology 99, 257–267.

Nedorostova, L., Kloucek, P., Kokoska, L., Stolcova, M., Pulkraibek, J., 2009. Antimicrobial properties of selected essential oils in vapour phase against foodborne bacteria. Food Control 20, 157-160.

Negi, P.S., 2012. Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. International Journal of Food Microbiology 156, 7-17.

Newell, D.G., Fearnley, C., 2003. Sources of *Campylobacter* colonization in broiler chickens. Applied and Environmental Microbiology 69, 4343-4351.

Nilson, A.M., Holley, R.A., 2012. Use of deodorized yellow mustard powder to control *Escherichia coli* O157:H7 in dry cured Westphalian ham. Food Microbiology 30, 400–407.

Nørrung, B., 2000. Microbiological criteria for *Listeria monocytogenes* in foods under special consideration of risk assessment approaches. International Journal of Food Microbiology 62, 217-221.

Obaidat, M.M., Frank, J.F., 2009a. Inactivation of *Escherichia coli* O157:H7 on the intact and damaged portions of lettuce and spinach leaves using allyl isothiocyanate, carvacrol and cinnamaldehyde in vapor phase. Journal of Food Protection 72, 2046-2055.

Obaidat, M.M., Frank, J.F., 2009b. Inactivation of *Salmonella* and *Escherichia coli*O157:H7 on sliced and whole tomatoes by allyl isothiocyanate, carvacrol, and cinnamaldehyde in vapor phase. Journal of Food Protection 72, 315-324.

Ohta, Y., Takatani, K., Kawakishi, S., 1995. Decomposition rate of allyl isothiocyanate in aqueous solution. Bioscience, Biotechnology and Biochemistry 59, 102–103.

Ohtsuru, M., Hata, T., 1972. Molecular properties of multiple forms of plant myrosinases. Agricultural and Biological Chemistry 36, 2495-2503.

Ohtsuru, M., Kawatani, H., 1979. Studies of the myrosinase from *Wasabia japonica*: Purification and some properties of *Wasabia* myrosinase. Agricultural and Biological Chemistry 43, 2249-2255.

Okrend, A.J., Johnston, R.W., Moran, A.B., 1986. Effect of acetic acid on the death rates at 52 °C of *Salmonella* Newport, *Salmonella* Typhimurium and *Campylobacter jejuni* in poultry scald water. Journal of Food Protection 49, 500–504.

Olaimat, A.N., Holley, R.A., 2013. Effects of changes in pH and temperature on the inhibition of *Salmonella* and *Listeria monocytogenes* by allyl isothiocyanate. Food Control 34, 414-419.

Olaimat, A.N., Holley, R.A., 2014a. Control of *Salmonella* on fresh chicken breasts by κ-carrageenan/chitosan-based coatings containing allyl isothiocyanate or deodorized Oriental mustard extract plus EDTA. Food Microbiology 48, 83-88.

Olaimat, A.N., Holley, R.A., 2014. Inhibition of *Listeria monocytogenes* and *Salmonella* by combinations of Oriental mustard, malic acid and EDTA. Journal of Food Science 79, M614-621.

Olaimat, A.N., Fang, Y., Holley, R.A., 2014a. Inhibition of *Campylobacter jejuni* on fresh chicken breasts by κ-carrageenan/chitosan-based coatings containing allyl isothiocyanate or deodorized Oriental mustard extract. International Journal of Food Microbiology 187, 77-82.

Olaimat, A.N., Sobhi, B., Holley, R.A., 2014b. Influence of temperature, glucose and iron on sinigrin degradation by *Salmonella* and *L. monocytogenes.* Journal of Food Protection 77, 2133-2138.

Oliveira, M.M.M., Brugnera, D.F., Alves, E., Piccoli, R.H., 2010, Biofilm formation by *Listeria monocytogenes* on stainless steel surface and biotransfer potential. Brazilian Journal of Microbiology 41, 97-106.

Olsen, S.J., Patrick, M., Hunter, S.B., Reddy, V., Kornstein, L., MacKenzie, W.R., Lane, K., Bidol, S., Stoltman, G.A., Frye, D.M., Lee, I., Hurd, S., Jones, T.F., LaPorte, T.N., Dewitt W., Graves, L., Wiedmann, M., Schoonmaker-Bopp, D.J., Huang, A.J., Vincent, C., Bugenhagen A., Corby, J., Carloni, E.R., Holcomb, M.E., Woron, R.F., Zansky, S.M., Dowdle, G., Smith, F., Ahrabi-Fard, S., Ong, A.R., Tucker, N., Hynes, N.A., Mead P., 2005. Multistate outbreak of

Listeria monocytogenes infection linked to delicatessen turkey meat. Clinical Infectious Diseases 40, 962-967.

Oussalah, M., Caillet, S., Saucier, L., Lacroix, M., 2007. Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*. Food Control 18, 414-420.

Pagotto, F., Lai-King, N., Clark, C., Farber, J., 2006. Canadian listeriosis reference service. Foodborne Pathogens and Disease 3, 132-137.

Painter, J.A., Hoekstra, R.M., Ayers, T., Tauxe, R.V., Braden, C.R., Angulo, F.J., Griffin, P.M., 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998–2008. Emerging Infectious Diseases 19, 407-415.

Palaniappan, K., Holley, R.A., 2010. Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. International Journal of Food Microbiology 140, 164-168.

Palop, M.L., Smiths, J.P., Brink, B., 1995. Degradation of sinigrin by *Lactobacillus agilis* strain R16. International Journal of Food Microbiology 26, 219-229.

Pang, Y-H., Sheen, S., Zhou, S., Liu, L., Yam, K.L., 2013. Antimicrobial effects of allyl isothiocyanate and modified atmosphere on *Pseudomonas aeruginosa* in fresh catfish fillet under abuse temperatures. Journal of Food Science 78, M555–M559.

Panisello, P.J., Rooney, R., Quantick, P.C., Stanwell-Smith, R., 2000. Application of foodborne disease outbreak data in the development and maintenance of HACCP systems. International Journal of Food Microbiology 59, 221-234.

Park, H.J., 1996. Gas and mechanical barrier properties of carrageenan-based films. Korean Journal of Food Science & Industry 29, 47–53.

Park, S.F., 2002. The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. International Journal of Food Microbiology 74, 177–188.

Park, S.H., Choi, M.R., Park, J.W., Park, K.H., Chung, M.S., Ryu, S., Kang, D.H., 2011. Use of organic acids to inactivate *Escherichia coli* O157, H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on organic fresh apples and lettuce. Journal of Food Science 76, M293–M298.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A.V., Moule, S., Pallen, M.J., Penn, C.W., Quail, M.A., Rajandream, M.A., Rutherford, K.M., van Vliet, A.H., Whitehead S., Barrell B.G., 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403, 665-668.

Pearson, A.D., Greenwood, M.H., Feltham, R.K.A., Healing, T.D., Donaldson, J., Jones, D.M., Colwell, R.R., 1996. Microbial ecology of *Campylobacter jejuni* in a United Kingdom chicken supply chain: intermittent common source, vertical transmission, and amplification by flock propagation. Applied and Environmental Microbiology 62, 4614-4620.

Pechacek, R., Velisek, J., Hrabcova, H., 1997. Decomposition products of allyl isothiocyanate in aqueous solutions. Journal of Agricultural and Food Chemistry 45, 4584–4588.

Pellicer, K., Copes, J., Giannuzzi, L., Zaritzky, N., 2011. Behavior of *Listeria monocytogenes* type1 355/98 (85) in meat emulsions as affected by temperature, pH, water activity, fat and microbial preservatives. Food Control 22, 1573-1581.

Pérez-Diaz, I.M., McFeeters, R.F., 2010. Preservation of acidified cucumbers with a natural preservative combination of fumaric acid and allyl isothiocyanate that target lactic acid bacteria and yeasts. Journal of Food Science 75, 204-208.

Petrou, S., Tsiraki, M., Giatrakou, V., Savvaidis, I.N., 2012. Chitosan dipping or oregano oil treatments, singly or combined on modified atmosphere packaged chicken breast meat. International Journal of Food Microbiology 156, 264–271.

Pinheiro, A.C., Bourbon, A.I., Quintas, M.A.C., Coimbra, M.A., Vicente, A.A., 2012a. κ-Carrageenan/chitosan nanolayered coating for controlled release of a model bioactive compound. Innovative Food Science and Emerging Technologies 16, 227–232.

Pinheiro, A.C., Bourbon, A.I., Medeiros, B.G.S., da Silva, L.H.M., da Silva, M.C.H., Carneiroda-Cunha, M.G., Coimbrad, M.A., Vicente, A.A., 2012b. Interactions between κ-carrageenan and chitosan in nanolayered coatings—Structural and transport properties. Carbohydrate Polymers 87, 1081–1090.

Prakash, O., Rai, A.K., Singh, J., Singh, P.M., 2013. Effect of heavy metal ions and carbohydrates on the activity of cauliflower (*Brassica oleracea* var. botrytis) myrosinase. Journal of Stress Physiology & Biochemistry 9, 108-117.

Public Health Agency of Canada, 2010. C-EnterNet 2008 annual report. National integrated enteric pathogen surveillance program. Available at: http://www.phac-aspc.gc.ca/c-enternet/pubs/2008/index-eng.php (Accessed on October 05, 2014).

Public Health Agency of Canada, 2011. *Salmonella enterica* spp., pathogen safety data sheet - infectious substances. Available at: http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/salmonella-ent-eng.php (Accessed on September 29, 2014).

Rabea, E.I., Badawy, E.T., Stevens, C.V., Smagghe, G., Steurbaut, W., 2003. Chitosan as antimicrobial agent: Applications and mode of action. Biomacromolecules 4, 1457–1465.

Raffa, R.G., Raivio, T.L., 2002. A third envelope stress signal transduction pathway in *Escherichia coli*. Molecular Microbiology 45, 1599–1611.

Rask, L., Andreasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B., Meijer, J., 2000. Myrosinase: gene family evolution and herbivore defense in *Brassicaceae*. Plant Molecular Biology 42, 93–113.

Raybaudi-Massilia, R.M., Mosqueda-Melgar, J., Martin-Belloso, O., 2009. Antimicrobial activity of malic acid against *Listeria monocytoenes*, *Salmonella Enteritidis* and *Escherichia coli* O157:H7 in apple, pear, and melon juices. Food Control 20, 105-12.

Rhee, M.S., Dougherty, R.H., Kang, D.H., 2002. Combined effects of mustard flour, acetic acid, and salt against *Escherichia coli* O157:H7 stored at 5 and 22 °C. Journal of Food Protection 65, 1632–1635.

Rhee, M.S., Lee, S.-Y., Dougherty, R.H., Kang, D.H., 2003. Antimicrobial effects of mustard flour and acetic acid against *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* serovar Typhimurium. Applied and Environmental Microbiology 69, 2959–2963.

Rowbury, R., 2011. Miracle molecules of our age: ethylenediaminetetraacetic acid. Science Progress 94, 232–240.

Ruiz-Palacios, G.M., 2007. The health burden of *Campylobacter* infection and the impact of antimicrobial resistance: playing chicken. Clinical Infectious Diseases 44, 701–703.

Ryan, K.J., 2004. Enterobacteriaceae In: Ryan, K.J., Ray, C.G. (Eds). *Sherris* Medical Microbiology: An Introduction to Infectious Disease. (pp. 343-371). McGraw-Hill, New York, USA.

Sánchez-Ortega, I., García-Almendárez, B.E., Santos-López, E.M., Amaro-Reyes, A., Barboza-Corona, J.E., Regalado, C., 2014. Antimicrobial edible films and coatings for meat and meat products preservation. The Scientific World Journal 2014, 248935 (18 pages).

Sangsuwan, J., Rattanapanone, N., Auras, R.A., Harte, B.R., Rachtanapun, P., 2009. Factors affecting migration of vanillin from chitosan/methyl cellulose films. Journal of Food Science 74, C549–C555.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M.., 2011. Foodborne illness acquired in the United States— major pathogens. Emerging Infectious Diseases 17, 7-15.

Scharff, R.L., 2010. Health-related costs from foodborne illness in the United States. The produce safety project at Georgetown University. Available: http://www.pewtrusts.org/en/research-and-analysis/reports/0001/01/01/healthrelated-costs-from-foodborne-illness-in-the-united-states (Accessed on September 23, 2014).

Schlech, W.F., Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S., Broome, C.V., 1983. Epidemic listeriosis-evidence for transmission by foods. New England Journal of Medicine 308, 203-206.

Sellam, A., Poupard, P., and Simoneau, P., 2006. Molecular cloning of *AbGst1* encoding a glutathione transferase differentially expressed during exposure of *Alternaria brassicicola* to isothiocyanates. FEMS Microbiology Letters 258, 241-249.

Sharma, A., Garg, S.K., 1996. Myrosinase activity in *Brassica* spp. Crop Research 11, 106-110.

Shin, I.S., Masuda, H., Naohide, K., 2004. Bactericidal activity of wasabi (*Wasabia japonica*) against *Helicobacter pylori*. International Journal of Food Microbiology 94, 255–261.

Shin, J., Harte, B., Ryser, E., Selke S., 2010. Active packaging of fresh chicken breast, with allyl isothiocyanate (AITC) in combination with modified atmosphere packaging (MAP) to control the growth of pathogens. Journal of Food Science 75, M65-M71.

Shofran, B.G., Purrington, S.T., Breidt, F., Fleming, H.P., 1998. Antimicrobial properties of sinigrin and its hydrolysis products. Journal of Food Science 63, 621–624.

Siahaan, E.A., Meillisa, A., Woo, H-C., Lee, C-W., Han, J-H., Chun B-S., 2013. Controlled release of allyl isothiocyanate from brown Algae *laminaria japonica* and mesoporous silica MCM-41 for inhibiting food-borne bacteria. Food Science and Biotechnology 22(S), 19-24.

Siripatrawan, U., Noipha, S., 2012. Active film from chitosan incorporating green tea extract for shelf-life extension of pork sausages. Food Hydrocolloids 27, 102–108.

Smith, V.H., Jimmerson, J., 2005. Mustard seed, Briefing No. 59, Agricultural Marketing Policy Center, Linfield Hall, Montana State University. Available at: http://www.ampc.montana.edu/briefings/briefing59.pdf (Accessed on October 07, 2014)

Smulders, F.J., Greer, G.G., 1998. Integrating microbial decontamination with organic acid in HACCP programmes from muscle foods: prospects and controversies. International Journal of Food Microbiology 44, 149–169.

Springett, M.B., Adams, J.B., 1989. Properties of Brussels sprouts thioglucosidase. Food Chemistry 33, 173-186.

Stoin, D., Pirsan, P., Radu, F., Poiana, M-A., Alexa, E., Dogaru, D., 2009. Studies regarding the myrosinase enzymatic activity from black mustard (*Brassica nigra*) seeds. Journal of Food, Agriculture and Environment 7, 44-47.

Su, L.H., Chiu, C.H., 2007. *Salmonella*: clinical importance and evolution of nomenclature. Chang Gung Medical Journal 30, 210-219.

Suzuki, H., Yamamoto, S., 2009. *Campylobacter* contamination in retail poultry meats and by-products in the world: a literature survey. Journal of Veterinary Medical Science 71, 255–261.

Tajkarimi, M.M., Ibrahim, S.A., Cliver, D.O., 2010. Antimicrobial herb and spice compounds in food. Food Control 21, 1199-1218.

Tani, N., Ohtsuru, M., Hata, T., 1974. Purification and general characteristics of bacterial myrosinase produced by *Enterobacter cloacae*. Agricultural and Biological Chemistry 38, 1623-1630.

Taylor, E.V., Herman, K.M., Ailes, E.C., Fitzgerald, C., Yoder, J.S., Mahon, B.E., Tauxe, R.V., 2012a. Common source outbreaks of *Campylobacter* infection in the USA, 1997–2008. Epidemiology and Infection 141, 987–996.

Taylor, T.M., Joerger, R., Palou, E., López-Malo, A., Avila-Sosa, R., Calix-Lara, T., 2012b. Alternatives to traditional antimicrobials for organically processed meat and poultry. In Ricke, S.C. (Ed.) Organic Meat Production and Processing. (pp. 211–230). Iowa State University Press, Ames, USA.

Thomas, M.K., Majowicz, S.E., Sockett, P.N., Fazil, A., Pollari, F., Doré, K., Flint, J.A., Edge, V.L., 2006. Estimated numbers of community cases of illness due to *Salmonella*, *Campylobacter* and verotoxigenic *Escherichia coli*. Canadian Journal of Infectious Diseases & Medical Microbiology 17, 229–234.

Thomas, M.K., Murray, R., Flockhart, L., Pintar, K., Pollari, F., Fazil, A., Nesbitt, A., Marshall B., 2013. Estimates of the burden of foodborne illness in Canada for 30 specified pathogens and unspecified agents, circa 2006. Foodborne Pathogens and Disease 10, 639-648.

Tiwari, B.K., Valdramidis, V.P., O'Donnell, C.P., Muthukumarappan, K., Bourke, P., Cullen, P.J., 2009. Application of natural antimicrobials for food preservation. Journal of Agricultural and Food Chemistry 57, 5987–6000.

Todd, E.C.D., Notermans, S., 2010. Surveillance of listeriosis and its causative pathogen, *Listeria monocytogenes*. Food Control 22, 1484-1490.

Tripathi, S., Mehrotra, G.K. Dutta, P.K., 2010. Preparation and physicochemical evaluation of chitosan/poly(vinyl alcohol)/pectin ternary film for food-packaging applications. Carbohydrate Polymers 79, 711–716.

Tsao, R., Yu, Q., Friesen, I., Potter, J., Chiba, M., 2000. Factors affecting the dissolution and degradation of Oriental mustard-derived sinigrin and allyl isothiocyanate in aqueous media. Journal of Agriculture and Food Chemistry 48, 1898–1902.

Uda, Y., Kurata, T., Arakawa, N., 1986. Effects of pH and ferrous ion on the degradation of glucosinolates by myrosinase. Agricultural and Biological Chemistry 50, 2735-2740.

Ullah, A., Orij, R., Brul, S., Smits, G.J., 2012. Quantitative analysis of the modes of growth inhibition by weak organic acids in *Saccharomyces cerevisiae*. Applied and Environmental Microbiology 78, 8377–8387.

U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2005. Dietary guidelines for Americans, 2005. Available at: http://www.health.gov/dietaryguidelines/dga2005/document/. (Accessed on October 12, 2014).

U.S. Food and Drug Administration, FDA, 2003. *L. monocytogenes* risk assessment: I. Introduction. FDA/Center for Food Safety and Applied Nutrition, USDA/Food Safety and Inspection Service. Available at: http://www.fda.gov/Food/ScienceResearch/ResearchAreas/RiskAssessmentSafetyAssessment/ucm184052.htm. (Accessed on October 12, 2014).

U.S. Food and Drug Administration, FDA, 2011. GRAS Notice No. GRN 363 by Del Monte Foods on calcium disodium EDTA and disodium EDTA to be used in packaged cooked sweet corn products to the U.S. Food and Drug Administration. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=363 (Accessed on October 21, 2014).

Vaara, M., 1992. Agents that increase the permeability of the outer membrane. Microbiology and Molecular Biology Reviews 56, 395–411.

van Beilen, J.W.A., Teixeira de Mattos, M.J., Hellingwerf, K.J., Brul, S., 2014. Distinct effects of sorbic acid and acetic acid on the electrophysiology and metabolism of *Bacillus subtilis*. Applied and Environmental Microbiology 80, 5918-5926.

Vig, A.P., Rampal, G., Thind, T.S., Arora, S., 2009. Bio-protective effects of glucosinolates – a review. LWT-Food Science and Technology 42, 1561–1572.

Ward, S.M., Delaquis, P.J., Holley, R.A., Mazza, G., 1998. Inhibition of spoilage and pathogenic bacteria on agar and pre-cooked roast beef by volatile horseradish distillates. Food Research International 31, 19-26.

Weiss, J., Gibis, M., Schuh, V., Salminen, H., 2010. Advances in ingredient and processing systems for meat and meat products. Meat Science 86, 196–213.

Wen, X., Dickson J.S., 2012. Survival of *Campylobacter jejuni* and *Salmonella enterica* Typhimurium in vacuum-packed, moisture-enhanced pork. Journal of Food Protection 75, 576–579.

White, D.G., Zhao, S., Simjee, S., Wagner, D.D., McDermott, P.F., 2002. Antimicrobial resistance of foodborne pathogens. Microbes and Infection 4, 405-412.

Wiktelius, E., Stenberg G., 2007. Novel class of glutathione transferases from cyanobacteria exhibit high catalytic activities towards naturally occurring isothiocyanates. Biochemistry Journal 406, 115–123.

Wilson, A.E., Bergaentzlé, M., Bindler, F., Marchioni, E., Lintz, A., Ennahar S., 2013. *In vitro* efficacies of various isothiocyanates from cruciferous vegetables as antimicrobial agents against foodborne pathogens and spoilage bacteria. Food Control 30, 318-324.

Winther, M., Nielsen, P.V., 2006. Active packaging of cheese with allyl isothiocyanate, an alternative to modified atmosphere packaging. Journal of Food Protection 69, 2430–2435.

Wreesmann, C.T.J., 2014. Reasons for raising the maximum acceptable daily intake (ADI) of EDTA and the benefits for iron fortification of foods for children 6–24 months of age. Maternal and Child Nutrition 10, 481–495.

Wu, C., 2013. Use of completely and partially deodorized yellow and Oriental mustards to control *Escherichia coli* O157:H7 in dry fermented sausage. MSc Thesis, Department of Food Science, University of Manitoba, Canada.

Wu, H., Zhang, X., Zhang, G.A., Zeng, S.Y., Lin, K.C., 2011. Antifungal vapour-phase activity of a combination of allyl isothiocyanate and ethyl isothiocyanate against *Botrytis cinerea* and *Penicillium expansum* infection on apples. Journal of Phytopathology 159, 450-455.

Xiao, D., Srivastava, S.K., Lew, K.L., Zeng, Y., Hershberger, P., Johnson, C.S., Trump, D.L., Singh, S.V., 2003. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G2/M arrest and inducing apoptosis. Carcinogenesis 24, 891–897.

Yonezawa, Y., Nishikawa, K., Nishioka, H., 1999. DNA damage and intracellular generation of reactive oxygen species by allyl isothiocyanate in *Escherichia coli.* Journal of Environmental Medicine 1, 27–31.

Yuki, N., Koga, M., 2006. Bacterial infections in Guillain-Barre and Fisher syndromes. Current Opinion in Neurology 19, 451-457.

Zhang, Y., 2010. Allyl isothiocyanate as a cancer chemopreventive phytochemical. Molecular Nutrition & Food Research 54, 127-135.

Zhou, F., Baoping, J.I., Zhang, H., Jiang, H., Yang, Z., Li, J., Li, J., Ren, Y., Yan, W., 2007. Synergistic effect of thymol and carvacrol combined with chelators and organic acids against *Salmonella* Typhimurium. Journal of Food Protection 70, 1704-9.

Zou, Y., Jung, L-S., Lee, S.H., Kim, S., Cho, Y., Ahn, J., 2013. Enhanced antimicrobial activity of nisin in combination with allyl isothiocyanate against *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* Typhimurium and *Shigella boydii*. International Journal of Food Science & Technology 48, 324-333.

Zou, Y., Woo, J., Ahn, J., 2012. Cellular and molecular responses of *Salmonella* Typhimurium to antimicrobial-induced stresses during the planktonic-to-biofilm transition. Letters in Applied Microbiology 55, 274–282.

Zrybko, C.L., Fukuda, E.K., Rosen, R.T., 1997. Determination of glucosinolates in domestic and wild mustard by high-performance liquid chromatography with confirmation by electrospray mass spectrometry and photodiode-array detection. Journal of Chromatography A 767, 43-52.