MECHANISM OF ACTION AND UTILIZATION OF ISOTHIOCYANATES FROM MUSTARD AGAINST *ESCHERICHIA COLI* O157:H7

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By

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THESIS FORMAT

The present thesis is comprised of five manuscripts at different stages of publication in peer-reviewed scientific journals (Chapters 3-7), and these are indicated below. The formatting of manuscripts was altered to standardize their presentation as is required for this thesis. Chapter 1 gives an overall introduction to the subjects of study in this thesis. Chapter 2 presents a comprehensive literature review of all the work described in the following chapters and Chapter 8 ends this work with overall conclusions. Lastly, bibliographic references are documented in alphabetical order.

Chapter 3 was originally published with the authorship of F.B. Luciano, Hosseinian, F. S., Beta, T. and Holley, R.A. in 2008, is entitled "Effect of free-SH containing compounds on allyl isothiocyanate antimicrobial activity against *Escherichia coli* O157:H7" and appears in the Journal of Food Science 73: M214-220.

Chapter 4 was originally published with the authorship of F.B. Luciano and Holley, R.A. in 2010, is entitled "Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7" and appears in the International Journal of Food Microbiology 131: 240-245.

Chapter 5 was originally submitted for publication with the authorship of F.B. Luciano and Holley, R.A. in 2010, is entitled "Bacterial degradation of glucosinolates and its influence on the growth of *E. coli* O157:H7 in a dry-fermented sausage model –

Part 1" to Fleischwirtshaft International, and the manuscript was accepted for publication on April 8th, 2010.

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Chapter 7 was originally submitted for publication with the authorship of F.B. Luciano, Belland, J. and Holley, R.A. in 2010, is entitled "Multi-valent origin of the bactericidal activity of yellow mustard powder toward *Escherichia coli* O157:H7 during dry sausage ripening" and is under review by the International Journal of Food Microbiology.

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ABSTRACT

E. coli O157:H7 has been found to survive in dry sausages and cause disease. Isothiocyanates have been studied for their capacity to eliminate pathogens from foods and are attractive from the consumer perspective because of their natural origin. There is a need to better understand how isothiocyanates kill microorganisms and their behaviour in food matrices. It was found that glutathione and cysteine naturally present in meat can react with AIT, forming a conjugate with no or low bactericidal activity against an *E. coli* O157:H7. In addition, AIT presented higher anti-*E. coli* activity at lower pH values; therefore, it should be more efficient in acid foods. AIT was also found to inhibit the activity of thioredoxin reductase and acetate kinase; hence, enzymatic inhibition may represent a way in which AIT kills *E. coli* O157:H7. Mustard powder is used as a spice (active myrosinase) and/or binder (inactive myrosinase) in meat products. Both of these powders killed *E. coli* O157:H7 in dry fermented sausage. This was not expected since the powder lacking myrosinase is not able to produce isothiocyanates. Starter cultures and, more actively, *E. coli* were found to consume significant amounts of glucosinolates. *Pediococcus pentosaceus* UM 121P and *Staphylococcus carnosus* UM 123M (higher myrosinase-like activity) were compared against *P. pentosaceus* UM 116P + *S. carnosus* UM 109M for their ability in reducing *E. coli* viability in dry sausage. Sausage batches containing powders of hot mustard (active myrosinase), cold mustard (inactive myrosinase), autoclaved mustard (inactive myrosinase) and no powder (control) were prepared. Both pairs of starters yielded similar results. Reduction >5 log CFU/g of *E. coli* O157:H7 occurred after 31 d for hot powder and 38 d for cold powder; there was no reduction in the control. *E. coli* O157:H7 itself has greater effect on glucosinolate

degradation than either pair of starters, which may be more important in determining its survival. Autoclaved powder caused >5 log CFU/g reduction after 18 d. This may be the result of synergistic/additive interaction among *E. coli* O157:H7 myrosinase-like activity, the presence of newly formed/released antimicrobials (e.g. phenolic acids, maillard reaction compounds) in the autoclaved powder and the multiple hurdles present in the dry sausage. Autoclaved mustard powder has potential as a novel food ingredient for the meat industry.

Chapter 1

Introduction

Escherichia coli was first isolated in 1855 from children's faeces by the German bacteriologist Theodor Escherich and was recognized as a commensal bacterium from the human gastrointestinal tract (Escherich, 1988). Two outbreaks of illness occurred in 1982 involving a new *E. coli* strain called O157:H7 (Riley et al., 1983). The first outbreak occurred in Oregon and three months later 21 patients were diagnosed with the same problem in Michigan. Both outbreaks were related to undercooked hamburger consumption from the same fast food chain (Buchanan and Doyle, 1997). To date human infections attributed to *E. coli* O157:H7 have been reported in several developed and developing countries. Most outbreaks have been associated with the consumption of undercooked meats. Dry fermented sausages are traditional products that are consumed raw and manufactured without thermal processing. Preservation of these products is a result of various factors: pH drop produced by the fermenting bacteria commonly known as lactic acid bacteria (LAB), reduction of the water activity (a_w) during the drying process, and addition of antimicrobial compounds such as salt, nitrite and spices. Although dry-cured sausages offer diverse hurdles against the growth of microbial pathogens, some of these microorganisms appear able to overcome these barriers. *E. coli* O157:H7 is well known to be pH and salt tolerant (Hunte et al., 2005; Lin et al., 1996). In addition, it has been reported to have a very low infectious dose and infections lead to high mortality rates, mainly in infants, elderly and immunocompromised people. In 1994, *Escherichia coli* O157:H7 sickened 18 individuals following consumption of pre-sliced

dry salami (Hinkens et al., 1996). Other *E. coli* O157:H7 outbreaks involving 39 and 143 people were related to the consumption of Genoa salami and Hungarian plus Cervelat salami, respectively (MacDonald et al., 2004; Williams et al., 2000). Both beef and pork meat were suspected to be the potential sources of *E. coli* O157:H7 during these outbreaks, as they are major ingredients in dry-cured sausages recipes. The occurrence of these outbreaks led food regulatory agencies in both Canada and the US to adopt strict rules for the manufacture of fermented meat products, where the process should result in at least 5-log reduction of the *E. coli* O157:H7 population (CFIA, 1999; Reed, 1995).

Glucosinolates are found in plants belonging to the family *Brassicaceae* (Nielsen and Rios, 2000). These compounds are located within vacuoles and are released when the plant suffers mechanical damage (e.g. wounding, cutting). Once in the cytoplasm, they are hydrolysed by myrosinase, resulting in the formation of three main groups of substances: nitriles, thiocyanates and isothiocyanates (Delaquis and Mazza, 1995). The last group contains diverse compounds with strong antimicrobial activity and they have been used in food products (Lin et al., 2000; Nielsen and Rios, 2000; Obaidat and Frank, 2009). Among them, allyl isothiocyanate (AIT) has been the most extensively researched. Although the antimicrobial activity of AIT varies widely, this volatile compound has been shown to limit the survival of *E. coli* including serotype O157:H7 in meat and meat products, including dry sausage (Chacon et al., 2006a, 2006b; Muthukumarassamy et al., 2003; Nadarajah et al., 2005). AIT is also an electrophilic compound that can react with a wide range of nucleophiles such as amines, thiols, hydroxyls and sulfites (Cejpek et al., 2000; Jiao et al., 1996; Kawakishi and Kaneko, 1985; Verma, 2003). It is suggested that AIT inhibits enzymes and can disrupt the microbial cell membrane, leading to bacterial

death (Kojima and Ogawa, 1971; Lin et al, 2000). However, little work has been conducted to understand how AIT acts against bacteria and whether the reaction of AIT with intrinsic components of food products can occur, reducing its antimicrobial activity. In addition, yellow mustard flour and its ρ-hydroxybenzyl isothiocyanate (ρ-HBIT) were also found to kill *E. coli* O157:H7 (Ekanayake et al., 2006; Graumann and Holley, 2008). The latter isothiocyanate is less pungent than AIT and it is not volatile, which are advantages when it is used as a food ingredient. Interestingly, the utilization of yellow mustard powder with inactivated myrosinase was found to cause a rapid decline in *E. coli* O157:H7 viability in dry fermented sausage (Graumann and Holley, 2008). This was surprising because the thermally-treated mustard powder could not produce ρ-HBIT, and the antimicrobial effect developed from unknown component(s) of the powder.

The main objective of this thesis was to better understand some of the mechanisms which influence the antibacterial activity of isothiocyanates toward *E. coli* O157:H7. Some of the possible interactions of AIT with substances found in food products and how they affect its antimicrobial activity were also explored. In addition, some hypotheses were postulated to understand how yellow mustard powder with inactivated myrosinase was able to kill *E. coli* O157:H7 in dry sausage. In the long term, the aim of this work was to offer the industry a novel and consistent method able to cause a > 5 log reduction in the population of *E. coli* O157:H7 in dry sausage.

Chapter 2

Review of Literature

2.1 *Escherichia coli* O157:H7

2.1.1 Characteristics and pathology

The common habitat of commensal *Escherichia coli* is the mammalian colon, more specifically in its mucous layer. This bacterium is well adapted to the intestinal environment, where it is a highly successful competitor at this crowded site, and is the main facultative anaerobe of the human intestinal microflora (Sweeney et al., 1996). Usually, humans host *E. coli* as an inoffensive microorganism which is harmlessly present as part of the colon microflora from birth (Escherich, 1988). However, several highly adapted *E. coli* clones acquired specific virulence genes that increased their ability to adapt to new environments and cause a broad spectrum of disease. Among these strains, the most successful combinations of virulence factors have persisted to yield specific, aggressive *E. coli* strains that are able to establish illnesses in healthy humans (Nataro and Kaper, 1998). Generally, infection by these strains may cause enteric disease characterized by diarrhea, urinary tract infections and sepsis/meningitis (Kaper et al., 2004). The common *E. coli* intestinal pathogens have been divided into six different categories: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (LeBlanc, 2003).

In 1982, two hemorrhagic colitis outbreaks were related to a new *E. coli* strain

classified as O157:H7 (Riley et al., 1983). The first outbreak occurred in Oregon and involved 26 cases. Three months later 21 patients were diagnosed with the same problem in Michigan. The cause of both outbreaks was related to the consumption of undercooked hamburgers produced by the same fast food chain (Buchanan and Doyle, 1997). The isolates were EHEC, and produced a combination of symptoms, where hemorrhagic colitis (bloody diarrhea) was a common event (Peacock et al., 2001). Strains of *E. coli* are normally named after their respective somatic (O) and flagellar (H) antigens, and O157:H7 is the most problematic and recurrent strain of EHEC in foodborne illness outbreaks (Buchanan and Doyle, 1997). Another common feature among EHEC strains is the production of either/both Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), also known as verotoxin 1 (VT1) and verotoxin 2 (VT2) (Levine, 1987). The term verotoxin originated from the cytopathogenic effect of this toxin against Vero cells (African green monkey kidney cells) (Panos et al., 2006). Stx 1 is identical to the toxin encountered in *Shigella*, and it has 56% homology with Stx 2. There is a high probability that the gene coding the Shiga toxin(s) was acquired through a bacteriophage, directly or indirectly from *Shigella*. The toxins consist of proteins composed of a single A subunit and five B subunits (Mead and Griffin, 1998). The B subunits provide for specific binding to the glycolipid globotriaosylceramide (Gb_3) on the surface of eukaryotic cells (Levine, 1987). After bacterial attachment, endocytosis of the toxin occurs and the A subunit enzymatically inactivates the 28S ribosomal unit. This inactivation blocks protein synthesis and kills the cell (Mead and Griffin, 1998). The Gb_3 receptor can be found in higher concentrations on the surface of epithelial enterocytes, vascular endothelial cells, smooth muscle cells, renal endothelial cells and erythrocytes (Chart, 2000).

Shiga toxin is produced in the colon and can reach the vascular system, traveling by the bloodstream to the kidney. At this site, the toxin is able to attach and damages renal endothelial cells. Initially, it occludes the local microvasculature through a combination of direct toxicity and induction of local inflammatory factors such as cytokines and chemokines (Andreoli et al., 2002). This process results in renal inflammation which, depending on the severity, can lead to the hemolytic uremic syndrome (HUS). HUS is commonly characterized by the sudden onset of hemolytic anemia with fragmentation of red blood cells, thrombocytopenia and acute renal failure after acute gastroenteritis (Jones et al., 2000).

Usually there is an incubation period of 1–7 days after pathogen intake. Then, abdominal cramps appear and are followed by non-bloody diarrhea. The bloody diarrhea starts by the second to third day of illness (Tarr and Neill, 2001). More problematic complications, such as HUS can be found 2–14 days after the initial diarrhea. HUS is more common in people with sub optimally functioning immune systems, like children and the elderly. Nowadays, it is the leading cause of acute renal failure in children (Dundas et al., 2001). After infection, people can continue shedding *E. coli* O157:H7 in their feces for up to 2 months (Panos et al., 2006).

Among the reported outbreaks in the US, HUS was found in 6% of patients and among these 1.2% of cases resulted in death (Boyce et al., 1995). According to the Centers for Disease Control and Prevention (CDC), a third of the patients that had HUS were left with different degrees of permanent renal damage, and 8% had other long term complications such as hypertension (Peacock et al., 2001).

During the first onset of disease, the treatment followed is dependent upon the

patient's reaction to the bacteria and normally it is just supportive. In some cases, patients show limited symptoms and recover without treatment. Many others just have diarrhea and completely recover in 5-10 days (Peacock et al., 2001). The use of antibiotic therapy is contraindicated. Their administration has been correlated with higher rates of subsequent risk of HUS and with increased numbers of deaths in the more severe cases (Carter et al., 1987). It is hypothesized that antibiotics cause the injured bacteria to release the Shiga toxin in the intestine, increasing the extent of tissue damage. Antibiotics can also destroy the normal intestinal flora, and perhaps, permit antibiotic resistant *E. coli* O157:H7 to flourish (Wong et al., 2000).

2.1.2 Habitat and Outbreaks

The principal reservoir of *E. coli* O157:H7 is the bovine intestinal tract and initial outbreaks have been associated with consumption of meat patties (McClure and Hall, 2000). Later it was found that diverse foods were also associated with outbreaks all over the world, including sausages, unpasteurized milk, lettuce, cantaloupe melon, spinach, apple cider and radish sprouts — the latter was responsible for an outbreak affecting > 8,000 people in Japan (Armstrong et al., 1996). Presumably, these food products were contaminated directly or indirectly (via irrigation water) by cattle feces. Facilitated by its low infectious dose (less than 100 cells), *E. coli* O157:H7 has also been found to cause numerous outbreaks associated with drinking and recreational water (Chalmers et al., 2000), person-to-person transmission and by direct contact with cattle (Peacock et al., 2001).

Human infections attributed to *E. coli* O157:H7 have been reported in over 30

countries, reaching alarming annual incidence rates of > 8 per 100,000 inhabitants in some areas of Canada (Table 2.1) (PHAC, 2007), the United States and Scotland (Dundas and Todd, 2000). In 2006, the average EHEC isolation rate in Canada was 3.1 per 100,000 people and 87.4% of these were of the O157:H7 serotype (PHAC, 2007). Other strains such as O26 and O111 have also been involved in foodborne illness outbreaks and are more prominent than O157:H7 in other countries (Bettleheim, 1996). In 1997, Hudson Foods recalled 25 million pounds of ground beef after an *E. coli* O157:H7 outbreak occurred that was caused by contamination at its Nebraska beef processing plant. In 2000, the province of Ontario had one of the largest outbreaks in North America, when seven people died and 2300 were intoxicated as a result of contaminated municipal drinking water (Deisingh and Thompson, 2004).

It has been estimated that 76 million people are poisoned by foodborne pathogens every year in the US alone. From this number, 325,000 are hospitalized and more than 5,000 die (Deisingh and Thompson, 2004). *E. coli* O157:H7 plays an important role, causing an average of 76,000 illnesses and 60 deaths yearly (Mead et al., 1999). In Canada, 999 cases of human illnesses caused by *E. coli* O157:H7 were reported in 2006 (PHAC, 2007). In Canada some provinces have shown much higher rates over the national average frequency in the past few years (see Table 2.1).

Table 2.1 – Incidence of *E. coli* O157:H7 per 100,000 inhabitants in each Canadian Province/Territory from 2002 to 2006 (PHAC, 2007).

Province / Territory	2002	2003	2004	2005	2006
Alberta	8.5	5.8	9.1	5.7	6.0
British Columbia	3.5	2.7	4.9	2.1	3.5
Manitoba	3.5	6.5	10.4	1.6	8.8
Nova Scotia	2.5	1.7	1.3	0.3	0.5
New Brunswick	2.0	2.5	3.5	2.1	2.1
Newfoundland	1.7	1.0	1.0	1.0	0.0
Ontario	3.6	3.3	2.6	2.3	2.7
Québec	3.4	1.6	2.2	1.7	2.0
Prince Edward Island	22.6	9.5	10.9	6.5	15.9
Saskatchewan	4.1	5.5	5.3	3.1	2.7
Northwest Territories	9.6	2.4	12.9	3.2	0.0
Nunavut	3.5	0.0	0.0	7.0	0.0
Yukon Territory	0.0	0.0	0.0	0.0	3.2
Canada	**4.0**	**3.2**	**3.4**	**2.4**	**3.1**

2.2 Dry fermented meat products

Dry fermented uncooked meat products have been consumed all over the world for centuries and are characterized by the growth of microorganisms that produce lactic acid, which drops the pH and generates a distinctive flavour (Getty et al., 2000). Peculiarities of process, recipes and fermenting microorganisms are as diverse as the number of countries where these products are manufactured (Lebert et al., 2007). Generally, dry fermented sausages are formulated with salt, curing salt (nitrite/nitrate), sugar, spices, herbs, lactic acid bacteria (LAB), lean meat and fat. Growth of LAB and metabolite production during sausage ripening will depend on product composition and process parameters (temperature, humidity, use of smoke or not) (Drosinos et al., 2006). Knowledge regarding the characteristics of the LAB used for manufacturing dry sausages is of paramount importance in achieving the desired sensory quality and also in enhancing food safety.

Traditionally, the fermentation of dry sausages was the result of adventitious LAB growth. In other words, the bacteria responsible for acidification and flavour development were present in the meat as contaminants and no external inoculum was used. More recently, with advances in molecular methods, these bacteria have been identified and a number have been made commercially available as starter cultures, usually as mixtures of more than one organism. Mixture composition is dependent on the type of final product desired, but normally a combination of LAB and a Gram-positive, catalase positive cocci are used (Aymerich et al., 2003). Among the LAB, *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Pediococcus pentosaceus* and *Pediococcus acidilactici* are most commonly used for production of dry fermented

sausages (Ammor and Mayo, 2007). The Gram-positive, catalase positive cocci have the ability to degrade H_2O_2 produced by the LAB, which prevents discoloration problems and delays rancidity. They are also responsible for reducing nitrate to nitrite (Työppönen et al., 2003). *Staphylococcus xylosus*, *Staphylococcus carnosus*, *Staphylococcus equorum*, *Staphylococcus succinus* and *Staphylococcus saprophyticus* are the most frequently used organisms for dry fermented sausage production (Mauriello et al., 2004; Drosinos et al., 2007).

2.3 Pathogenic bacteria in dry sausage

Fermented sausages exhibit a number of barriers, known as "hurdles", to the growth of opportunistic pathogenic bacteria. These hurdles consist of low pH, high salt level, presence of organic acids and nitrite, low water activity (a_w), presence of competitive resident microflora, and antimicrobial effects of spices, herbs and smoke (Työppönen et al., 2003). These factors are able to prevent the growth of common foodborne pathogens such as *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium botulinum* (Työppönen et al., 2003). However, *Listeria monocytogenes* (Farber and Peterkin, 1991) and *Escherichia coli* O157:H7 (Hinkens et al., 1996) are able to persist in this harsh environment due to their ability to resist stresses caused by low pH and high salt content. Both these bacteria have very low infectious doses and have caused foodborne illness outbreaks related to the consumption of meat products, although human illness caused by *Listeria monocytogenes* contamination of dry cured fermented meat products has not yet been reported. In 1994, *E. coli* O157:H7 from dry fermented sausages was responsible for 18 cases of foodborne illnesses in the United States, and this led American and Canadian

health authorities to require a minimum 5 log reduction of *E. coli* O157:H7 during dry fermented sausage manufacture (Hinkens et al., 1996; Palanichamy et al., 2008). Several countries have reported outbreaks of enterohemorrhagic *E. coli* in dry sausages including Norway (Schimmer et al., 2008), Australia (Incze, 1998) as well as Canada (MacDonald et al., 2004).

2.4 Mechanisms of pH resistance and salt tolerance

Enteric bacteria possess protective mechanisms which allow them to survive the acidic environment of the stomach and colonize the gastrointestinal tract. For example, *E. coli* was found to persist at pH 2.5 for several hours (Foster, 2004), and this was the result of a complex pH tolerance system. This is a common feature in both non-pathogenic and pathogenic *E. coli* strains, which contributes to the low infectious dose of the latter (Large et al., 2005; Lin et al., 1996). Bacteria like *Salmonella* that have much lower acid resistance require much higher numbers to cause human illnesses (Lin et al., 1995).

It is recognized that *E. coli* has at least three acid resistance systems whose mechanisms have not been totally described. Two systems are activated at low pH in the presence of glucose and are dependent on glutamate or arginine (Hersh et al., 1996; Lin et al., 1995). In these systems, arginine and/or glutamate decarboxylases are utilized. These enzymes cause decarboxylation of amino acids, forming end products that serve as transporters of H^+ ions from the cytoplasm to the exterior of the bacterial cell. For glutamate, two decarboxylases have been described, GadA and GadB, and these react with an amino acid forming γ-aminobutyric acid (GABA) with the consumption of H^+. Then, antiporters expel GABA from the cytoplasm in exchange for more glutamate. The

arginine-dependent system also works by decarboxylation, where agmatine is formed by the arginine-decarboxylase AdiA which uses arginine plus H^+ as substrates. Similarly to the glutamate system, agmatine is also transported to the exterior of the cell and more arginine is internalized by an antiporter system. The glutamate-dependent and arginine-dependent systems are able to protect the bacterial cell at very low pH for several hours if amino acids are available (Foster, 2004).

A third system operates in *E. coli* to provide it with pH resistance when glucose is not present in the environment. This is called the oxidative system and it requires the activation of the gene *rpoS*, or alternative sigma factor, which is a gene common in different bacteria, and is associated with several types of stress (Castanie-Cornet et al., 1999; Lin et al., 1996) including osmotic shock, oxidative stress, starvation and extreme temperatures (Fang et al., 1992; Hengge-Aronis et al., 1991; Suh et al., 1999; Wiedman et al., 1998). Consequently, the activation of *rpoS* by a single stress can provide protection against several other hurdles, and this is called the *rpoS* stress response (Lin et al., 1996). Numerous enzymes responsible for combating oxidative stress (i.e. superoxide dismutase, alkyl hydroperoxide dismutase, and glutathione synthetase) are induced when cells are challenged with low pH (Blankenhorn et al., 1999; Stancik et al., 2002; Maurer et al., 2005). The composition of the bacterial cell membrane was found to be altered when pH was lowered. Reduction in pH increased the levels of saturated fatty acids and decreased the content of unsaturated fatty acids, leading to enhanced membrane rigidity (Yuk and Marshall, 2004).

The salt tolerance of *E. coli* O157:H7 is mostly derived from a mechanism common to several living organisms that involves the antiporter Na^+/H^+ system which is composed

of integral membrane proteins (Hunte et al., 2005). The most common antiporter in *E. coli* is NhaA, which expells Na^+ ions from the interior of the cell in exchange for protons or the reverse (Padan et al., 2004). Apparently, NhaA expression is downregulated under acidic conditions (Gerchman et al., 1999), and other factors may contribute to the salt tolerance of this bacterium at lower pH values (i.e. *rpoS* system) (Lin et al., 1996). Interestingly, *E. coli* O157:H45 showed a higher acid-resistance when salt (4%) was added to the media, with cells having a higher internal pH than those only challenged by acid pH. Thus salt contributed to bacterial survival, offering protection against acidic conditions (Casey and Condon, 2002). In theory, if the increased acid resistance induced by 4% sodium chloride in *E. coli* O157:H45 also occurs in the O157:H7 serotype, it could represent a mechanism contributing to the survival of the latter pathogen in fermented sausage.

2.5 Utilization of natural antimicrobials in food products

2.5.1 Food spoilage and food safety

It is estimated that > 30% of the food produced in the world is lost by spoilage, which is largely caused by microbial growth. This represent an annual cost of almost US$ 50 billion in the US alone (Nelleman et al., 2009). Problems concerning food losses due microbial contamination start at the farm and continue throughout storage, processing, wholesale or retail distribution and consumer utilization (Huis in't Veld, 1996). There are techniques available to help reduce spoilage losses (e.g. gamma irradiation, heat treatment, high pressure treatment and utilization of antimicrobials) that produce food

technically free of microorganisms or decrease their viability to very low levels (Crawford and Ruff, 1996). However, there is strong public opinion polarized against use of processes like irradiation and utilization of chemically synthesized antimicrobials, but it is highly supportive of minimally processed foods that are perceived as natural, fresh and environmentally friendly (Brul et al., 2002). In addition, consumers are demanding products that are easy to prepare, of consistent quality and conveniently packaged. Considering these characteristics together, the development of new products has become a real challenge for food scientists (Roller, 1999).

Food pathogens are also major contributors to food waste worldwide. Although these microorganisms usually do not form visible signs of food spoilage (i.e. slime formation, off-odours, and off-flavours), they may cause severe illnesses, and contaminated food products must be recalled and destroyed. The cost of recall and medical care due to foodborne pathogens was recently estimated to be US$ 152 billion per year in the US alone (Sharff, 2010). Life-threatening pathogens like *Listeria monocytogenes* and *Clostridium botulinum* generate an average cost of US$1.6 and US$ 0.7 million per infected person, respectively. *E. coli* O157:H7 infections were estimated to have an average cost of US$ 15,000/patient. Multiplying this value by the number of *E. coli* O157:H7 cases in the US, the organism generates an annual bill of approximately US$ 1 billion for tax-payers (Sharff, 2010). In 2008, Maple Leaf Foods was involved in one of the worst foodborne illness outbreaks in Canadian history. About 200 types of products that were manufactured at their meat processing facility in Toronto were recalled. In addition, there were 57 confirmed cases and 22 deaths (PHAC, 2009). The cost to Maple Leaf Foods surpassed US$ 50 million, and this included the recall process,

product loss, lawsuits and loss of market share (Greenberg and Elliott, 2009). Analyzing these numbers, the urgent need to minimize the risk of food contamination is clear. However, consumers continue to request products mildly processed which maintain their fresh flavour and have extended shelf-life (Burt, 2004). Hence, interest in the use of natural products that can reduce microbial growth is increasing (Schuenzel and Harrison, 2002; Skandamis and Nychas, 2002). Some of the most common natural antimicrobials are weak organic acids, bacteriocins and essential oils.

2.5.2 Weak organic acids

Weak organic acids include some of the classic preservative agents used in food (i.e. acetic, lactic, benzoic and sorbic acid). These compounds are able to limit bacterial and fungal growth, and they also inhibit the formation of bacterial spores (Blocher and Busta, 1985). After several foodborne illness outbreaks, the combination of lactate and diacetate was extensively adopted as an alternative to control the growth of *Listeria monocytogenes* in meat products (Mbandi and Shelef, 2002; Stekelenburg, 2003). The antimicrobial activity of weak organic acid molecules is enhanced at low pH, where these preservatives become undissociated. In this molecular form, they can easily cross the cytoplasmic membrane and reach the cell interior. Inside the cell, these acids return to their ionic form due the higher pH of the cytoplasm, which results in accumulation of charged structures inside the cell which drastically reduces the ability of these substances to be externalized (Brul and Coote, 1999).

The mechanism of action of weak acids against microbial growth is not totally understood. It has been postulated that they attack from many fronts including: membrane

disruption (Stratford and Anslow, 1998); inhibition of essential metabolic reactions (Krebs et al., 1983); disturbance of intracellular pH homeostasis, and the accumulation of toxic anions (Eklund, 1985). Although living organisms naturally produce all these weak organic acids, many of them are chemically synthesized by industry.

2.5.3 Bacteriocins

Some strains of LAB have the capacity to produce bacteriocins. These are peptides or proteins with bactericidal or bacteriostatic effects against other bacteria (Goff et al., 1996; Benkerroum et al., 2005; Alves et al., 2006). The mechanism of bacteriocin action against bacterial cells is still not fully understood, but they appear to form pores in the cytoplasmic membrane causing the release of metabolites and collapse of the proton motive force (Montville et al., 1995). The more effective action of bacteriocins against Gram-positive bacteria is thought to be related to the presence of an outer membrane in Gram-negatives, which serves as a protective shield, preventing the interaction of bacteriocins with the cytoplasmic membrane (Työppönen et al., 2003). Helander and colleagues (1997) suggested that an edible permeabilizing agent be used to help these bacteriocins traverse the outer membrane of Gram-negatives and reach their supposed target(s).

To date the bacteriocin nisin, synthesized by *Lactococcus lactis*, is the most studied and is the only one approved for use as a food ingredient (Hansen, 1994). Nisin is a peptide composed of 34 amino acids, and was first described in 1928 (Breukink and Kruijj, 1999). Since the early 1960s, nisin has been allowed as a food additive in the UK and nowadays it is permitted in more than 45 other countries (Harris et al., 1992). Its

bactericidal action is predominantly against Gram-positive bacteria; however, it can be active against Gram-negative organisms if their outer membrane is damaged. Thus, nisin can have additive or synergistic effects with other antimicrobials or chemicals that damage the Gram-negative outer membrane (Breukink and Kruijj, 1999). Early studies reported the antimicrobial activity of nisin to be a result of enzymatic inhibition, which could lead to bacterial death (Reddy et al., 2004). However, other studies have shown that treatment with nisin results in efflux of small cytoplasmic molecules such as ATP and amino acids, which seems to occur after membrane damage by nisin. It is now generally accepted that the bacterial membrane is the target of nisin action, where the agent creates pores, eventually causing structural collapse by changing the inner/outer ion gradient, depleting vital molecules and the proton motive force, with loss of bacterial viability (Tiwari et al., 2009). Numerous other bacteria have been found to produce bacteriocins including: *Lactobacillus sakei* (Leroy and de Vuyst, 1999); *Enterococcus faecalis* and *Enterococcus faecium* (Herranz et al., 2001; Ananou et al., 2005); *Lactobacillus buchneri* (Minor-Pérez et al., 2005); *Pediococcus acidilactici* (Nieto-Lozano et al., 2005), and *Pediococcus pentosaceus* (Todorov and Dicks, 2005).

2.5.4 Essential Oils

In the 16th century the scientist Paraceulsus firstly introduced the term essential oil, which is derived from "Quinta essential" representing the active component of a medicine (Burt, 2004). Currently, essential oils are defined as aromatic hydrophobic liquids extracted from different plants and plant materials, such as roots, fruits, bark, herbs, flowers and seeds. Steam distillation is the most common method of extraction

industrially (Holley and Patel, 2005). More than 3000 essential oils are known, and many have been found to have antimicrobial activity (Burt and Reinders, 2003; Tiwari et al., 2009); however, it is just recently that this research area has gained more interest due to the ideology of "ecologically correct consumerism" and popular concern about the potentially harmful activity of synthetic additives (Sacchetti et al., 2005).

Compounds containing phenolic groups are reported to be the most effective antimicrobials among the essential oils (Dorman and Deans, 2000). Oils from clove, oregano, rosemary, thyme, sage and vanillin were consistently shown to have bactericidal activity, and were usually more effective against Gram-positive than Gram-negative bacteria (Marino et al., 2001). However, some non-phenolic compounds have also been described as strong antimicrobials [i.e. cinnamaldehyde and allyl isothiocyanate (AIT)] (Delaquis and Mazza, 1995; Tajkarimi et al., 2010). It is of interest that AIT, a component of brown/black mustard, horseradish and wasabi oil fractions, generally had greater activity against Gram-negative bacteria (Ward et al., 1998). Although the antimicrobial activity of AIT can vary widely, the volatile compound has been shown to limit the survival of *E. coli* including serotype O157:H7 in meat products (Chacon et al., 2006a; Muthukumarasamy et al., 2003; Nadarajah et al., 2005). In addition, AIT is also effective against many species of fungi (Nielsen and Rios, 2000).

Other isothiocyanates were also found to have strong antimicrobial activity including: benzyl isothiocyanate (Manici et al., 1997; Smolinska et al., 2003); phenethyl isothiocyanate (Sexton et al., 1999); methyl isothiocyanate (Lin et al., 2000), and ρ-hydroxybenzyl isothiocyanate (ρ-HBIT) (Ekanayake et al., 2006). Very few studies have examined the potential for use of ρ-HBIT (found in yellow mustard) to eliminate

microbial pathogens from food products, and this appears mainly due to difficulties in its extraction (Buskov et al., 2000) and stability of the purified compound (Kawakishi et al., 1967). However, this compound showed promising bactericidal activity against *Pseudomonas aeruginosa* and several foodborne pathogens including *Escherichia coli*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Salmonella* Enteritidis, *Listeria monocytogenes*, *Shigella boydii* and *Clostridium perfringens* (Ekanayake et al., 2006).

Essential oils are generally composed of some major compounds (> 85% of the oil) plus minor substances that occur at very low levels (Tajkarimi et al., 2010). Their mechanism of antimicrobial activity appears to involve multiple targets (Holley and Patel, 2005). An important feature of essential oils is their hydrophobicity, which enables their insertion in the bacterial cytoplasmic membrane to cause structural alterations that increase cell permeability. This disturbance can also disrupt the cellular proton motive force, destroy transmembrane transport systems, alter the ion gradient inside the cell and inhibit vital enzymes, leading to cell death (Gill and Holley, 2006; Sikkema et al., 1994, 1995). The exact way in which these essential oils kill microorganisms is still largely unknown.

Usually the antimicrobial potency of essential oils observed *in vitro* is not as strong in food products, and levels necessary to achieve satisfactory antimicrobial activity may generate undesirable taste (Burt, 2004). Actually, the strong flavour and aroma present in these oils can be either pleasant or distasteful depending on the type of food in which they are used. Hence, it is more reasonable to incorporate these antimicrobials in products traditionally associated with herbs or spice use (e.g. meat, fish, poultry and their products). In addition, it has been challenging for food microbiologists to develop novel

techniques to optimize the antimicrobial activity of essential oils while minimizing the concentrations needed to achieve microbial inhibition (Brul and Coote, 1999). High concentrations of an essential oil may be necessary because the antimicrobial may react with components of the food product or become lost because of the volatility of the active agent in the absence of suitable protective packaging. Several studies have been done to prevent losses by volatilization, where microencapsulation has shown satisfactory results (Li et al., 2007; Shaikh et al., 2006; Varona et al., 2009). It is a technique widely used for pharmaceutical products to control delivery rates of drugs, but it is also used in the food industry for flavour stabilization. Microencapsulation has been shown to be a useful technique to ensure better effectiveness of essential oils as antimicrobials by improving their stabilization and reducing losses by volatilization (Chacon et al., 2006a; Gamage et al., 2009).

2.5.5 Formation and occurrence of isothiocyanates in mustard

The presence of secondary metabolites called glucosinolates and the enzyme myrosinase (EC 3.2.1.147) are phytochemical characteristics of all *Brassicaceae* (*Cruciferae*) species (Chung et al., 2005). These elements are thought to form a mechanism of defence by *Brassicaceae* plants that is activated upon tissue damage, including infestation by insects, microbial infection or by other mechanical damage (Mithen, 2001). In homeostasis, the enzyme myrosinase is physically separated from glucosinolates. The latter was found to be compartmentalized in vacuoles (Grob and Matile, 1979), while myrosinase was located in the cytoplasm of the plant cell. Upon tissue damage, glucosinolates are released from vacuoles and interact with myrosinase in

the presence of moisture, which cleaves the thio-glucose bond of the secondary metabolites. The degradation of a glucosinolate molecule results in the formation of glucose plus an intermediate thiohydroximate-*O*-sulphonate component (Choubdar et al., 2010). This intermediate compound is extremely unstable and rearranges (via Lossen rearrangement), releasing sulphate plus one or more of the following: isothiocyanates, thiocyanates, nitriles, epithonitriles, oxazolidine-thiones, and elementary sulphur. The formation of each one of these products will depend on the structural characteristics of the glucosinolate cleaved, pH and the presence of other substances in the environment (Fig. 2.1) (Vig et al., 2009). Lüthy and Matile (1984) called this myrosinase-glucosinolate system the "mustard oil bomb", where myrosinase is located in the cytoplasm and waits for the disruption of vacuoles containing glucosinolate to form pungent metabolites with fungicidal, bactericidal, herbicidal and insecticidal properties (Bodnaryk, 1991; Mithen, 2001; Sarwar et al., 2001).

About 100 different glucosinolates have been identified, which have a thioglucoside component as a common structure and differ at their side chains which could be aromatic, heteroaromatic or aliphatic (Hansen et al., 1995). In addition, glucosinolates have been found to be derived from amino acids through chain elongation, oxidation, desaturation and hydroxylation processes. Their compartmentalization in vacuoles may be facilitated by the glucose and sulphate residues characteristic of their structure, which may help in the translocation of these molecules through the vacuole membrane using an active transport system (Mithen, 2001).

Myrosinase is not an enzyme exclusive to the plant kingdom since myrosinase-like activity has also been detected in fungi and bacteria (Bones and Rossiter, 1996).

Researchers have shown that myrosinase is found as a family of different isoenzymes that may vary between species and also among different organs of the same plant (Lenman et al., 1993; MacGibbon and Allison, 1970). Mustard species have intense myrosinase activity, but glucosinolate content differs among varieties. *Sinapis alba* (white or yellow mustard) seeds contain almost exclusively ρ-hydroxybenzyl glucosinolate (sinalbin), whereas *Brassica nigra* (black mustard) and *Brassica juncea* (brown and oriental mustard) seeds have allyl glucosinolate (sinigrin) as the principal glucosinolate (Cui and Eskin, 1998). Extracts of yellow mustard were found to contain at least 14 different myrosinase isoenzymes, and their activity was found to be 10-fold higher than the myrosinase activity in *Brassica campestris* and *Brassica napus* (Bones, 1990).

Mustard seeds and mustard powder have been used as common spices in fermented sausages. The most commercially used botanical species of this crop, *Sinapis alba and Brassica juncea*, contain high levels of glucosinolates. Yellow mustard has the ability to form ρ-hydroxybenzyl isothiocyanate up to 2.5% of its weight, while oriental mustard can contain 0.8% allyl isothiocyanate (Fig. 2.2) (Cui and Eskin, 1998). At these levels, the mustard added to food products has the potential to form quantities of isothiocyanates sufficient to be bactericidal to pathogenic bacteria (Chacon et al., 2006b, Ekanayake et al., 2006; Graumann and Holley, 2008; Nadarajah et al., 2005). These characteristics make mustard an excellent ingredient for sausage production, having the ability to improve flavour, texture and the safety of these products.

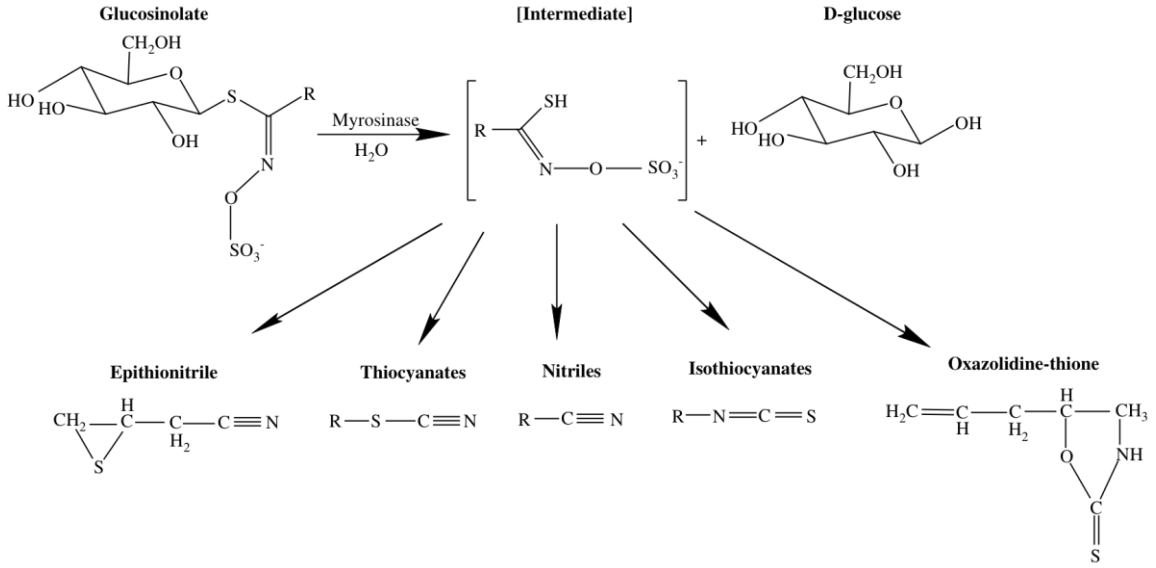


Fig. 2.1 – Reaction of glucosinolates with the enzyme myrosinase and the glucosinolate hydrolysis products (Vig et al., 2009).

2.5.6 Isothiocyanates: mechanism of action

Very little is known about the specific mechanism by which isothiocyanates kill bacteria. Different from the phenolic essential oils (e.g. carvacrol, eugenol, thymol), isothiocyanates are believed to act against bacteria through the highly reactive carbon atom of the isothiocyanate group. Zsolnai (1966) first reported that thiol-containing molecules like thioglycolate and cysteine could interact with the isothiocyanate group and reduce its antibacterial activity, which could prevent bactericidal action of the essential oil. The author hypothesized that the antimicrobial action of isothiocyanates may be linked to the inhibition of bacterial sulfhydryl-enzymes. However, there are very few reports demonstrating the interaction of isothiocyanates with enzymatic systems. Kojima and Ogawa (1971) observed that allyl, methyl, phenyl and β-phenylethyl isothiocyanates were able to reduce oxygen uptake in yeast, and cytochrome c oxidase was inhibited by allyl isothiocyanate. Allyl isothiocyanate was also found to react with the amino acids arginine and lysine, and to cause the oxidative cleavage of disulfide bonds in cystine residues (Kawakishi and Kaneko 1985, 1987).

Other studies have shown that AIT is able to damage the cytoplasmic membrane of *E. coli* K-12, allowing the leakage of cellular metabolites (Lin et al., 2000). On the other hand, no leakage of ATP was observed when *Listeria monocytogenes* was challenged by AIT. However, the cytoplasmic levels of ATP were diminished, which could have been the result of inhibition of enzymes related to ATP formation or depletion of proton motive force (Ahn et al., 2001). In addition, AIT was found to modify the internal structure of *L. monocytogenes* when compared to non-treated cells when analyzed by transmission electron microscopy (Ahn et al., 2001). Studies have shown that AIT,

phenethyl isothiocyanate (Kassie and Knasmüller, 2000) and methyl isothiocyanate (Kassie et al., 2001) were able to cause DNA damage in *Salmonella*, *E. coli* and human cells (Hep G2), but its genotoxic activity was attenuated by the presence of proteic substances. Therefore, it seems that the reactive isothiocyanate group is essential for the antimicrobial activity of these essential oils and differences in potency are related to the nature of their side chains.

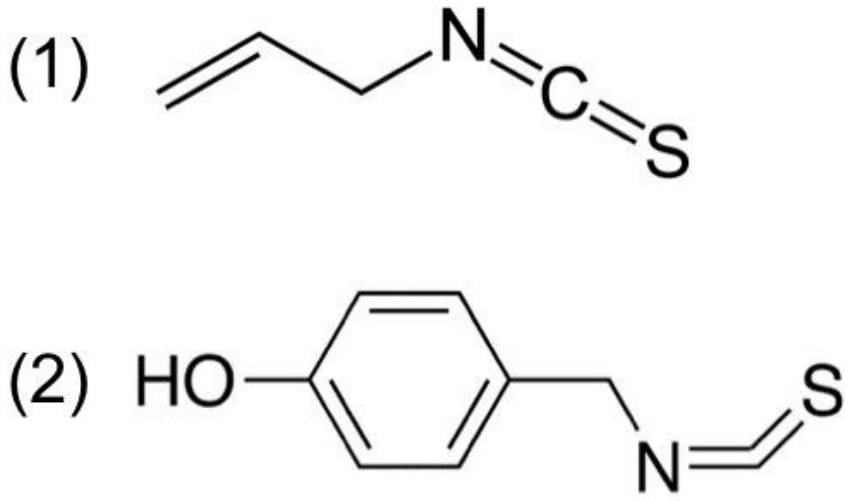


Fig. 2.2 – Molecular structure of the principal isothiocyanates formed in *Brassica juncea* and *Sinapis alba*, which are (1) allyl isothiocyanate and (2) ρ-hydroxybenzyl isothiocyanate, respectively.

Chapter 3

Effect of Free-SH Containing Compounds on Allyl Isothiocyanate Antimicrobial Activity Against *Escherichia coli* O157:H7

3.1 Abstract

Escherichia coli O157:H7 contamination is a significant meat safety issue in many countries. Allyl isothiocyanate (AIT) is a natural compound found to limit the survival of *E. coli* O157:H7 and other pathogens in meat and meat products. In the present study, it was found that glutathione and cysteine naturally present in meat can interfere with AIT antimicrobial activity. Spectroscopy, HPLC, and LC-MS were used to confirm that glutathione was able to react with AIT and formed a conjugate with no or low bactericidal activity against the tested organisms. The same reaction also occurred at pH values of 4.9 and 5.8 at 25 and 4 ◦C, respectively, which broadly represent storage conditions in raw beef (pH 5.8) and during fermented sausage (pH 4.9) manufacture. Reactions observed help to explain the reduction in antimicrobial potency of AIT in food (meat) systems.

3.2 Introduction

Glucosinolates are a group substances found within cell compartments of plants that belong to the family *Cruciferaceae* (for example, broccoli, mustard, horseradish, wasabi) (Nielsen and Rios, 2000). When these intracellular compartments are disrupted after damage to some extent, glucosinolates are hydrolyzed by the membrane-bound

enzyme myrosinase, resulting in the formation of 3 main groups of substances: nitriles, thiocyanates, and isothiocyanates (Delaquis and Mazza, 1995). The last group contains diverse compounds with well-established antimicrobial activity, such as benzyl isothiocyanate, phenethyl isothiocyanate, and, more important for this study, allyl isothiocyanate (AIT) (Delaquis and Sholberg, 1997; Hashem and Saleh, 1999; Shin et al., 2004). These substances have a characteristic strong flavor and high volatility (Kawakishi and Kaneko, 1987).

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 (Lin et al., 2000). Additionally, the utilization of AIT in food systems has been approved in Japan provided it has a natural plant source (Sekiyama et al., 1994). On the other hand, Canada and the United States have not yet permitted the use of isothiocyanates as food antimicrobials, but they can be found unintentionally in foods with added condiments such as mustard, wasabi, and horseradish (Delaquis and Sholberg, 1997). It has been estimated that 76 million people fall ill from foodborne pathogens in the United States each year. Of these, 325,000 are hospitalized and more than 5,000 die. This is estimated to cost US$ 5 to 6 billion in medical expenses and lost productivity (Deisingh and Thompson, 2004). *E. coli* O157:H7 causes about 20,000 illnesses and 250 deaths yearly in the United States, and it is also an important foodborne pathogen in Canada and some European countries (Peacock et al., 2001). To date, human infections attributed to *E. coli* O157:H7 have been reported in over 30 countries, with most outbreaks associated with the consumption of undercooked meats, especially ground beef or hamburger patties (Nadarajah et al., 2005).

Allyl isothiocyanate has been demonstrated to have high bactericidal activity in

vapor and liquid forms against various food pathogens of concern, including *Listeria monocytogenes, Staphylococcus aureus, Salmonella* Typhimurium, and *E. coli* O157:H7 (Isshiki et al., 1992; Lin et al., 1999; Rhee et al., 2003). Lin and others (2000) tested liquid AIT against 3 different bacterial species and found that 500 μg/mL (approximately 0.5 μL/mL) was needed to achieve a 3 to 4 log reduction of *E. coli* O157:H7 (*in vitro*) at different stages of bacterial growth. This level had a similar effect when applied against *Salmonella* Montevideo; however, 2,500 μg/mL of AIT were required for *L. monocytogenes*. Delaquis and Sholberg (1997) evaluated the activity of AIT in the vapour form and showed that 1,000 μg/L of air were needed to yield a ≥ 5.65 log reduction of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* growth on tryptic soy agar disks at 35 ◦C.

Isothiocyanates are well known as powerfully reactive species that go through nucleophilic addition reactions (Verma, 2003). The isothiocyanate group has a highly electrophilic central carbon atom (——N==C==S) which can react readily and under mild conditions with hydroxyls, amines, and thiols, generating products such as carbamates, thiourea, and thiocarbamates, respectively (Zhang and Talalay, 1994). In this context, AIT reacted with glutathione, amino acids, proteins, water, alcohol, and sulfites (Kawakishi and Namiki, 1969; Cejpek et al., 2000) and was able to disintegrate the cystine disulfide bond through an oxidative process (Kawakishi and Namiki, 1982).

The antimicrobial effects of AIT have been largely reported in raw meat and meat products (Ward et al., 1998; Muthukumarasamy et al., 2003; Nadarajah et al., 2005; Chacon et al., 2006a, 2006b). Compounds containing free thiol, amine, and hydroxyl groups are commonly present in meat (Faustman and Cassens, 1991) and theoretically

can react with AIT. Ward and others (1998) and Nadarajah and others (2005) showed that the percentage of residual AIT during storage was significantly reduced when applied to meat in comparison with agar models. This may have been due to reactions of AIT and components in meat. Moreover, these results suggest that higher doses of AIT might be needed for meat products to overcome interference and achieve the same bactericidal effects found in agar and broth models. Levels of ≤ 500 μg of AIT/kg were found not objectionable by a sensory panel (Chacon et al., 2006b). The goal of the present study was to determine if there was interaction of AIT with some of these substances and whether the reaction would be beneficial, detrimental, or neutral in terms of AIT antibacterial activity against *E. coli* O157:H7.

3.3 Material and Methods

3.3.1 Chemicals

Allyl isothiocyanate was purchased from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.), cysteine from Fluka (Buchs, Switzerland); 5,5′ -dithiobis (2-nitrobenzoic acid) (DTNB), glutathione, glycine, and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The solvents: methanol, acetonitrile, and acetic acid were HPLC grade (Fisher Scientific Co., Fair Lawn, N.J., U.S.A.).

3.3.2 Bacterial strains

A 5-strain cocktail of *E. coli* O157:H7 was used during these experiments. The strain LCDC 7283 (pathogenic, hamburger isolate) was provided by Dr. R. Khakria, Laboratory Centre for Disease Control, Ottawa, Canada. Strains 02-0628, 02-0627, 00-

0351, and 02-0304 (nonpathogenic, human isolates) were supplied by Rafiq Ahmed, Natl. Microbiology Laboratory, Public Health Agency, Canadian Centre for Human and Animal Health, Winnipeg, MB, Canada. The organisms were subcultured overnight twice at 37 ◦C in Luria broth (Difco Laboratories, Sparks, Md., U.S.A.) prior to the experiments. Cells were cultured again and 0.1-mL samples at mid-exponential phase (optical density approximately 0.6, at 600 nm) were used (approximately 7 log CFU/mL of each culture). Cultures were handled in a sterilized laminar flow hood and all the biological materials were autoclaved for 15 min at 121 ◦C before disposal.

3.3.3 Amine, thiol, hydroxyl, and AIT interaction

Fresh Luria-Bertani (LB) broth, a highly nutritive medium for growth of *E. coli*, was prepared (pH 7.4) containing one of the following reactants: 5 mM glutathione (GSH), 5 mM cysteine, 5 mM glycine, or 5 mM ascorbic acid. The mixtures were filter-sterilized through a 0.22-μm membrane (Fisher Scientific). Subsequently, 9.9-mL aliquots were added to screw-capped tubes, followed by 0.1 mL *E. coli* O157:H7 cocktail inoculum (OD approximately 0.6) and 2.5 mM AIT. Standards containing reactant + inoculum and AIT + inoculum were also prepared. Cell density was measured after 24 h (600 nm, Novaspec Plus spectrophotometer, Biochrom, Cambridge, U.K.) at 37 ◦C. Samples were also inoculated on tryptic soy agar (Difco) for bacterial enumeration after incubation of agar plates at 37 ◦ C for 24 h. An excess of each of the 4 reactants was used to verify whether there was a reduction in AIT antimicrobial activity under the previously described test conditions.

3.3.4 GSH and cysteine meat simulation

To make the approach industrially relevant, concentrations of GSH (1.75 mg/kg) and cysteine (4 mg/kg) found in fresh meat (Valencia et al., 2001) were used in tests following the procedures outlined in the previous section, using different concentrations of AIT (50 to 500 μL/L). This concentration range was chosen because the highest was able to inhibit *E. coli* O157:H7 growth in fermented salami (Chacon et al., 2006b), while the lowest was found to be the minimum AIT inhibitory concentration (MIC) using the conditions described in the previous section in the absence of GSH and cysteine (results not shown).

3.3.5 Measurement of free thiol content in AIT-GSH solutions

Equimolar concentrations (0.1 mM) of AIT and GSH were dissolved in 10 mM phosphate buffer, pH 7, and kept at 37 ◦C with 200 rpm agitation for 30 min, 1, 2, 4, 6, 12, and 24 h. Standards containing either 0.1 mM GSH or 0.1 mM AIT were prepared and kept under the same conditions to determine the behaviour of these compounds alone. The thiol content was determined using the method of Ellman (1959). Briefly, 1 mL of the reaction sample or standard solutions was transferred to a photometer cuvette and 33 μL of a 10 mM DTNB solution were added. Color developed within 2 min, and the absorbance was measured at 412 nm with the Novaspec spectrophotometer.

3.3.6 Product identification and AIT-GSH reaction at pH and temperatures that reflect beef and fermented sausage environments

The products and the behavior of the equimolar (0.1 mM) AIT-GSH reaction

mixtures were examined using high-performance liquid chromatography (HPLC), and the products of this reaction were identified using liquid chromatography coupled with mass spectroscopy (LC-MS) as described in the following section. The reaction was followed for 24 h at 37 ◦C and 200 rpm in a 10 mM phosphate buffered (pH 7) aqueous solution. To reflect conditions in a fresh beef environment, the pH of the buffered solution was decreased to 5.8 with HCl and the reaction was followed at 4 to 6 ◦C for 7 d. A pH of 4.9 was used to better understand changes in the fermented sausage environment and the solution was maintained at 25◦C for 4d.

3.3.7 HPLC and LC-MS apparatus and operating conditions

Analyses were conducted on an HPLC (Waters 2695, Waters Corp., Milford, Mass., U.S.A.) system equipped with a photodiode array detector (Waters 996), Empower software, and autosampler (Waters 717 plus). The separation of the compounds was performed at room temperature using a Symmetry C18 column (Waters Co., 4.6 × 250 mm i.d. 5 μm). Elution was carried out isocratically for 40 min at a flow rate of 0.5 mL/min, using a solvent system containing 40% (v/v) acetonitrile (0.1%, v/v acetic acid) and 60% water (0.1% acetic acid). The injection volume used was 10 μL. A dual absorbance detector was used to simultaneously measure the absorbance at 220 and 254 nm to verify the presence of AIT, GSH, and reaction products.

LC-MS was used to identify the by-products from the reaction between AIT and GSH. LC separation was performed on an ACQUITYTM UPLC system consisting of a binary pump, a sample manager, and a PDA detector set at 254 nm (Waters Corp.). An ACQUITYTM UPLC BEH C18 column 1.0 × 100 mm, i.d. 1.7 μm was used for

detection of the reaction products with a flow rate of 0.2 mL/min. Five-microliter samples were injected into the LC and were run isocratically in a solvent system composed of 60% water (solvent A) and 40% (v/v) acetonitrile (solvent B), both containing 0.1% (v/v) formic acid. The eluting stream from the LC was introduced into a Waters Quatro MicroTM API mass spectrometer (Waters Corp.) equipped with an ESI Multi-Mode Ionization probe. All spectra were obtained in both positive and negative mode ESI and the scan was set at 100 to 600 m/z. Mass spectroscopy parameters were as follows: capillary voltage: 3 kV; cone voltage: 30 V; extractor voltage: 3.3 V; source temperature: 100 ◦C; desolvation temperature: 210 ◦C; cone gas (nitrogen) flow: 50 L/h; desolvation gas (nitrogen) flow: 600 L/h.

3.3.8 Statistical analyses

The data reported are the average values from a minimum of 3 experiments and are represented by means ± SEM. Differences among treatments were analyzed by Tukey's test. A P value of 0.05 was used as the cutoff for statistical significance.

3.4 Results and Discussion

3.4.1 Effect of reduced glutathione, cysteine, ascorbic acid, and glycine on allyl isothiocyanate antimicrobial activity

Allyl isothiocyanate is a very reactive nucleophile with potent antimicrobial activity, and was found to react with compounds commonly present in foods which contain electrophile groups such as thiols, amines, and hydroxyls (Kawakishi and

Kaneko, 1987; Cejpek et al., 2000). However, whether or not these substances react with AIT to interfere with its antimicrobial activity was unclear.

In this study, it was found that both reduced glutathione and cysteine (5 mM) interacted with AIT (2.5 mM) in such a way that AIT lost its antimicrobial properties, but this did not happen with glycine or ascorbic acid at 5 mM. As shown in Fig. 3.1, there was no significant difference ($P > 0.05$) in *E. coli* O157:H7 growth between the control group and AIT-treated tubes when the broth also contained either GSH or cysteine. However, the same result was not seen when glycine or ascorbic acid were added to the medium, where AIT prevented bacterial growth as shown by no increase in OD measurements. Additionally, when samples of test broths (50 μL) were plated on tryptic soy agar the absence of bacterial growth was confirmed in the AIT-treated tubes containing glycine and ascorbic acid (results not shown). The later reactants contain free amine and hydroxyl groups, respectively, whereas cysteine and GSH are reactive thio-substances. These findings suggest that the thiol–AIT interaction resulted in the reduced antimicrobial activity of AIT.

Some studies have shown that isothiocyanates are able to react with a diverse range of compounds such as amino acids and peptides (Kawakishi and Kaneko, 1985; Jiao et al., 1996; Cejpek et al., 2000), aromatic and aliphatic amines, acids (Verma, 2003), and proteins (Kawakishi and Kaneko, 1987). However, in these studies, reactions were conducted under different conditions compared with those in the present study, which included use of high temperature (Kawakishi and Kaneko, 1987; Verma, 2003), acidification or alkalinization (Cejpek et al., 2000; Verma, 2003), addition of alcohol (Verma, 2003), or long incubation periods (Cejpek et al., 2000). Additionally, in contrast

with the literature, it seemed that either AIT did not react with ascorbic acid or glycine under the conditions used during the present experiments or the products formed after the reaction retained their antimicrobial activity.

3.4.2 AIT antimicrobial activity in the presence of glutathione and cysteine concentrations found in raw meat

Since glutathione and cysteine seemed to interfere with the antimicrobial activity of AIT against *E. coli* O157:H7 (5-strain cocktail), the next issue addressed in this study was to determine whether concentrations of these substances normally found in food products (more specifically meat products) could also reduce AIT antimicrobial activity.

As shown in Fig. 3.2, the amount of GSH and cysteine found in meat neutralized the inhibitory effect of AIT at levels up to 100 μL/L, but not when AIT was present at ≥ 250 μL/L. These results can explain, at least in part, differences of AIT concentrations needed to reduce *E. coli* O157:H7 numbers in meat and agar model systems, where levels of AIT necessary to inhibit bacterial growth in meat models were much higher (Ward et al., 1998). Meat is a good source not only of GSH and cysteine, but also contains other thio-compounds (for example, proteins, nonproteic sulfhydryl substances) (Faustman and Cassens, 1991). Therefore, it is likely that the levels of AIT needed to ensure its effective antimicrobial activity must overcome the influence of the background concentration of thio-compounds present in the food product.

3.4.3 Confirmation of AIT-GSH reaction and conjugate formation

The method of Ellman (1959) was used to evaluate the evidence for nucleophilic

attack by the thio-reactants against the AIT molecule. This colorimetric method quantifies the thiol content of a solution, where higher thiol concentrations yield more intense yellow color development. The rationale of this experiment was that if decreases in colour intensity occurred after AIT and GSH were mixed together, it would mean that the thiol group of glutathione reacted with the essential oil (AIT) and therefore was lost.

The reaction between AIT and GSH was followed for 24 h (Fig. 3.3), and a significant difference ($P < 0.05$) was observed within in the 1st hour in comparison with the control solution that contained only GSH and buffer. The difference remained significant for the whole test period, but notably the presence of free-thiol groups decreased very rapidly after 6 h and this decrease continued for 24 h.

These results confirmed the expectation that glutathione reacted with AIT to cause the loss of the glutathione thiol group during the reaction. Glutathione has also been implicated in causing a decrease in nisin antimicrobial activity through an enzymatic reaction (Rose et al., 1999). However, with AIT, its reduced activity was found to be caused by a nonenzymatic reaction, because the reaction occurred in an aqueous solution using pure substrates.

The results from HPLC analyses (Fig. 3.4) showed the formation of 1 main product derived from the AIT-GSH reaction immediately after mixing which had a retention time of approximately 5.5 min (Fig. 3.4A). The results of this analysis at 12 to 24 h coincided with the reaction behavior found previously by the colorimetric method used (Ellman, 1959), with greater accumulation of this new product being measured at 24 h (Fig. 3.4B). The rate of reaction product formation from AIT + GSH is represented by changes in the area under the curve (AUC) in Fig. 3.5. LC-MS analysis (Fig. 3.6) identified the

molecular weight of this product as 406.15, which closely coincides with the molecular weight that would be predicted for an AIT-GSH conjugate. The formation of this conjugate is supported by studies of other researchers (Kawakishi and Kaneko, 1985; Bruggeman et al., 1986; Jiao et al., 1996), but not its lack of antimicrobial activity against *E. coli* O157:H7. Additionally, the molecular structure of the proposed conjugate observed does not include the thiol group, supporting the interpretation of the previous results (Figure 3.3).

Reaction with microbial proteins and cleavage of disulfide bonds have been hypothesized as the mechanisms of antimicrobial action of AIT (Delaquis and Mazza, 1995). Allyl isothiocyanate was demonstrated to uncouple the oxidative phosphorylation of yeasts through the inhibition of carriers in the electron transport chain (Kojima and Ogawa, 1971). In addition, Lin and others (2000) showed that AIT increases the leakage of cellular metabolites from *E. coli* O157:H7 and *Salmonella* Montevideo, indicating cell membrane damage. All these findings seem to be related to the high reactivity of AIT. Therefore, intrinsic substances found in meat such as GSH, cysteine, and proteins, could act as protective shields for contaminating microorganisms.

3.4.4 Reaction at pH values simulating beef and fermented sausage conditions

AIT was found to react with glutathione when raw beef and sausage pH and temperature environments were simulated (Fig. 3.7 and 3.8, respectively). The concentration of AIT decreased significantly ($P < 0.05$) in both systems when compared with the standard solution, presumably due to the conjugate formation. Consequently, the availability of the essential oil was reduced in such environments, which may have

caused loss of its antimicrobial activity. These findings reinforce the assumptions that AIT inhibitory activity against *E. coli* O157:H7 might be affected by intrinsic compounds present in raw meat and meat products.

Utilization of AIT to enhance meat safety has been widely studied (Ward et al., 1998; Hasegawa et al., 1999; Muthukumarasamy et al., 2003; Nadarajah et al., 2005; Chacon et al., 2006a, 2006b). Consequently, the knowledge of factors influencing its mechanism of action and the presence of agents capable of causing interference with its action is fundamental to improve AIT utilization. Furthermore, AIT is a pungent essential oil, a characteristic that can be objectionable to some palates, and it was found to be unacceptable at levels of 750 ppm or higher when added to fermented dry sausages (Chacon et al., 2006b). However, these sausages are usually consumed raw, and when AIT is used in fresh ground meat, cooking would reduce its flavor and taste impact because of its volatility. Nevertheless, *E. coli* O157:H7 outbreaks are commonly associated with the consumption of under cooked foods (Nadarajah et al., 2005). Therefore, it would be ideal to generate effective antimicrobial activity at low concentrations of AIT, which would cause only mild effects on food taste.

Few studies have been conducted to evaluate the results of reducing the amount of AIT used to inhibit growth of pathogenic bacteria. Nadarajah and others (2005) reported the use of AIT dispersed on paper filter to inhibit *E. coli* O157:H7 growth in modified atmosphere-packaged ground beef patties. The conditions used in that study achieved better results with lower levels than used in a previous study on cooked roast beef slices (Ward et al., 1998). Although AIT was used on different meat products in both studies, interference from meat constituents was reduced by using volatilized AIT. In the study by

Nadarajah and others (2005), it was shown that after being volatilized, AIT bactericidal activity against *E. coli* O157:H7 was reduced at lower storage temperatures and higher initial numbers of the pathogen. In other studies (Chacon et al., 2006a, 2006b; Li et al., 2007), microencapsulated AIT was used to optimize the effectiveness of AIT. Microencapsulation is a well-known procedure used to gradually release medicines (Lu et al., 2001), and is used to stabilize or to trap flavor and odor (Li et al., 2007). Moreover, controlled time-releasing systems could theoretically contribute in reducing the amount of AIT necessary to achieve bactericidal effects since interfering glutathione, cysteine, and other sulphydryl compounds are lost during meat aging (Khan and Lentz, 1977; Faustman and Cassens, 1991). Microencapsulation might also reduce the negative sensory properties exhibited by AIT as well as sustain its antimicrobial activity. Some studies have been conducted to examine these possibilities (Chacon et al., 2006a, 2006b; Li et al., 2007). When microencapsulated with gum acacia and added to fermented salami (Chacon et al., 2006b), AIT showed bactericidal activity against *E. coli* O157:H7 at levels as low as 500 μL/L. It was still perceived to be a strong flavor at that concentration, but the salami was considered acceptable by a sensory panel. The authors also noted that 90% of the panelists ate spicy food at least 4 times a year, and showed a preference for that kind of product. It was suggested that the treated sausage could be marketed "as a specialty product, such as a hot salami." The same gum acacia-microencapsulated AIT was found to significantly inhibit *E. coli* O157:H7 growth in chopped beef at levels ≥ 1481 μL/L (Chacon et al., 2006a). It is probable that the level necessary was higher than used with salami because of composition differences between the products, such as moisture content, pH, presence of lactic acid bacteria, and fat content. Moreover, as

hydrophilic compounds, gum acacia microcapsules can be damaged and partially dissolved in high moisture environments. Li and others (2007) used α- and β-cyclodextrin in the form of microcapsules to control AIT release characteristics, but the system was not used to test AIT bactericidal activity in food systems.

Although studies have evaluated delayed release of AIT in meat products, the issues related with sensory acceptability are not yet resolved. Different materials for microencapsulation and perhaps other time-delayed releasing techniques might be a good choice for AIT application in meat products. This approach could reduce undesirable early reactions of AIT with naturally occurring sulphydryl compounds.

3.5 Conclusion

Combination of glutathione and cysteine at levels found in fresh beef can neutralize the antimicrobial activity of AIT at levels that were otherwise bactericidal. This effect was found during tests in nonmeat broths to be due to formation of an AIT-GSH conjugate, which can also occur at normal beef and fermented sausage storage conditions and pH. The reduction of this reaction should be a first step to optimize AIT effectiveness in meat and meat products. Moreover, better understanding of the mechanism by which AIT kills *E. coli* O157:H7 can also contribute to its enhanced utilization in food systems.

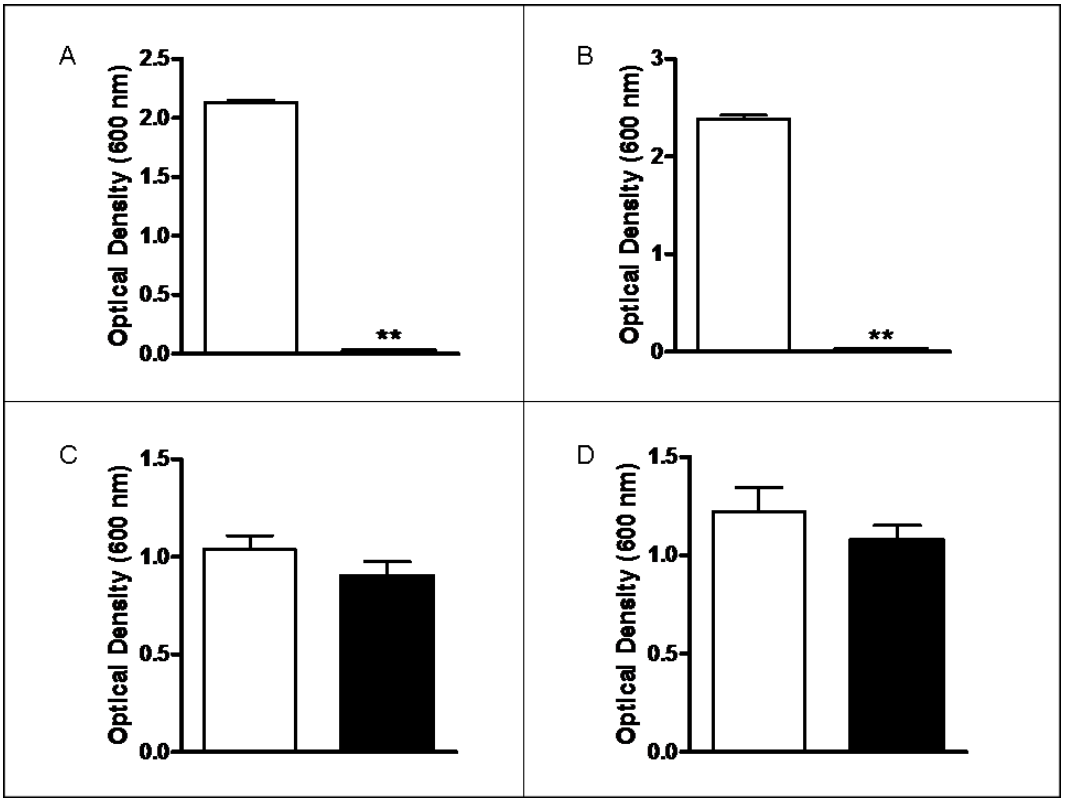


Fig. 3.1 - Effect of the presence of 4 separate reactants at 5 mM on the antimicrobial effectiveness of 2.5 mM AIT against a 5-strain *E. coli* O157:H7 cocktail measured by changes in OD_{600} after 24h at 24^{o}C. A) Glycine. B) Ascorbic acid. C) Glutathione. D) Cysteine. Reactant control □; AIT + reactant ■. ** = $P < 0.01$ was considered a highly significant difference.

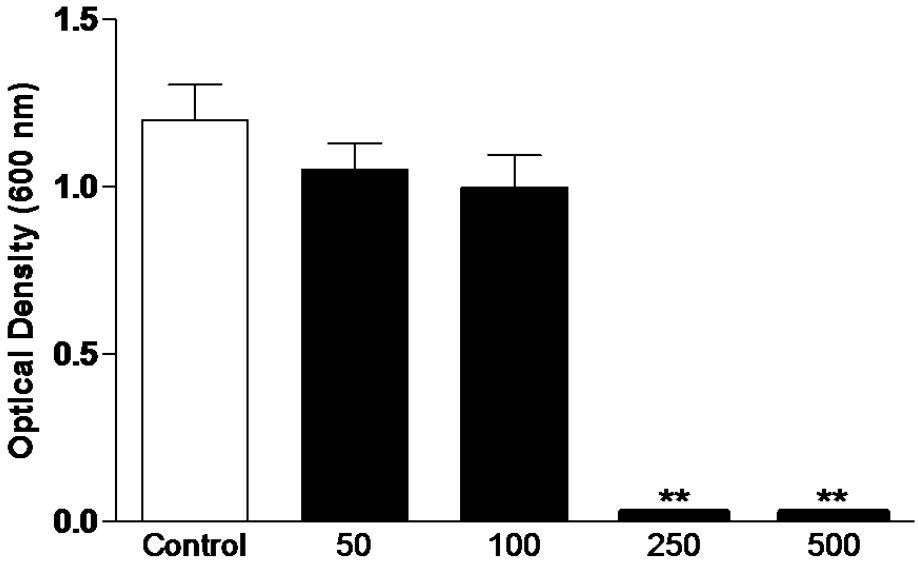


Fig. 3.2 - Antimicrobial activity of AIT (μL/L) against *E. coli* O157:H7 after ≤ 24h at 37^{o}C in Luria broth with GSH (1.75 mg/kg) and cysteine (4 mg/kg) at concentrations found in beef. ** = $P < 0.01$ was considered a significant difference.

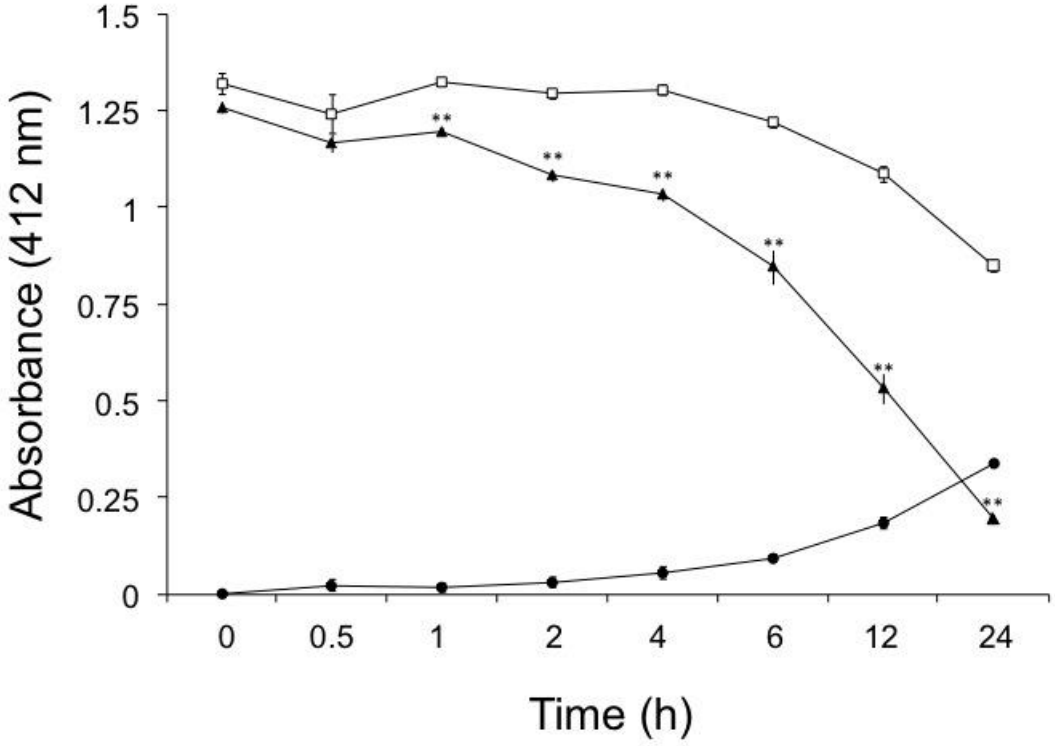


Fig. 3.3 - Changes in the absorption caused by the loss of the thiol group when AIT was added to a solution containing GSH (▲) at equimolar concentrations (0.1 mM) for 24h at 37^{o}C. Standards of AIT (●) and GSH (□) were used for comparison. ** = $P < 0.01$ was considered a significant difference.

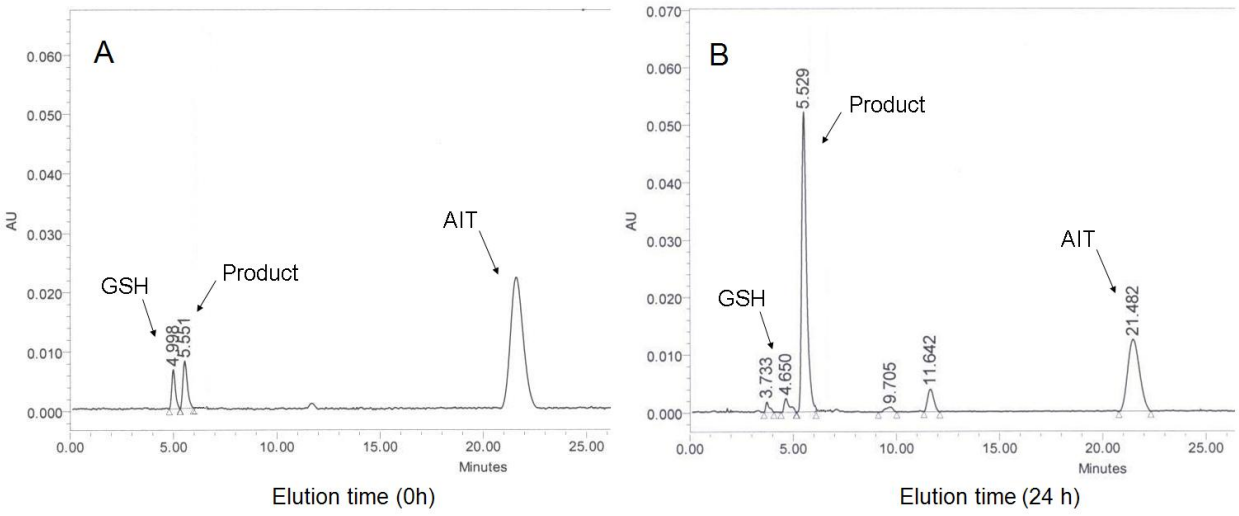


Fig. 3.4 - Formation of the reaction product from mixing AIT and GSH detected by HPLC after 0 (A) and 24 h (B) at 37 ^{o}C.

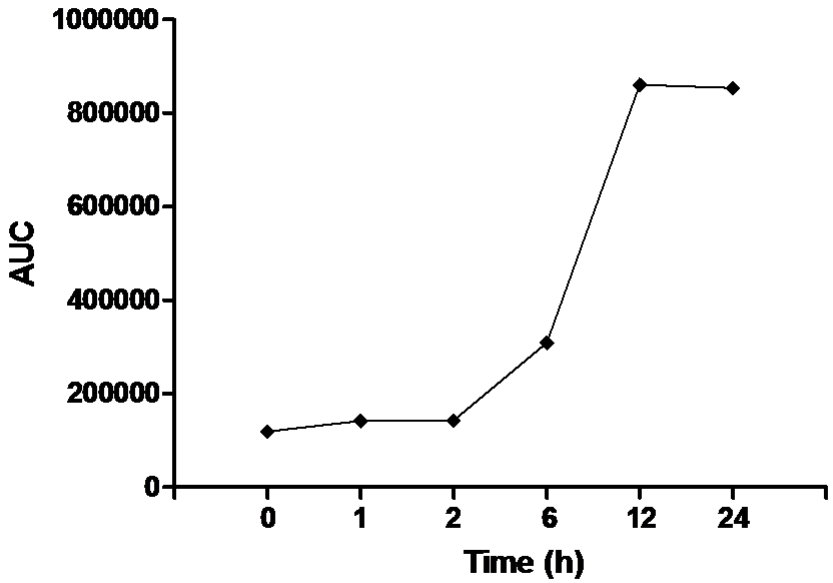


Fig. 3.5 - Development of AIT + GSH reaction product at 37°C measured by HPLC and represented by the area under the curve (AUC).

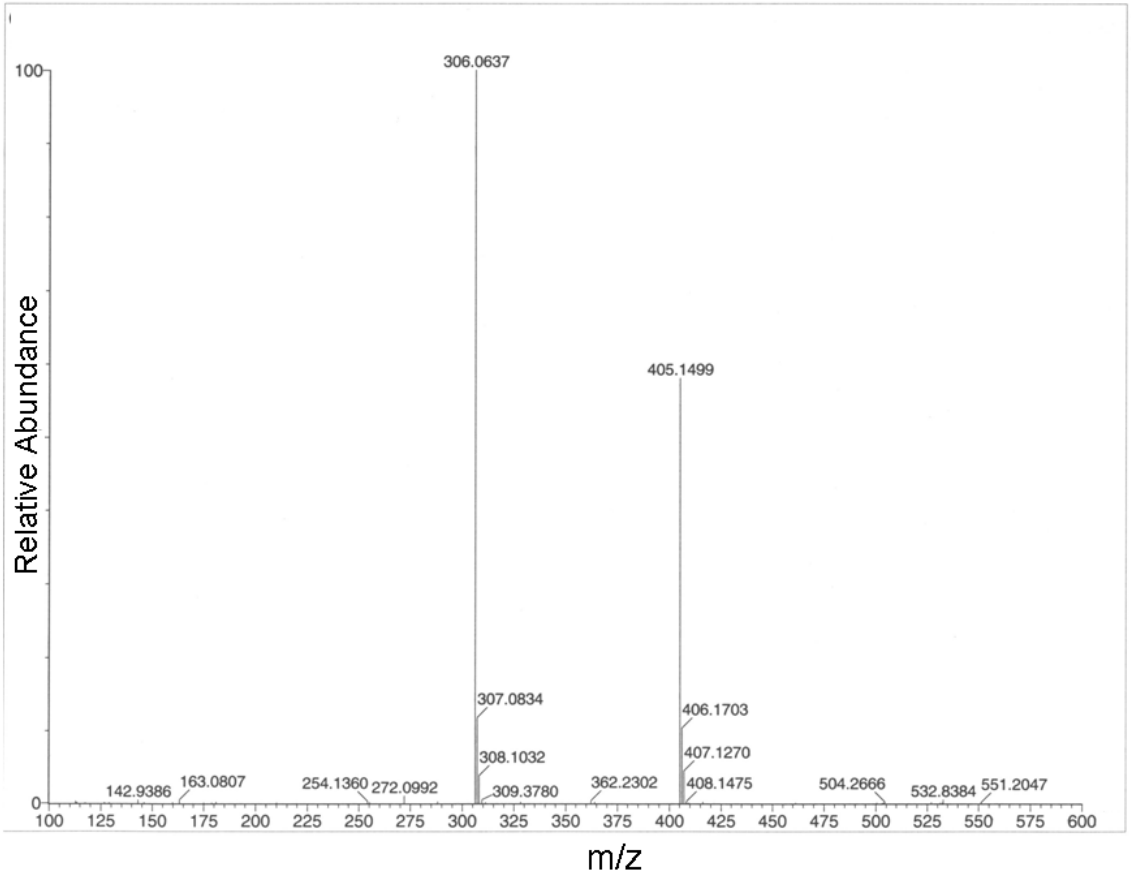


Fig. 3.6 - MS analysis indicating the presence of an AIT-GSH conjugate (m/z -1 = 405.15) after reaction of AIT and GSH for 24h at 37 ^{o}C.

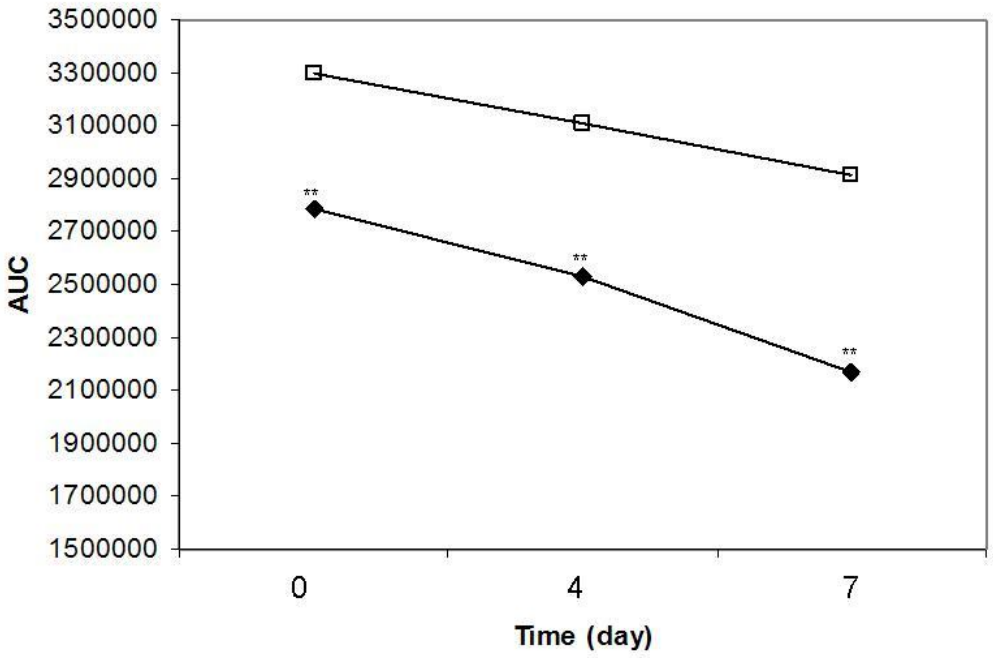


Fig. 3.7 - Results from HPLC analysis of AIT (□) (0.1 mM) and AIT+GSH (◆) (0.1 mM) behaviour at 4-6 ^{o}C and pH 5.8, simulating beef pH conditions. Values shown are levels of AIT recovery. AUC = area under the curve. ** = $P < 0.01$ was considered a highly significant difference.

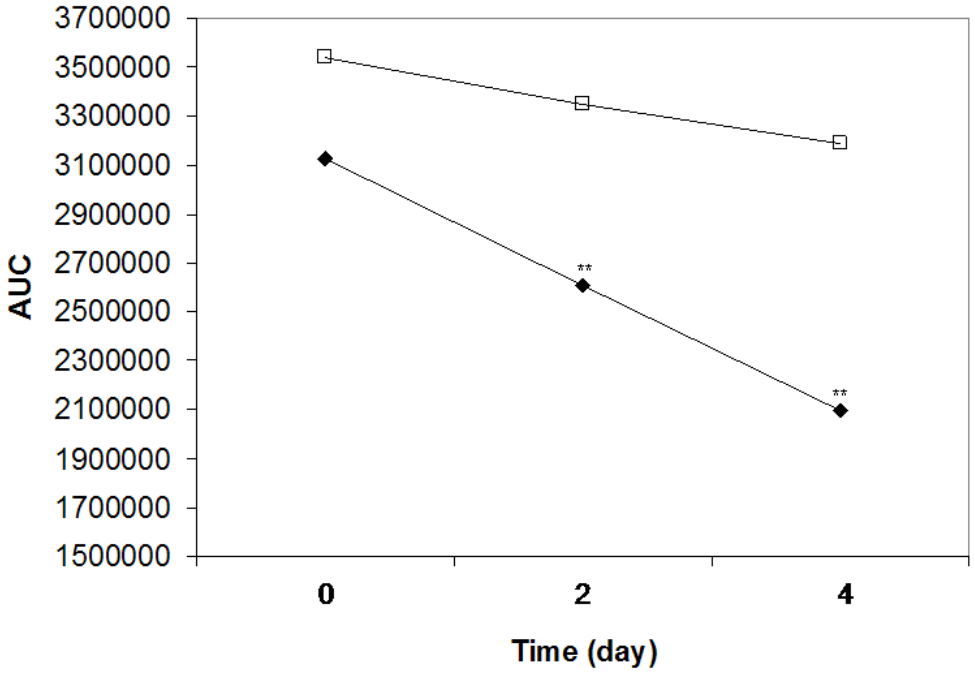


Fig. 3.8 - Sausage pH conditions simulated in a time-course study by adding AIT (□) or AIT+GSH (◆) in a 10 mM phosphate buffered solution at 25 ^{o}C and pH 4.9 for 4 d. Values are levels of AIT recovery monitored by HPLC. AUC = area under the curve. ** = $P < 0.01$ was considered a highly significant difference.

Chapter 4

Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7

4.1 Abstract

Allyl isothiocyanate (AIT) is derived from the glucosinolate siningrin found in plants of the family *Brassicaceae*. It is a well-recognized antimicrobial agent against a variety of microorganisms, including foodborne pathogens such as *E. coli* O157:H7. The efficiency of this natural agent in reducing *E. coli* O157:H7 numbers in food products has been reported. However, few reports have examined the mechanism by which AIT, and perhaps most of the isothiocyanates, kill *E. coli* O157:H7. In the present report, AIT showed greater antimicrobial activity at low pH values. For example, at pH 4.5 and 5.5 the MIC was 25 μL/L, while at pH 8.5, 500 μL/L was required to inhibit bacterial growth. This mustard-derived compound exhibited a high decomposition rate in water at 37^{o}C. Its degradation profile contained 3 major products and of these, diallylthiourea represented the largest (~ 80%) component. The decomposition products did not show antimicrobial activity towards *E. coli* O157:H7, even when combined with a sub-lethal dose of AIT (10 μL/L). AIT may only be antimicrobial in its original form and any further degradation in water is undesirable. AIT interactions with thioredoxin reductase and acetate kinase were also subjects of this study. AIT at 10 to 100 μL/L was able to significantly inhibit both enzymes, but only 1 μL/L was needed to decrease the activity of thioredoxin reductase. From these results, it can be postulated: 1) AIT is a more effective antimicrobial at low

pH values and its degradation reduces this activity; 2) decomposition products in water might not participate in the antimicrobial action of AIT; and 3) AIT seems to have a multi-targeted mechanism of action, perhaps inhibiting several metabolic pathways and damaging cellular structures.

4.2 Introduction

Isothiocyanates naturally occur in plants of the family *Brassicaceae*. The enzyme myrosinase is released after injury or disruption of cell membrane or walls and catalyzes cleavage of the glucose molecule from glucosinolates, generating isothiocyanates, thiocyanates and nitriles (Delaquis and Mazza, 1995). Allyl isothiocyanate (AIT) is derived from the glucosinolate sinigrin and is responsible for the characteristic pungency found in horseradish and mustard pastes (Cejpek et al., 2000). Several researchers have described the antimicrobial properties of AIT against various foodborne pathogens (Isshiki et al., 1992; Lin et al., 1999; Rhee et al., 2003), but the specific mechanism of its microbiocidal activity is still unknown.

Zsolnai (1966) reported that thioglycolate and cysteine could diminish the antibacterial action of isothiocyanates and related this event to the possible reaction of the isothiocyanate with the thiol groups of these compounds. He hypothesized that the antimicrobial action of isothiocyanates may be linked to the inhibition of sulfhydryl-enzymes. Subsequently, there has only been a single report where the interaction of isothiocyanates with enzymatic systems has been verified. In that work, Kojima and Ogawa (1971) observed a reduction of oxygen uptake when allyl, methyl, phenyl and β-phenylethyl isothiocyanates were tested against 3 different species of yeast. The authors

also showed significant enzymatic inhibition of cytochrome c oxidase by allyl isothiocyanate. However, the results of this work raise questions, since the levels used to achieve both enzymatic and oxygen uptake inhibition were 200 times greater than the actual minimum inhibitory concentration of the isothiocyanates for those organisms.

Reactions involving isothiocyanates also were studied by Kawakishi and Kaneko (1985, 1987), where they showed that allyl isothiocyanate was able to slowly cause disulfide bond oxidative cleavage in cystine residues and to react with the terminal amino groups of lysine and arginine. However, these authors did not relate these findings to the possible reaction of AIT with microbial proteins. In addition, the reactions were analyzed at temperatures over 37 ^{o}C with intensive stirring, which can facilitate AIT-protein interactions.

Lin et al. (2000) showed that AIT caused damage in the cell membrane of *E. coli* K-12, leading to leakage of cellular metabolites. However, Ahn and colleagues (2001) found no leakage of ATP or damage in the cell wall when AIT was tested against *Listeria monocytogenes*, but internal levels of ATP were reduced. In addition, the authors observed that the AIT-treated cells had altered internal structure in comparison to non-treated bacteria when analyzed by transmission electron microscopy.

Degradation of allyl isothiocyanate in water has been reported (Chen and Ho, 1998; Kawakishi and Namiki, 1969; Ohta et al., 1995; Tsao et al., 2000), but no study relating this degradation to AIT antimicrobial activity has been published. Kawakashi and Namiki (1969) demonstrated that AIT was degraded in aqueous solutions at 37^{o}C, forming a variety of by-products which produced a garlic-like odor. This decomposition was slower than with other isothiocyanates such as ρ-hydroxybenzyl isothiocyanate and produced

negligible amounts of SCN^-. The latter authors also showed that AIT reacted with water and the main products of this reaction were identified as allyl dithiocarbamate, diallyl tetra- and penta-sulphide, sulfur and N,N'-diallylthiourea. In addition, the degradation process was dependent on temperature and pH, where AIT was more easily decomposed at higher temperatures and alkaline pH values (Tsao et al., 2000).

The purposes of the present study were to evaluate the antibacterial activity of AIT against *E. coli* O157:H7 at different pH values; to examine the participation of eventual degradation products in this activity; and to test AIT for inhibitory action against two enzymes important in the metabolism of *E. coli*: thioredoxin reductase, implicated in ribonucleotide synthesis; and acetate kinase, related to energy metabolism.

4.3 Material and Methods

4.3.1 Chemicals

Allyl isothiocyanate was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA); diallylthiourea from Alfa Aesar (Karlsruhe, Germany); 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), acetyl phosphate, adenosine 5′-diphosphate sodium salt (ADP), adenosine 5′-triphosphate disodium salt (ATP), adenosine 5'-triphosphate (ATP) bioluminescent assay kit, bovine serum albumin (BSA), *Escherichia coli* acetate kinase (AK), *Escherichia coli* thioredoxin reductase (TR), ethylenediaminetetraacetic acid (EDTA), HEPES buffer, and reduced β-nicotinamide adenine dinucleotide 2′-phosphate tetrasodium salt (NADPH) were purchased from Sigma Chemical Co (St. Louis, MO, USA). The solvent components, methanol, acetonitrile and acetic acid, were HPLC grade

(Fisher Scientific Co., Fair Lawn, NJ, USA). Other chemicals noted were of analytical grade.

4.3.2 Bacterial strains

Experiments involving bacterial growth used a five strain mixture of *E. coli* O157:H7. The strain LCDC 7283 (pathogenic, hamburger isolate) was provided by Dr. R. Khakria, Laboratory Centre for Disease Control, Ottawa, Canada. Strains 02-0628, 02-0627, 00-0351 and 02-0304 (non-pathogenic, human isolates) were supplied by Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency, Canadian Centre for Human and Animal Health, Winnipeg, MB, Canada. Each bacterial strain was transferred into fresh Luria-Bertani (LB) broth (Difco Laboratories, Sparks, MD, USA) and allowed to grow overnight (16 h) at 37^{o}C. Then, 50 µl of each strain was added together to a single tube containing 9.75 ml LB broth and cultured for another 16 h at 37°C prior to the experiments. The organisms were cultured again and 0.1 ml samples at mid-exponential phase were used (optical density ~ 0.6, at 600 nm represented approximately 7 log CFU/ml). Cultures were handled in a sterilized laminar flow hood and all biological material was autoclaved at 121^{o}C for 20 min before disposal.

4.3.3 Determination of minimum inhibitory concentration at different pH values

Luria-Bertani broth was prepared following the manufacturer specifications and NaOH or HCl was used to reach the desired pH (4.5, 5.5, 6.5, 7.5, 8.5) before autoclaving. The pH values were verified after sterilization. Bacteria were grown overnight at each one of the experimental pH values for adaptation and were re-

inoculated in fresh broth. Aliquots of 0.1 ml were taken from the culture mixture at mid-exponential phase (optical density ~ 0.6) and added to screw-capped tubes containing 9.9 ml of LB broth at the corresponding pH value. Concentrations of allyl isothiocyanate (AIT) ranging from 10 µL/L to 500 µL/L were used to determine the minimum inhibitory concentration at specific pH values. The tubes were incubated at 37^{o}C at 200 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA) for 48 h. Absence of growth (no increase in measured OD) was considered as the MIC.

4.3.4 Antimicrobial activity of degradation products

Allyl isothiocyanate at 500 µL/L was added to deionized water and stored 48h at 37^{o}C. The AIT remaining was then extracted using hexane (adapted from Shahidi and Gabon, 1990) and the extracted aqueous phase (EAP) was used for identification analysis (HPLC and LC-MS) and tested against *E. coli* O157:H7. For the latter tests, 8.9 ml LB broth were added to screw-capped tubes, followed by 0.1 ml *E. coli* O157:H7 mixed inoculum (OD ~ 0.6). After the inoculum was added, the tubes were treated with one of the following solutions: 1 ml of the EAP, 1 ml EAP + AIT 10 µL/L, 1 ml of deionized water + AIT 10 µL/L or 1 ml of deionized water (control). Cell density was measured after 16 h (600 nm, Novaspec Plus spectrophotometer; Biochrom, Cambridge, England) at 37^{o}C. Diallylthiourea, diallylurea and allyl disulfide, the main degradation products found in the EAP, were also tested against the *E. coli* O157:H7 mixture. In this experiment, 4 mg diallylthiourea and 0.5 mg of diallylurea were dissolved in 10 µL ethanol in a microtube (1.5 ml, flat top microcentrifuge tubes; Fisher Scientific) and 990 µL of LB broth (stock solution) were added. Diallyl disulfide, the third decomposition

product, also was added to the tube (0.5 μL) and the mixture (degradation products solution) was filter sterilized (25 mm syringe filter, 0.22 μm pore size, Fisher Scientific). To test tubes containing 9.7 ml of LB; 0.1 ml of *E. coli* O157:H7 mixed inoculum (OD ~ 0.6) and 0.1 ml of the degradation products solution were added. All groups tested received 0.1 ml of a 1 ml/L AIT solution (sub-lethal level of 10 ppm) or an equal amount of water to bring the final volume to 10 ml. Control groups were prepared with bacteria treated with 10 μL/L of AIT or bacteria with no AIT exposure. All tests above were incubated at 37^{o}C for 16 h and were repeated 3 times and each experiment was done in triplicate (n = 9).

4.3.5 HPLC and LC-MS apparatus and operating conditions

Analyses were conducted on an HPLC (Waters 2695, Waters Corp., Milford, MA, USA) system equipped with a photodiode array detector (Waters 996), Empower software, and autosampler (Waters 717 plus). The separation of the compounds was done at room temperature using a Symmetry C18 column (Waters Co., 4.6 x 250 mm i.d. 5 μm). Elution was carried out isocratically for 40 min at a flow rate of 0.5mL/min, using a solvent system containing 40% (v/v) acetonitrile (0.1% v/v acetic acid) and 60% water (0.1% acetic acid). The injection volume used was 10 μl. A dual absorbance detector was used to simultaneously measure the absorbance at 220 and 254 nm in order to verify the presence of AIT and its degradation products.

LC-MS was used to identify the decomposition products of AIT in aqueous solution. LC separation was done on an ACQUITY™ UPLC system consisting of a binary pump, a sample manager, and a PDA detector set at 254 nm (Waters Corp.). An

ACQUITY™ UPLC BEH C18 column 1.0 x 100 mm, i.d. 1.7 μm was used for detection of the reaction products with a flow rate of 0.2 ml/min. Five μL samples were injected into the LC and analyses were done isocratically in a solvent system composed of 60% water (solvent A) and 40% v/v acetonitrile (solvent B), both containing 0.1% v/v formic acid. The eluting stream from the LC was introduced into a Waters Quatro Micro™ API mass spectrometer (Waters Corp.) equipped with an ESI Multi-Mode Ionization probe. All spectra were obtained in both positive and negative mode ESI and the scan was set at 50-300 *m/z*. Mass spectroscopy parameters were as follows: capillary voltage: 3 kV; cone voltage: 30 V; extractor voltage: 3.3 V; source temperature: 100^{o}C; desolvation temperature: 210^{o}C; cone gas (nitrogen) flow: 50 L/h; desolvation gas (nitrogen) flow: 600 L/h.

4.3.6 Changes in thioredoxin reductase activity

The thioredoxin reductase (TR) activity was spectrophotometrically measured using DTNB as a colorimetric marker (Holmgren and Björnstedt, 1995). Thioredoxin reductase reduces DTNB using NADPH to form 5'-thionitrobenzoic acid, which is yellow and has a maximum absorbance at 412 nm. For this assay, a chromogenic reaction mixture was prepared in a test tube containing 0.5 ml EDTA (0.02 M), 1 ml of 1 M monopotassium phosphate buffer (pH 7.0), 0.8 ml of a 25mg/ml DTNB solution (dissolved in ethanol), 0.1 ml of 20 mg/ml BSA and 0.05 ml of 40 mg/ml NADPH. The volume was completed to 10 ml with sterile deionized water. Purified thioredoxin reductase from *E. coli* in an ammonium sulfate suspension was purchased (Sigma) and aliquots of 10 μL were withdrawn and mixed with 990 μL phosphate buffer (0.1 M, pH 7.0) to prepare the

enzymatic solution. Assay cuvettes were prepared by adding 100 μL of the enzymatic solution and 100 μL of different concentrations of AIT (100, 10 and 1 μL/L in the 0.7 ml final solution) dissolved in phosphate buffer (0.1 M). The addition of 100 μl of the same buffer was used as control. Enzyme and AIT solutions were exposed together for 5 min in the cuvette before 500 μL of the chromogenic reaction mixture was added. A period of 2 min was allowed before the first measurement to facilitate reaction stabilization. The reaction was followed at 412 nm and 25^{o}C for 20 min. Non-competitive inhibition of TR action by AIT was confirmed by calculation of enzymatic kinetic parameters (Lineweaver-Burk Plot) using different concentrations of substrate and/or inhibitor (Nelson and Cox, 2004).

4.3.7 Bioluminescent acetate kinase assay

All stock solutions in this experiment were prepared using a buffer containing 50 mM HEPES and 10 mM $MgSO_4$ (pH 7.0). Acetate kinase (AK) from *E. coli* was purchased and dissolved following the manufacturer's instructions (Sigma). Concentrations of AK used were calculated to result in ~0.2 unit per assay (1 unit is the quantity of enzyme able to phosphorylate 1 μM of acetyl phosphate min^{-1} at pH 7.6 and 25 ^{o}C) as recommended by the manufacturer. The enzyme was added with different concentrations of AIT (100, 10 and 1 μL/L) in a capped microtube (1.5 ml, flat top microcentrifuge tubes; Fisher Scientific) and the same volume of HEPES/$MgSO_4$ buffer was used as control. After 5 min, ADP and acetyl phosphate were added to start the enzymatic reaction at concentrations of 1.25 μM and 2.5 mM, respectively, giving a final solution of 0.5 ml. The reaction was left for another 5 min before the ATP assay mix™

(Sigma) was added (10 µl containing luciferase, luciferin, $MgSO_4$, dithiothreitol, EDTA, BSA, and tricine buffer salts). The ATP assay mix™ had been dissolved and was used following the manufacturer recommendations to detect ATP in samples at concentrations ranging between $2x10^{-12}$ to $2x10^{-9}$ M. The light output was measured after 5 s using a Junior Luminometer (Berthold Technologies, Germany). A standard curve was prepared using different concentrations of ATP ($5x10^{-9}$ to $5x10^{-13}$ M) and the results were plotted as concentration of ATP produced. Non-competitive inhibition of acetate kinase action by AIT was confirmed by calculation of enzymatic kinetic parameters (Lineweaver-Burk Plot) using different concentrations of substrate and/or inhibitor (Nelson and Cox, 2004).

4.3.8 Statistical analyses

The data reported are the average values from a minimum of 3 experiments and are represented by means ± SEM. Differences among treatments were analyzed by Tukey's test. A *P* value of 0.05 was used as the cut-off for statistical significance.

4.4 Results and Discussion

4.4.1 Effect of pH on the antimicrobial activity of AIT

Allyl isothiocyanate showed minimum inhibitory concentrations as low as 25 µL/L at pH 4.5 (Table 4.1). This concentration was 20-fold lower than the MIC found at pH 8.5. The results in Table 4.1 indicate a gradual reduction of the antimicrobial activity when the pH was raised. Therefore, it is suggested that AIT might work better in more acid foods.

Tsao et al. (2000) found that AIT had higher stability at acid pH values, where its half-life at pH 6.0 was 34 days, but was only 26 days at pH 9.0. The degradation of AIT in aqueous solutions has been attributed to nucleophilic attack by water molecules against AIT (Kawakishi and Namika, 1969). In addition, it seems that higher availability of negatively charged hydroxide ions can directly influence the degradation of AIT, since these species can also work as electron donors (Ohta et al., 1995). Results from Table 4.1 could mean that the presence of unmodified AIT is essential for its optimal antibacterial activity against *E. coli* O157:H7, since the lower pH values (more stable AIT) achieved more effective levels of growth inhibition.

4.4.2 Formation of degradation products

After 48 h in water, AIT was degraded to form 3 major products. To show this, AIT was removed from the aqueous solution using hexane for the extraction (Shahidi and Gabon, 1990), and its successful removal was confirmed by HPLC. The aqueous phase was analyzed and results showed that the retention times (RT) for the products were approximately 9, 11 and 14 min (Fig. 4.1A). Among them, the product found at RT = 9.273 min had a significantly higher peak, representing ~ 80% (result not shown) of the area under the curve (AUC), and was identified as diallylthiourea with a molecular weight of 157 (m/z+1) (Fig. 4.1B). Chen and Ho (1998) followed the thermal degradation of AIT at 100 ^{o}C for 1 h, and suggested that the peak at m/z = 42 might represent the presence of one or more allyl groups and the portion at m/z = 57 could characterize CH_2=CH-CH=NH_2^+. Kawakishi and Namiki (1969) also found diallylthiourea as one of the major degradation products of AIT in water solutions and it was the main nonvolatile

compound from the thermal degradation of AIT (Chen and Ho, 1998). The other two peaks were identified as diallylurea (MW = 141) and diallyl disulfide (MW = 146) (results not shown), which also were found by others (Chen and Ho, 1998; Pechacek et al., 1997).

4.4.3 Participation of degradation products in antimicrobial activity

HPLC analysis showed that hexane extraction was able to totally remove AIT from the aqueous solution (result not shown), while its decomposition products were retained. Theoretically, AIT at 500 μL/L has the potential to generate 10 times the concentration of degradation products above those present at its minimum inhibitory concentration of 50 μL/L at pH 6.5. Therefore, the concentration of degradation products generated at the MIC of AIT would have been reached by adding 1 ml of the 500 μL/L solution to 9 ml of the *E. coli*-inoculated LB broth. As shown in Fig. 4.2, neither the degradation products nor their combination with a sublethal concentration of AIT were able to inhibit bacterial growth.

Reagent grade diallylthiourea (40 μg/ml), diallylurea (10 μg/ml) and diallyl disulfide (10 μg/ml) also were tested to verify their effect on *E. coli* O157:H7 growth. Levels used represented the theoretical amount of each one of the components formed from a total degradation of AIT at its MIC (50 μL/L, pH 6.5). Similar to the previous result, the purified compounds were not able to reduce bacterial growth by themselves or when combined with a sub-lethal dose of AIT (results not shown). These data confirmed the expectation that the decomposition of AIT in water reduced or totally abolished its antimicrobial activity towards *E. coli* O157:H7. Together, these results also agree with

the poor antimicrobial activity of AIT found at higher pH values. As mentioned previously, AIT is more easily degraded at basic pH, requiring significant higher levels in order to kill *E. coli* O157:H7.

4.4.4 Thioredoxin reductase inhibition

The results in Fig. 4.3 clearly show a dose dependent inhibitory activity of AIT against *E. coli* thioredoxin reductase. Even the lowest level (1 μL/L) used in these experiments, which was 50-fold lower than the MIC, resulted in significant enzymatic inhibition at 3 of the 4 evaluated time-points. The higher levels tested had a greater inhibitory effect, where 10 μL/L was able to reduce the enzymatic activity by levels ≥ 58%. In addition, the enzymatic inhibition occurred in a non-competitive fashion (Appendix 4.1). Previously, Zsolnai (1966) speculated that the antimicrobial activity of isothiocyanates could be related to intracellular inactivation of sulphydryl-enzymes. This assumption resulted from the observations where proteins and sulphydryl compounds (e.g., cattle serum, cysteine and thioglycolate) were able to suppress the antimicrobial activity of diverse isothiocyanates. Several researchers have shown that isothiocyanates can react with amino acids (Fig. 3.1) and proteins (Cejpek et al., 2000; Kawakishi and Kaneko, 1985, 1987; Luciano et al., 2008; Verma, 2003), but only one research group demonstrated the ability of AIT to inhibit a specific microbial metabolic pathway (where the essential oil reduced yeast capacity for oxygen uptake; Kojima and Ogawa, 1971). However, the data presented in the latter report showed that a level 200 times greater than the actual MIC was needed to effectively inhibit oxygen uptake, indicating that AIT was not a strong respiratory inhibitor. Ankri and Mirelman (1999) also reported that allicin

(which is an allyl sulfide derived from garlic with structural similarities to AIT), was able to inhibit thiol-containing enzymes such as cysteine proteinases, alcohol dehydrogenases and thioredoxin reductases of different microorganisms.

Thioredoxin reductase is responsible for reduction of the multi-task protein thioredoxin to its functional state (Holmgren, 1989). The thioredoxin system is well recognized for its essential role in DNA synthesis, where thioredoxin works as an electron donor during the reduction of ribonucleotides to deoxyribonucleotides (Reichard, 1993). Researchers have shown the ability of AIT in crossing the plasma membrane and reaching the cytoplasm of prokaryotic (Ahn et al., 2001) and eukaryotic cells (Li and Zhang, 2005). Therefore, the antibacterial activity of AIT against *E. coli* may be related, at least in part, to the inhibition of DNA synthesis. The enzymatic activity of thioredoxin reductase involves the participation of a disulfide bond which receives reducing equivalents from NADPH, forming a dithiol structure responsible for reducing thioredoxin (Holmgren and Björnstedt, 1995). AIT reaction with thiol compounds has been described previously (Fig. 3.1) (Kawakishi and Kaneko, 1987; Luciano et al., 2008; Zsolnai, 1966). These results combined with the present findings that AIT exhibited non-competitive inhibition against TR (Appendix 4.1), suggesting that AIT may have changed the conformation of the enzyme by interacting with cystine bonds or available electrophilic groups of the enzyme resulting in lower enzymatic activity.

4.4.5 AIT interaction with acetate kinase

AIT was able to significantly ($P < 0.01$) inhibit acetate kinase activity at levels of 100 μL/L and 10 μL/L, but not at 1 μL/L (Fig. 4.4). These results showed that the

antimicrobial action of AIT against *E. coli* O157:H7 could also be related to the inhibition of this enzyme, although the mustard-derived compound did not show the same inhibitory potency when compared to thioredoxin reductase. The enzymatic inhibition against AK also presented non-competitive behaviour (Appendix 4.2).

Acetate kinase is an amphibolic enzyme that catalyzes the conversion of acetate + ATP to acetyl phosphate + ADP and the reverse (Rose et al., 1954). Together with phosphotransacetylase, AK is used to form acetyl-CoA, which is involved in several metabolic pathways (Imgram-Smith et al., 2006). Nevertheless, AK is used to activate acetate as a carbon source and to produce ATP anaerobically (Koplove and Clooney, 1978). The current findings suggest that AIT interacts with AK, perhaps causing a conformational change of the enzyme resulting in the catalytic inhibition observed. This could be due cysteine bond cleavage (Kawakashi and Kaneko, 1985) or interaction with free thiol and/or amino groups (Kawakishi and Kaneko, 1987).

The capacity of AIT to inhibit two completely different enzymes such as AK and TR, suggests it may exert a broad spectrum of inhibitory action. In addition, isothiocyanates inhibited mammalian thioredoxin and glutathione reductases (Hu et al., 2007). Other studies in human cells showed a broad interaction of isothiocyanates and metabolic systems, where isothiocyanates: inhibited the activity of enzymes involved in the metabolism of xenobiotics such as cytochrome P450 2E1 (Moreno et al., 1999) and N-nitrosodimethylamine demethylase (Jiao et al., 1996); blocked the mitotic process (Smith et al., 2004; Xiao et al., 2003); and caused DNA damage (Kassie and Knasmuller, 2000; Murata et al., 2000). Prokaryotic and eukaryotic cells are physiologically different, but these studies in mammalian cells provide insight about the mechanism of

antimicrobial action of isothiocyantes. Nevertheless, further studies examining capacity of isothiocyanates to generate DNA damage and block cell division are recommended.

4.5 Conclusion

Action of AIT as an antibacterial agent against *E. coli* O157:H7 was improved at lower pH values. This fact can be related to the higher stability of AIT in more acidic environments (Pechacek et al., 1997; Tsao et al., 2000). In addition, degradation products of AIT in water were ineffective against *E. coli* O157:H7 growth. Therefore, the aqueous decomposition of AIT and basic conditions will limit its antimicrobial activity.

Furthermore, allyl isothiocyanate inhibited the catalysis of both thioredoxin reductase and acetate kinase, which are responsible for important metabolic reactions in bacteria. Thus, it seems that AIT, and perhaps other isothiocyanates, have multi-targeted antimicrobial activity, since they caused enzymatic inhibition and membrane damage (Lin et al., 2000).

Table 4.1 – Minimum inhibitory concentration of AIT against an *E. coli* O157:H7 cocktail at different pH values.

pH	Minimum Inhibitory Concentration
4.5	25 µL/L
5.5	25 µL/L
6.5	50 µL/L
7.5	250 µL/L
8.5	500 µL/L

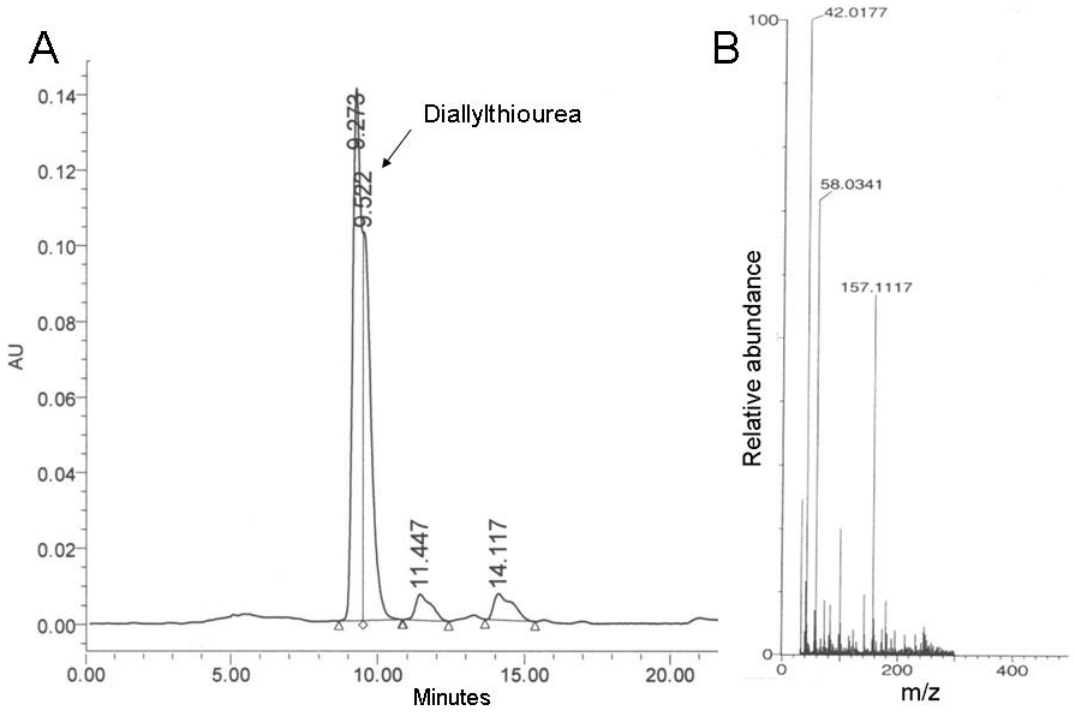


Fig. 4.1 - Formation of degradation products from AIT in water after 48 h at 37 ^{o}C detected by HPLC (A). The peak at retention time ~ 9 min was identified by MS analysis (B) as diallylthiourea (m/z + 1 = 157.11).

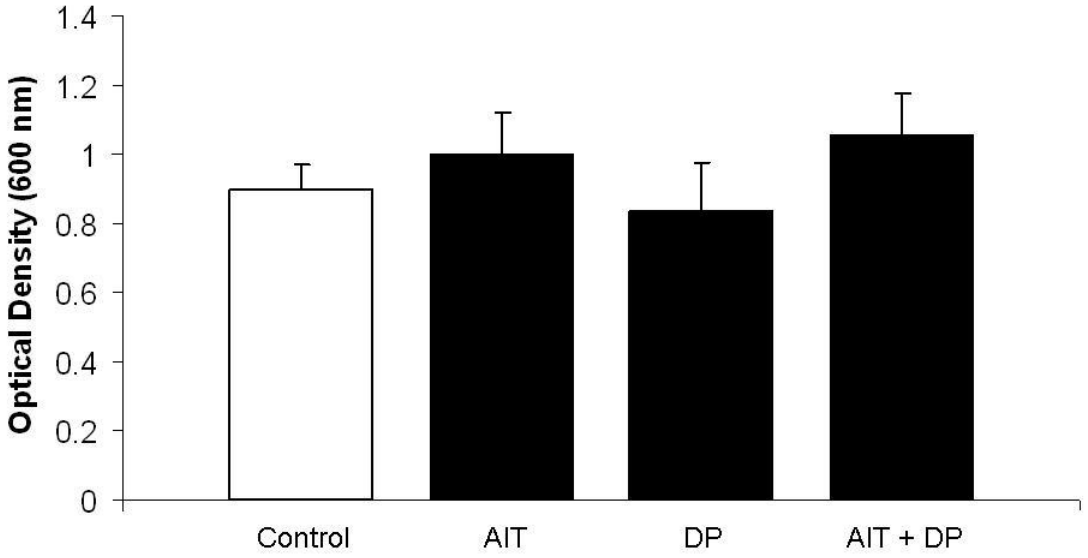


Fig. 4.2 - Effect of the degradation products (DPs) of AIT on the growth of a 5-strain *E. coli* O157:H7 mixture measured by changes in OD_{600} after 16h at pH 6.5 and 37^{o}C. DP and AIT + DP groups received 1 ml of the extracted solution; control and AIT groups received the same volume of deionized water. Groups containing AIT received a sub-lethal (10 μL/L) dose of the oil.

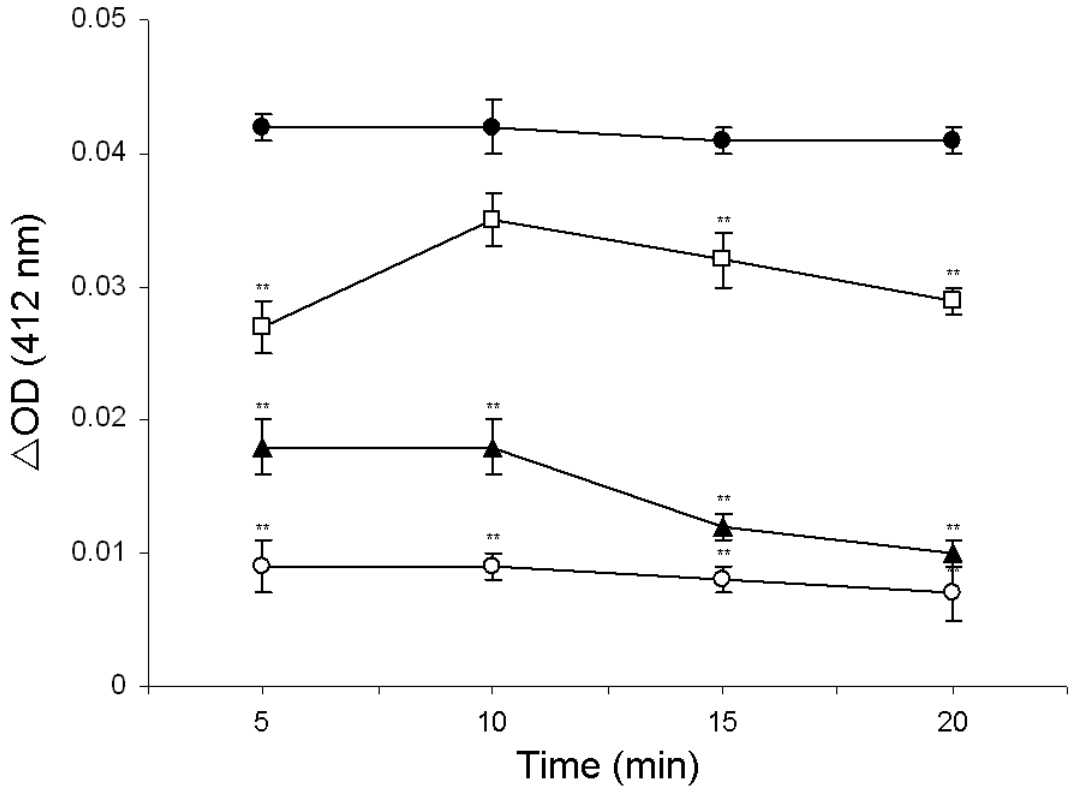


Fig. 4.3 – Effects of different levels of AIT on the enzymatic activity of thioredoxin reductase from *E. coli*. Degradation of DTNB was measured spectrophotometrically at 412 nm and followed for 20 min at pH 7.0. Groups were treated with 100 μL/L (○), 10 μL/L (▲) or 1 μL/L (□) of AIT. The control group (●) did not receive any treatment. ** = $P < 0.01$ was considered a highly significant difference.

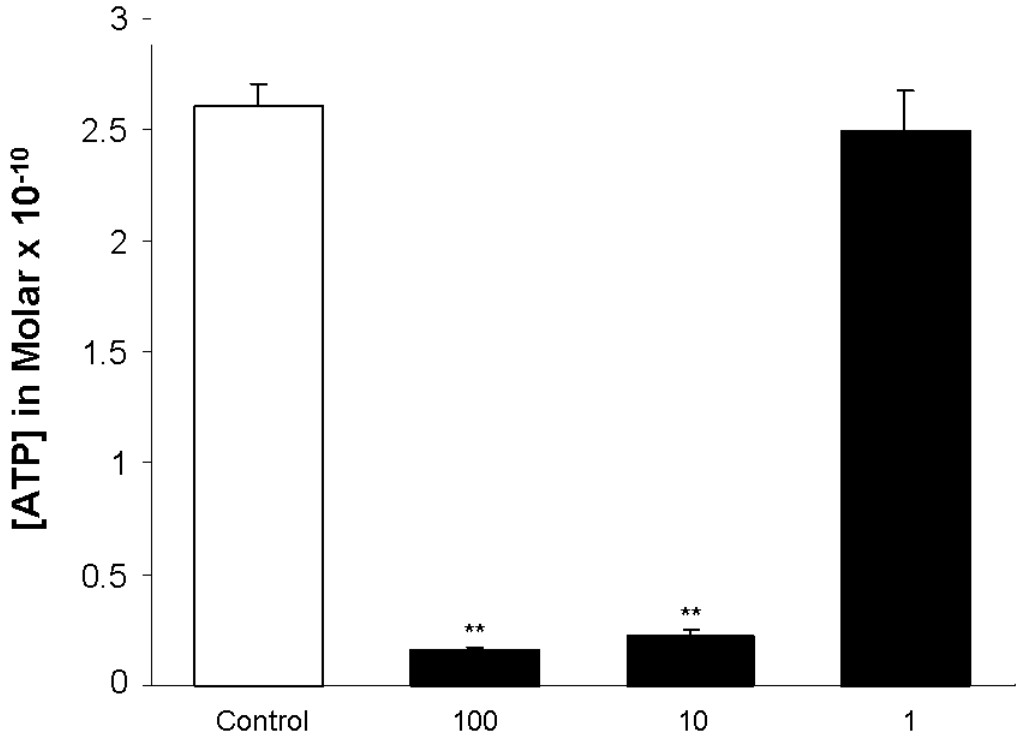
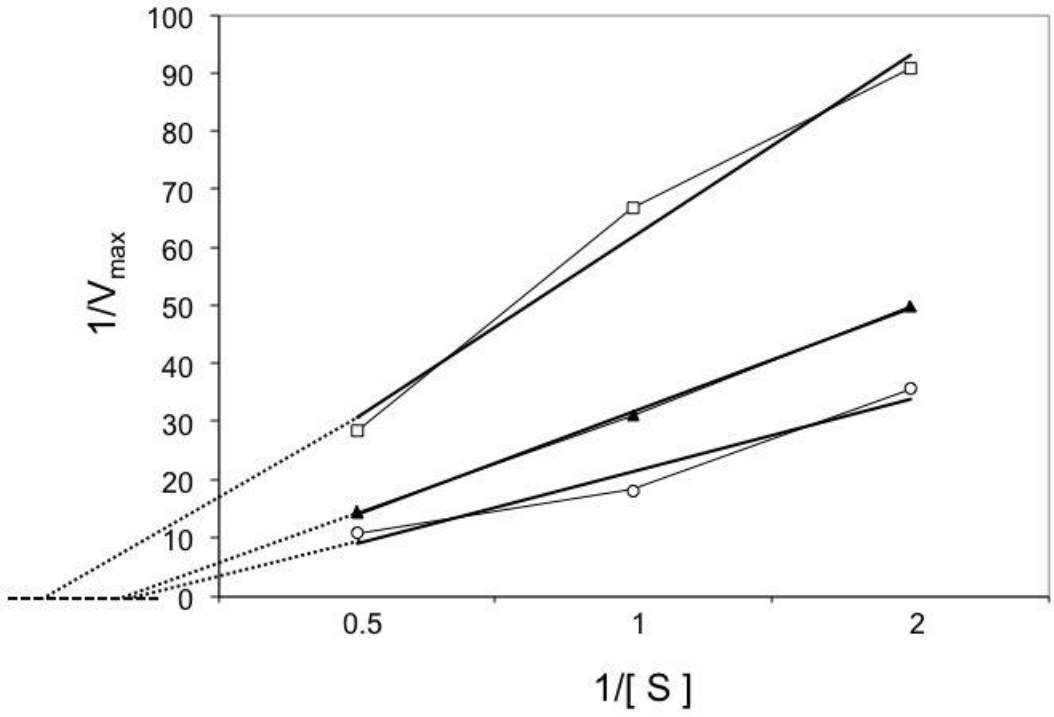


Fig. 4.4 – Acetate kinase activity in the absence (□) or presence (■) of different levels of AIT (μL/L) at pH 7.6. Light output of the samples was measured using a luminometer and compared to a standard curve. Numbers represent concentration of ATP produced. ** = $P < 0.01$ was considered a highly significant difference.

Appendix 4.1

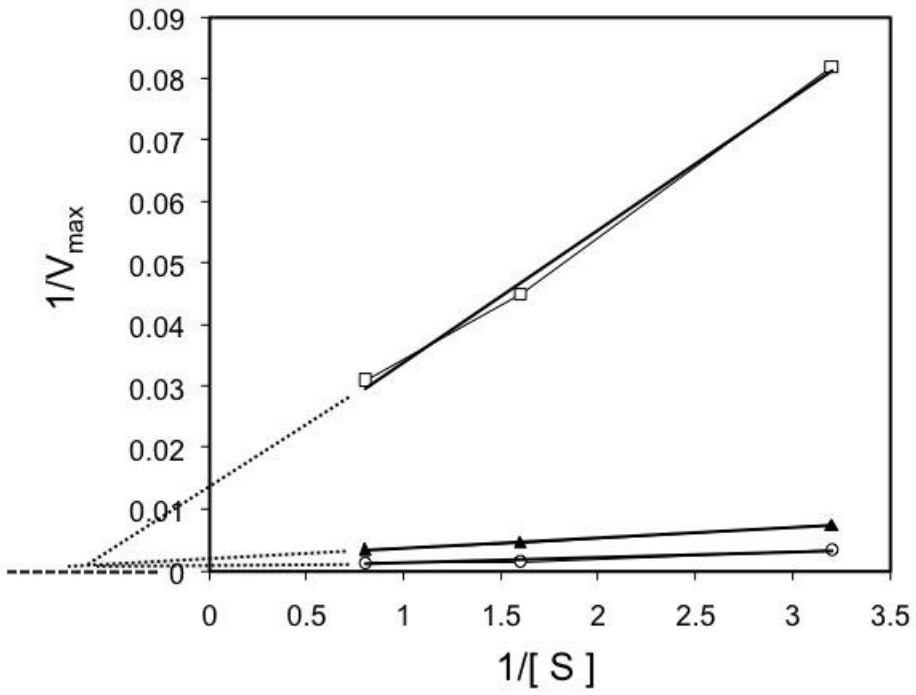
Lineweaver-Burke plot of the non-competitive inhibition of thioredoxin reductase caused by AIT.



Concentrations of AIT used were 100 ppm (□), 10 ppm (▲) and 1 ppm (○). V_{max} was measured by the consumption of DTNB (Abs = 412 nm). Substrate concentrations: 0.5 = 12.5 mg/ml DTNB + 20 mg/ml NADPH, 1 = 25 mg/ml DTNB + 40 mg/ml NADPH and 2 = 50 mg/ml DTNB + 80 mg/ml. Measurements were done following the method described in section 4.3.6.

Appendix 4.2

Lineweaver-Burke plot of the non-competitive inhibition of acetate kinase caused by AIT.



Concentrations of AIT used were 100 ppm (□), 10 ppm (▲) and 1 ppm (○). V_{max} was measured by the formation of ATP. Substrate concentrations used were: 2.5 μM ADP + 5 mM acetyl phosphate, 1.25 μM ADP + 2.5 mM acetyl phosphate and 0.624 μM ADP + 1.25 mM acetyl phosphate. S represents the concentration of ADP. Measurements were done following the method found in section 4.3.7.

Chapter 5

Bacterial degradation of glucosinolates and its influence on the growth of *E. coli* O157:H7 in a dry fermented sausage model – Part 1

5.1 Abstract

Illness outbreaks involving *Escherichia coli* O157:H7 and dry-cured sausage consumption have been reported in a number of countries. North American food regulatory agencies mandate a 5-log reduction of *E. coli* O157:H7 during the manufacture of these products. Curiously, the addition of 6% yellow mustard flour lacking the enzyme myrosinase (and therefore unable to produce antimicrobial isothiocyanates from the glucosinolate, sinalbin, in mustard) in dry sausage was able to cause significant ($P < 0.01$) reduction of the pathogen in only 6 days. Three hypotheses were postulated: 1) intrinsic meat enzymes were able to convert the glucosinolate to isothiocyanate; 2) the mustard present in the spice used (at 10%) in the sausage recipe was responsible; or 3) bacteria had myrosinase-like activity and formed the isothiocyanate. Changes in sinalbin levels were followed for 6 days using HPLC. Results showed that neither the meat enzymes nor the spice mix affected sinalbin concentrations. However, all bacteria tested were able to degrade sinalbin and form ρ-hydroxybenzyl isothiocyanate. This degradation occurred intracellularly. Separately, the bacteria showed different rates of glucosinolate breakdown, with *E. coli* O157:H7 > *Staphylococcus carnosus* > *Pediococcus pentosaceus*. These results suggest that the starter cultures may contribute to *E. coli*

O157:H7 death, and possibly that *E. coli* O157:H7 may commit suicide by degrading the glucosinolate and releasing the isothiocyanate into the sausage environment.

5.2 Introduction

Escherichia coli O157:H7 has been involved in foodborne illness outbreaks in over 30 countries, which were first reported in the early 1980's. Most of these outbreaks have been associated with the consumption of undercooked meats (LeBlanc, 2003). Dry fermented sausages are traditional products, usually consumed raw and manufactured without thermal processing. Preservation of these products is a result of various characteristics: pH drop produced by the fermenting bacteria, commonly lactic acid bacteria (LAB), reduction of the water activity (a_w) during the drying process, and addition of antimicrobial compounds such as salt, nitrite and spices (Lucke, 1986). Although dry-cured sausages offer diverse hurdles against the growth of microbial pathogens, some of these microorganisms can overcome these barriers. *E. coli* O157:H7 is well known to be pH and salt tolerant (Cheville et al., 1996). In addition, it has been reported to have very low infective doses and high mortality rates, mainly in infants, in the elderly and in the immunocompromised. In 1994, *E. coli* O157:H7 sickened 18 individuals in an outbreak related to the consumption of pre-sliced dry salami (Tilden et al., 1996). Other *E. coli* O157:H7 outbreaks involving 39 and 143 people were related to the consumption of Genoa salami and Hungarian (plus Cervelat) salami, respectively (Williams et al., 2000; MacDonald et al., 2004). Beef and to a less extent pork meat were the potential sources of *E. coli* O157:H7 during these outbreaks, and they are major ingredients in dry-cured sausages recipes. The occurrence of these outbreaks led food regulatory agencies in both Canada (CFIA, 1999) and the US (Reed, 1995) to adopt strict

rules for the manufacture of fermented meat products, requiring processes used to achieve at least a 5-log reduction of the *E. coli* O157:H7 population.

Glucosinolates are substances found within cell compartments of plants belonging to the family *Cruciferaceae* (e.g. broccoli, mustard, horseradish, wasabi) (Nielsen And Rios, 2000). When these intracellular compartments are disrupted by mechanical damage, the membrane-bound enzyme myrosinase is released and hydrolyzes glucosinolates, resulting in the formation of three main groups of substances: nitriles, thiocyanates and isothiocyanates (Delaquis and Mazza, 1995). The last group contains diverse compounds with well-established antimicrobial activity, such as benzyl isothiocyanate, phenethyl isothiocyanate, allyl isothiocyanate (AIT) and ρ-hydroxybenzyl isothiocyanate (ρ-HBIT) (Delaquis and Mazza, 1995; Nielsen and Rios, 2000; Ekanayake et al., 2006). The latter compound is the most relevant for the present study because ρ-HBIT is the only isothiocyanate formed after hydrolysis of sinalbin by myrosinase, and sinalbin is practically the sole glucosinolate found in yellow mustard flour (~2.5%) (Cui and Eskin, 1998).

Graumann and Holley (2008) evaluated the potential antimicrobial effect of 2, 4, and 6% (wt/wt) non-deheated (active myrosinase) yellow mustard flour (YMF) (*Sinapis alba*) or 6% (wt/wt) deheated (inactive myrosinase) YMF following addition to dry sausage batter inoculated with *E. coli* O157:H7 at about 7 log CFU/g. Mustard flour can be used as a spice (containing active myrosinase) and/or binder (inactive myrosinase) in dry-cured sausages. Diverse parameters were monitored throughout the study (water activity, pH, bacterial population) and it was found that all levels of non-deheated mustard powder resulted in significant reductions of *E. coli* O157:H7 during 30 days of

drying. However, only concentrations of 6% were able to achieve the 5-log reduction required by food regulatory agencies. Longer fermentation periods were necessary for a 5-log *E. coli* O157:H7 reduction by 2 and 4% yellow mustard powder. On the other hand, populations of the starter cultures used were not affected by the mustard powder. Interestingly, the 6% deheated mustard powder treatment resulted in the most rapid reduction of *E. coli* O157:H7 (significant drop after 6 days). This result was not expected since the "cold" or tasteless flour lacks the enzyme myrosinase and, therefore, is not able to produce isothiocyanates intrinsically. The aim of the present paper was to understand, at least partially, the mechanism by which the "cold" flour was able to reduce *E. coli* O157:H7 population.

5.3 Material and Methods

5.3.1 Bacterial strains

Experiments used a five strain mixture of *E. coli* O157:H7. The strain LCDC 7283 (pathogenic, hamburger isolate) was provided by Dr. R. Khakria, Laboratory Centre for Disease Control, Ottawa, Canada. Strains 02-0628, 02-0627, 00-0351 and 02-0304 (non-pathogenic, human isolates) were supplied by Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency, Canadian Centre for Human and Animal Health, Winnipeg, MB, Canada. *Staphylococcus carnosus* (UM 109M) and *Pediococcus pentosaceus* (UM 116P) were isolated from commercial lyophilized dry-sausage starter culture preparations (Trumark LTII and Trumark LTIIM, respectively; Rector Foods Ltd., Mississauga, Ontario, Canada).

5.3.2 Chemicals

Myrosinase and tetrabutylammonium hydrogen sulfate (TBA) were purchased from Sigma Chemical Co (St. Louis, MO, USA); sinalbin from C_2 Bioengineering (Copenhagen, DK); acetonitrile HPLC grade from Fisher Scientific Co. (Fair Lawn, NJ, USA) and the deheated yellow mustard flour was provided by G.S. Dunn (Hamilton, ON, Canada). Other chemicals noted were of analytical grade.

5.3.3 Microbial growth conditions

All bacteria were incubated in broth for 16h at 25^{o}C prior to the experiments. *E. coli* O157:H7 (5-strain cocktail) and *Staphylococcus carnosus* grew in Müller-Hinton broth (MHB) (Oxoid, Unipath, Nepean, ON); *Pediococcus pentosaceus* grew in deMan, Rogosa and Sharpe (MRS) broth (Oxoid). After the incubation period, 0.1 ml of each bacterial culture was used as the inoculum for subsequent experiments. The final volume of 10 ml in screw-capped tubes was used during all studies.

5.3.4 Degradation of glucosinolates

Levels of sinalbin were followed for 6 days (samples collected on days 0, 1, 3 and 6) in different sets of experiments: 1) Bacterial growth in MHB containing 0.1% sinalbin was examined (adapted from Brabban and Edwards, 1994). 2) Then, the effect of meat enzymes on the degradation of glucosinolates was evaluated using fresh meat exudates in the presence of sinalbin. For that purpose, lean cuts of pork and beef were treated in a stomacher (BagMixer 400, Intersciences Inc., Markham, Ontario, Canada) for 1.5 min in

M9 media (Oxoid) (10g of meat per 90 ml of M9 media), and the slurry formed was filtered-sterilized (0.22 µM Fisherbrand syringe filter; Fisher Scientific Co.). Sinalbin at 0.1% was added to this solution. 3) The spice mix used by Graumann and Holley (2008) was found to contain up to 10% of mustard powder and, therefore, was examined for the presence of myrosinase that could also have been responsible for conversion of glucosinolates to isothiocyanates. A level of 0.44% of the spice mix, which is used in dry-cured sausage recipes, was added to M9 media with 0.1% sinalbin.

5.3.5 HPLC analysis

Separation and quantification of the glucosinolates was performed using an HPLC equipped with a C18 column (Waters Co., 4.6 x 250 mm i.d. 5 µm). Elution was carried out isocratically for 20 min at a flow rate of 1 mL/min, using a solvent system containing 20% (v/v) acetonitrile and 80% water + 0.02M tetrabutylammonium hydrogen sulfate (pH 5.5). The injection volume used was 10 µl. A detector was used to measure the absorbance at 227 nm in order to verify and quantify the presence of the glucosinolates and its degradation products.

5.3.6 Challenging *E. coli* O157:H7 using media containing ingredients present in dry fermented sausage, mustard and starter culture

Müller-Hinton broth containing ingredients present in dry fermented sausages (2.91% salt, 0.31% pickle cure concentrate, 0.1% glucose, 0.05% sodium erythorbate) was prepared in the presence or absence of deheated yellow mustard (2%). Overnight cultures of *P. pentosaceus* UM 116P, *S. carnosus* UM 109M and *E. coli* O157:H7 (5-

strain mixture) were inoculated (0.1 ml each) in the experimental broth to achieve a final volume of 10 ml (screw-capped tubes), which was maintained at 25^{o}C and 200 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA). Bacterial populations were followed for 3 days.

5.3.7 Evaluation of extra- and intracellular enzymatic activity

E. coli O157:H7 (5-strain cocktail), *S. carnosus* and *P. pentosaceus* were cultivated separately in MH broth containing sinalbin (500 mg/L) for 6 days at 25^{o}C and 200 rpm. Degradation of glucosinolates by the bacteria was monitored by HPLC. The bacteria were transferred to microcentrifuge tubes (1.5 ml) and spun at 3000 rpm for 15 min. The supernatant was collected, filter sterilized and analyzed for sinalbin (0.5 mg/ml) degradation for 6 days. Any degradation caused by this fraction would be considered to have been from extracellular enzymatic activity. The bacterial pellet was washed with 50 mM phosphate buffer (pH 7.0) and centrifuged again using the conditions previously described. The washing supernatant was discarded and the cells were resuspended again in 1.5 ml of ice-cooled 50 mM phosphate buffer (pH 7.0). Harvested bacteria were treated with a 60 watt High Intensity Ultrasonic Processor (Vibra-Cell 60, Sonics & Materials Inc., Danbury, CT, USA). A 2 mm probe with 40% vibration output was used for 1.5 min to disrupt *E. coli* O157:H7 and for 5 min to destroy both *S. carnosus* and *P. pentosaceus* (with a cycle of 2 min sonication/2 min rest/2 min sonication/2 min rest/1 min sonication, to avoid heating). The solution was filter-sterilized (0.2 μm filters) and sinalbin at 0.5 mg/ml was added to the tubes. Any degradation of sinalbin by this fraction over the 6 days was considered to have been due to intracellular enzymatic activity.

5.4 Results And Discussion

Neither the meat exudates (Fig. 5.1) nor the spice mix (result not shown) were able to reduce the levels of sinalbin in the media. These results suggest that the enzymes present in meat are not able to hydrolyze sinalbin and form ρ-HBIT. In addition, the mustard present in the spice mix had very little or no myrosinase activity.

Glucosinolate consumption was followed using MHB to improve bacterial growth (Fig. 5.1). *S. carnosus* produced a 220.5 μM reduction of the sinalbin levels and *P. pentosaceus* lowered the levels by 16.4 μM. The 5-strain *E. coli* cocktail produced the highest amount of glucosinolate degradation (507.9 μM). Recent work (unpublished, this laboratory) has shown that the minimum inhibitory concentration of ρ-HBIT against *E. coli* O157:H7 at pH levels found in dry-cured sausages (pH 5.5) was ~ 600 μM. Formation of isothiocyanates by the starter cultures or *E. coli* O157:H7 is hard to follow due to the high level of instability of this class of compounds in aqueous systems. The transient formation of ρ-HBIT can be confirmed by the presence of more stable products derived from its reaction with water. These products were ρ-hydroxybenzyl cyanate, ρ-hydroxybenzyl alcohol and the cyanate ion (SCN^-) (Buskov et al., 2000). Conversion of glucosinolates to isothiocyanates by myrosinase was shown to have a yield of up to 90% (Kawakishi and Maramatsu, 1966). If the decomposition of sinalbin generated by these bacteria follows the same pattern, this reaction will indeed exert an extra hurdle against *E. coli* O157:H7 growth in the dry-sausage environment, and could possibly cause the death of the pathogen.

Myrosinase-like activity in bacteria has been described before (Brabban and

Edwards, 1994; Palop et al., 1995; Krul et al., 2002; Cheng et al., 2004). The starter cultures and, more importantly, *E. coli* O157:H7 also showed the capacity to degrade glucosinolates. Yellow mustard flour contains 2.5% sinalbin on average, and this results in 3.53 mM sinalbin in a dry sausage containing 6% YMF. This concentration should allow formation of ρ-HBIT in sufficient quantity to be bactericidal to *E. coli* O157:H7 when combined with other hurdles inherent in the dry sausage.

Müller-Hinton broth containing ingredients used to manufacture dry-cured sausage was used to inoculate *P. pentosaceus* UM 116P, *S. carnosus* UM 109 or the 5-strain cocktail of *E. coli* O157:H7. The treated group also received a yellow mustard flour extract (2%). The population of *E. coli* O157:H7 dropped in both treated and control groups, but it was significantly lower in the treated group (Fig. 5.2). After 3 days, the population of *E. coli* O157:H7 decreased > 6 log CFU/ml in the treated group, whereas in the control group this reduction was 3.4 log CFU/ml. In addition, *P. pentosaceus* numbers were maintained during this study, while *S. carnosus* showed a similar reduction for both groups. A third treatment containing yellow mustard flour extract and myrosinase showed a more abrupt reduction of *E. coli* O157:H7 numbers. These results suggest that the presence of the yellow mustard flour extract offers an extra hurdle to the survival *E. coli* O157:H7. *In vitro*, as in the results shown by Graumman and Holley (2008), this extra hurdle may be a result of the bacterial degradation of sinalbin and consequent formation of ρ-HBIT.

Separate analysis of extracellular culture filtrates and intracellular bacterial fractions prepared by sonication revealed that all bacteria studied had intracellular enzymatic activity capable of degrading sinalbin and forming SCN^- (degradation caused

by *E. coli* O157:H7 is shown on Fig. 5.3). However, no extracellular myrosinase-like activity was found (result not shown). This result suggests that bacteria must internalize sinalbin in order to form the isothiocyanate (p-BHIT), which may then react with cell components and/or enzymatic systems (Fig. 4.3 and Fig. 4.4), leading to cell death (Luciano and Holley, 2009).

5.5 Conclusion

The results presented in this paper suggest that *P. pentosaceus* UM 116P, *Staphylococcus carnosus* UM 109 and, more importantly, *E. coli* O157:H7 are able to break down sinalbin and presumably convert significant amounts of the glucosinolate to its corresponding isothiocyanate. This conversion helps to explain why deheated yellow mustard flour was able to kill *E. coli* O157:H7 in dry-cured sausages (Graumman and Holley, 2008). This pathogen may internally convert sinalbin to ρ-HBIT in order to acquire energy, since a molecule of glucose is released during this reaction. This event is not lethal when the bacteria is growing in MHB broth alone (results not shown), but together with other hurdles present in the dry sausage environment, it may cause *E. coli* O157:H7 to commit suicide.

Further understanding of the mechanisms involved in glucosinolate-to-isothiocyanate formation, and selection of starter cultures with higher capacity to conduct this conversion should contribute to consistent achievement of the 5-log reduction of *E. coli* O157:H7 required by food regulatory agencies.

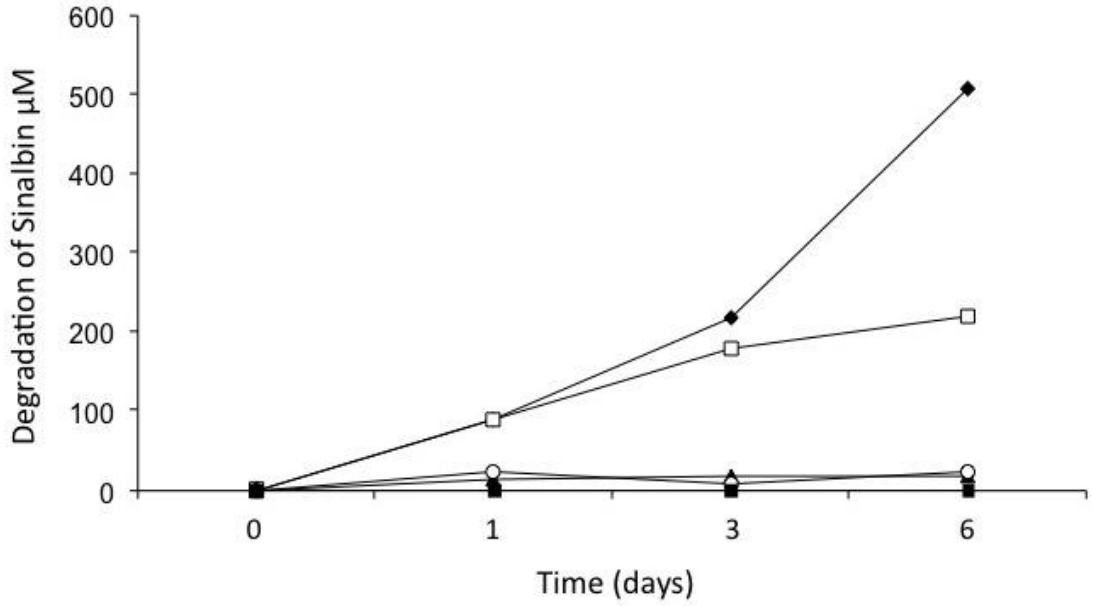


Fig. 5.1 - Hydrolysis of sinalbin by pork exudate (○), beef exudate (■), *Staphylococcus carnosus* (□), *Pediococcus pentosaceus* (▲) and *E. coli* O157:H7 cocktail (◆). Reduction of sinalbin levels was analyzed by HPLC and quantified by comparison to a 2.36 mM sinalbin standard. The commercial spice mix had no negative effect on sinalbin stability (result not shown).

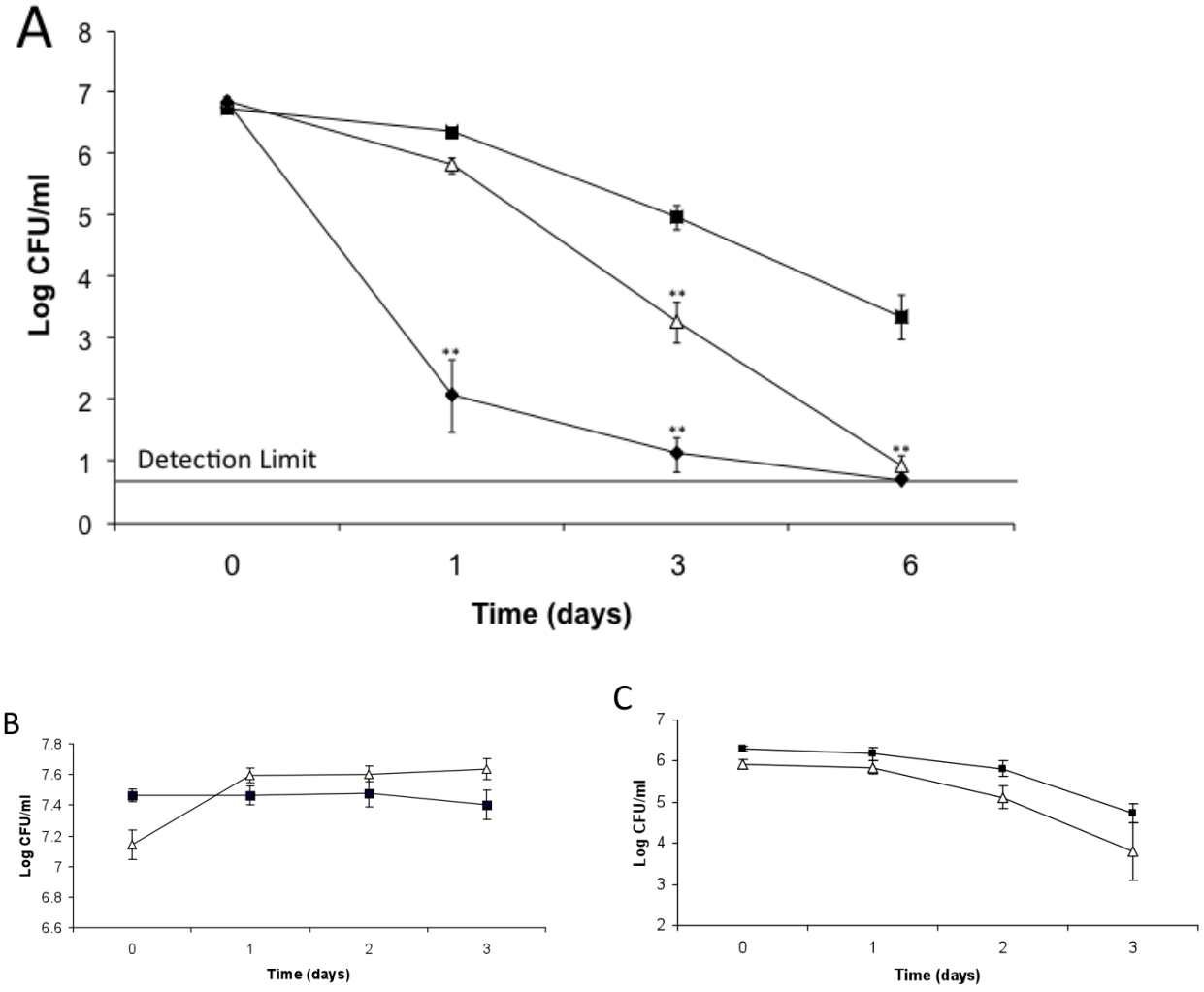


Fig. 5.2 - Bacterial population in Müller-Hinton broth containing the dry ingredients used for the production of dry-fermented sausages. A broth containing yellow mustard flour (△) was compared to a control (■) using the same incubation conditions (25^{o}C and 200 rpm). A) represents the population of *E. coli* O157:H7, *E. coli* O157:H7 was also incubated in the presence of myrosinase (0.2 U/tube) and YMF (♦); B) *P. pentosaceus*; and C) *S. carnosus*. Bacterial enumeration was done in VRB agar (*E. coli*), MSA agar (*S. carnosus*) and MRS agar (*P. pentosaceus*). ** = $P < 0.01$ was considered a highly significant difference.

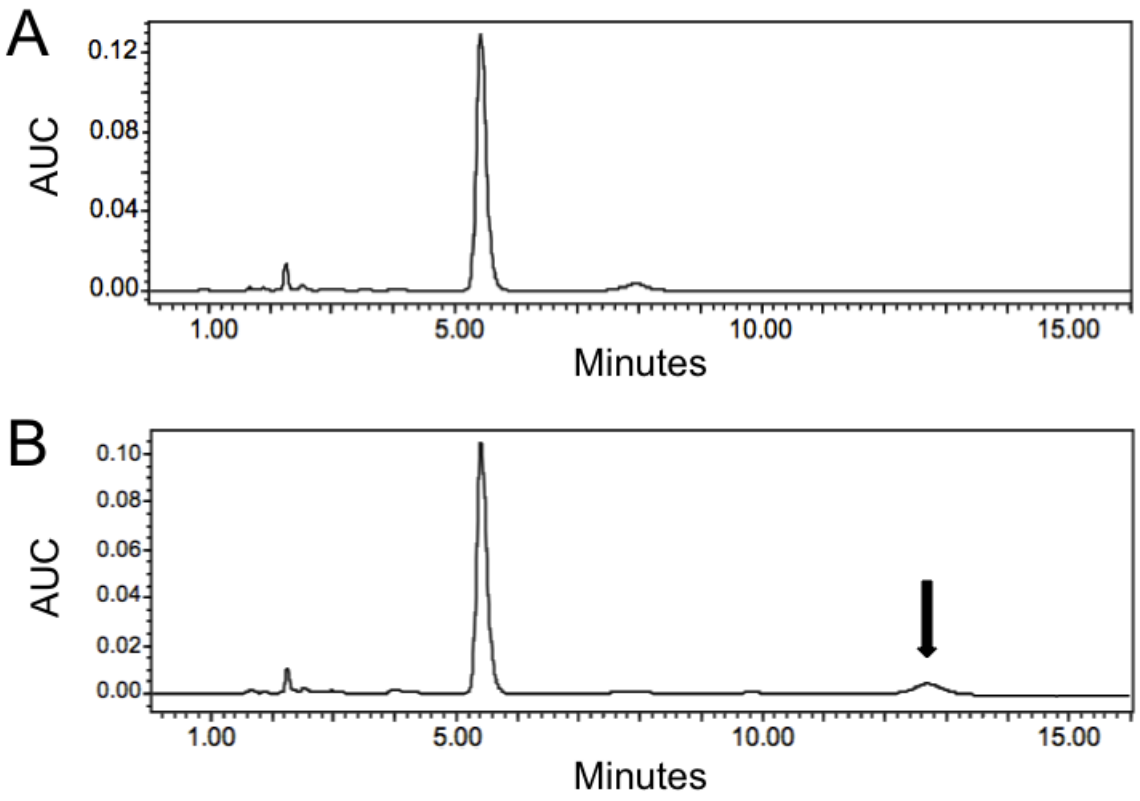


Fig. 5.3 – HPLC chromatogram of sinalbin (RT ~ 5.2 min) in the presence of the intracellular fraction from *E. coli* O157:H7 at 0h (A) and 72h (B). The arrow at RT = 12.9 min (B) represents the formation of the cyanate ion (SCN^-).

Chapter 6

Bacterial degradation of glucosinolates and its influence on the growth of *E. coli* O157:H7 in a dry fermented sausage model – Part 2

6.1 Abstract

Use of thermally deodorized (cold) yellow mustard flour in the production of dry fermented sausage has been shown to reduce the levels of *Escherichia coli* O157:H7 by > 5 log CFU/g. Although the cold flour (which contains heat-inactivated myrosinase) cannot form the antimicrobial ρ-hydroxybenzyl isothiocyanate (ρ-HBIT) from the native glucosinolate sinalbin, it was still able to eliminate *E. coli* O157:H7. Recently, it was found that *E. coli* O157:H7 and some starter cultures possessed myrosinase-like activity, and were able to degrade sinalbin to form ρ-HBIT. In the present study, these same bacteria were tested for their capacity to degrade a similar glucosinolate, sinigrin (found in brown/oriental mustard), and form allyl isothiocyanate (AIT). All three bacteria tested showed myrosinase-like activity towards sinigrin, with decomposition capacity in order of: *E. coli* O157:H7 > *Staphylococcus carnosus* > *Pediococcus pentosaceus*. The minimum bactericidal concentrations (MBCs) of AIT and ρ-HBIT were also tested. *S. carnosus* and *P. pentosaceus* were more sensitive to ρ-HBIT (0.59 and 5.92 mM, respectively), while *E. coli* O157:H7 was more susceptible to the action of AIT (1.04 mM). *E. coli* was also challenged following the *in vitro* formation of AIT or ρ-HBIT by commercial myrosinase. *E. coli* O157:H7 was more rapidly reduced by AIT, supporting the MBC results. In conclusion, it seems that ground, cold black or brown/oriental

mustard flour may also be used to prevent survival of *E. coli* O157:H7 in dry-fermented sausage.

6.2 Introduction

Glucosinolates (secondary metabolites found in plants of the family *Cruciferaceae*) are precursors of antimicrobial isothiocyanates. Glucosinolate to isothiocyanate transformation is part of the plant defense mechanism and it is dependent on myrosinase, which is a membrane-bound enzyme released after plant tissue is damaged (e.g. wounding, milling, grinding). Interestingly, some bacteria have been found to have myrosinase-like activity, and are able to form isothiocyanates from glucosinolates. Examples are *Lactobacillus agilis* R16 (Palop et al. 1995), unidentified strains of *Bacillus* and *Staphylococcus* (Brabban and Edwards, 1994), *Bacterioides thetaiotaomicron* (Elfoul et al., 2001) and more recently *Pediococcus pentosaceus* UM116P, *Staphylococcus carnosus* UM109M and *Escherichia coli* O157:H7 (Fig. 5.1).

A recent study by Graumann and Holley (2008) showed that the addition of 6% w/w cold (non-spice flavored) ground mustard (*Sinapis alba*) lacking myrosinase activity was able to significantly decrease a population of *Escherichia coli* O157:H7 in dry fermented sausage. Although this was an unexpected result, bacterially-derived myrosinase activity could explain, at least partially, the reasons for this outcome (Fig. 5.1). Surprisingly, it is possible that *E. coli* degraded sinalbin in an attempt to acquire energy, and simultaneously formed the antimicrobial ρ-hydroxybenzyl isothiocyanate (ρ-HBIT). Observation of active, *in situ*, formation of isothiocyanate by contaminating *E.*

coli O157:H7, would represent discovery of a unique method for the manufacture of dry sausage within regulatory guidelines for control of this pathogen without cooking.

Although isothiocyanates have been recognized as antimicrobial compounds for a long time (Zolsnai, 1966), very few studies have been conducted to quantify the concentration of ρ-HBIT needed to inhibit the growth or kill different species of bacteria. Ekanayake and colleagues (2006) tested ρ-HBIT at 60 to 360 ppm (0.35-2.13 mM) against *Escherichia coli*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Pseudomonas aeroginosa*, *Salmonella* Enteritidis, *Listeria monocytogenes*, *Shigella boydii* and *Clostridium perfringens*. Overall, addition of 60-120 ppm generated an average 2-4 log CFU reduction for each organism, while 360 ppm resulted in a > 6 log reduction. Tests were carried out at 6.5^{o}C using 0.5% peptone water as the nutritional medium. Additionally, sinalbin and its respective isothiocyanate were tested against the fungus *Fusarium culmorum* (Manici et al., 1997). Sinalbin itself did not prevent microbial growth, but its derivative ρ-HBIT caused a 31% reduction in growth of *Fusarium culmorum* when applied at 2 mg/ml.

In contrast with ρ-HBIT, allyl isothiocyanate (AIT) derived from sinigrin, which is the major glucosinolate found in black (*Brassica nigra*) and brown/oriental (*Brassica juncea*) mustard, has been more extensively studied (Zolsnai, 1966; Kojima and Ogawa, 1971; Delaquis and Sholberg, 1997; Ward et al., 1998; Hasegawa et al., 1999; Lin et al., 2000; Chacon et al., 2006a). Its antimicrobial properties have been demonstrated against a variety of pathogenic bacteria, including *E. coli* O157:H7 (Fig. 5.2), *Listeria monocytogenes* (Lin et al., 2000), *Bacillus cereus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella* Enteritidis and *Vibrio parahaemolyticus* (Isshiki et

al., 1992). A disadvantage of this isothiocyanate is its extremely pungent flavor, which limits its use at higher concentrations. To understand the potential for broader application of the antimicrobial glucosinolate/isothiocyanate hypothesis, an objective of the present work was to investigate whether *E. coli* O157:H7, *Staphylococcus carnosus* and *Pediococcus pentosaceus* were able to degrade sinigrin as well as sinalbin. In addition, the bactericidal activity of AIT and ρ-HBIT were examined against *E. coli* O157:H7, and the meat starter cultures *Staphylococcus carnosus* and *Pediococcus pentosaceus*. It was anticipated that results from the work conducted could serve as proof in principle that mustard glucosinolates could be used as a dry sausage ingredient and antimicrobial precursor for "self-control" of *E. coli* O157:H7 contamination in these products.

6.3 Material and Methods

6.3.1 Bacterial strains

Experiments used a five strain mixture of *E. coli* O157:H7. The strain LCDC 7283 (pathogenic, hamburger isolate) was provided by Dr. R. Khakria, Laboratory Centre for Disease Control, Ottawa, Canada. Strains 02-0628, 02-0627, 00-0351 and 02-0304 (non-pathogenic, human isolates) were supplied by Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency, Canadian Centre for Human and Animal Health, Winnipeg, MB, Canada. *Staphylococcus carnosus* (UM 109M) and *Pediococcus pentosaceus* (UM 116P) were isolated from commercial lyophilized dry-sausage starter culture preparations (Trumark LTII and Trumark LTIIM, respectively; Rector Foods Ltd., Mississauga, Ontario, Canada).

6.3.2 Chemicals

Sinigrin, allyl isothiocyanate, myrosinase (EC 3.2.1.147) and tetrabutylammonium hydrogen sulfate (TBA) were purchased from Sigma Chemical Co (St. Louis, MO, USA); sinalbin from C_2 Bioengineering (Copenhagen, DK); ρ-hydroxybenzyl isothiocyanate was from Toronto Research Chemicals (North York, ON, Canada); Dimethyl sulfoxide (DMSO) and acetonitrile HPLC grade were from Fisher Scientific Co. (Fair Lawn, NJ, USA). Other chemicals used were of analytical grade.

6.3.3 Degradation of sinigrin

Bacteria grew in Müller-Hinton broth (MHB) (Oxoid, Unipath, Nepean, ON) containing 0.1% sinigrin (adapted from Brabban and Edwards, 1994), and the glucosinolate levels were examined in samples collected at days 0, 1, 3 and 6. Test was done in duplicate. Separation and quantification of sinigrin was performed using an HPLC equipped with a C18 column (4.6 x 250 mm i.d. 5 μm, Waters Co., Milford, MA, USA). Elution was carried out isocratically for 20 min at a flow rate of 1 mL/min, using a solvent system containing 20% (v/v) acetonitrile and 80% water + 0.02M tetrabutylammonium hydrogen sulfate (pH 5.5). The injection volume used was 10 μl. A detector was used to measure the absorbance at 227 nm in order to verify and quantify the presence of sinigrin.

6.3.4 *In vitro* formation of isothiocyanates

E. coli O157:H7 was grown overnight and re-inoculated in Müller-Hinton broth (MHB) (10^7-10^8 CFU/ml; optical density = 0.60-0.65). A 0.1 ml sample was drawn from this tube and added to screw-capped tubes containing a mixture of 9.9 ml of MHB, myrosinase (0.2 Units per tube), ingredients used in dry-fermented sausage (0.291g salt, 0.031g pickle cure concentrate, 0.01g glucose, 0.005g sodium erythorbate) and either sinigrin or sinalbin (2.5 mM). Viability of *E. coli* O157:H7 was followed for 4 d by surface plating on violet red bile agar (VRB) (Oxoid).

6.3.5 Minimum bactericidal concentration

The minimum bactericidal concentration (adapted from Kim et al., 1995) of AIT and ρ-HBIT were tested against *E. coli* O157:H7 (5-strain cocktail), *S. carnosus* and *P. pentosaceus* (in broth contained in capped glass tubes). Degradation products of ρ-HBIT in water [ρ-hydroxybenzyl alcohol (ρ-HBA), ρ-hydroxybenzyl cyanide (ρ-HBC) and cyanide ion (SCN^-)] (Choubdar et al., 2010) were also tested against *E. coli* O157:H7 to a maximum concentration of 40 mM. The aromatic compounds ρ-HBIT, ρ-HBA, ρ-HBC were dissolved in DMSO at a final concentration <0.1% to facilitate their dispersion. *E. coli* O157:H7 was grown in Luria Broth (Oxoid), *S. carnosus* in tryptic soy broth (Oxoid), and *P. pentosaceus* in de Man, Rogosa and Sharpe broth (Oxoid). Broth was adjusted to pH 5.5 using 0.1 M HCl, and bacteria grew at 25^{o}C and 200 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA). Bacteria were plated after 18h and the minimum bactericidal concentration (MBC) of the antimicrobials was calculated. The population of *E. coli* was enumerated using VRB agar (Oxoid),

Staphylococcus using mannitol salt agar (Oxoid) and *Pediococcus* using de Man, Rogosa and Sharpe agar (Oxoid). Test was done three times in triplicates (n=9).

6.4 Results and Discussion

Glucosinolate consumption was followed using MHB as growth media (Fig. 6.1). *S. carnosus* produced a 425 μM reduction of the sinigrin levels and *P. pentosaceus* reduced the levels by 297 μM after 6 d. The 5-strain *E. coli* cocktail yielded the greatest extent of sinigrin degradation (1.02 mM) during the period analyzed (6 d). These numbers were superior to those found for the degradation of sinalbin (Fig. 5.1), where *E. coli* O157:H7 decomposed 507.9 μM of the glucosinolate, *S. carnosus* 220.5 μM and *P. pentosaceus* 16.4 μM. A previous study showed that the minimum inhibitory concentration of allyl isothiocyanate against *E. coli* O157:H7 at pH levels found in dry-cured sausages (pH 4.9 to 5.5) was ~ 250 μM (Fig. 4.1). Quantification of allyl isothiocyanate formation by these bacteria is difficult because of the significant instability of this compound in aqueous systems (Kawakishi and Namiki, 1969). Conversion of glucosinolates to isothiocyanates by myrosinase is capable of yields reaching 90% (Kawakishi and Muramatsu, 1966). If the decomposition of sinigrin generated by these bacteria follows the same pattern, this reaction should easily establish an effective hurdle against *E. coli* O157:H7 survival in the dry sausage environment, and should eliminate the pathogen.

The sensitivity of the three species of bacteria to AIT and ρ-HBIT was also tested. It was clear that *P. pentosaceus* possess greater resistance to isothiocyanates than *E. coli* O157:H7 and *S. carnosus* (Table 6.1). The latter two organisms had similar sensitivity to AIT (MBC was 1.04 mM), while *S. carnosus* showed lower resistance to ρ-HBIT than *E.*

coli O157:H7. In contrast with the other two organisms, *E. coli* O157:H7 was more susceptible to the inhibitory activity of AIT than ρ-HBIT. This result was corroborated by the antimicrobial activity of the isothiocyanates found when they were formed *in vitro* upon the action of myrosinase. As shown in Fig. 6.2, *E. coli* was not detectable after 2 d when challenged by myrosinase plus sinigrin (resulting in the formation of AIT). Similar reduction was achieved only after 3 d when the glucosinolate present was sinalbin (forming ρ-HBIT). Although both antimicrobials led to bacterial death, inhibitory action against the pathogen was faster by the aliphatic isothiocyanate (AIT). Kim and Lee (2009) reported that aromatic isothiocyanates had stronger antibacterial activity against *E. coli* than aliphatic derivates, which is contrast with the findings of the present study. Kim and Lee (2009) added the isothiocyanates (dissolved in methanol) to paper disks, which were put aside to allow the solvent to dry and they were subsequently applied on the surface of agar containing the bacteria. The zone of inhibition produced was used to indicate antimicrobial potency. Their results showed strong inhibition of all bacteria tested by the aromatic isothiocyanates and no inhibition was observed by the aliphatic compounds. However, the authors did not take in consideration that the aliphatic isothiocyanates were volatile and could have been lost during the period that the paper disk dried before use. The present results indicate that these isothiocyanates do exert mild to strong antimicrobial activity when tests are done under hermetic conditions.

ρ-Hydroxybenzyl isothiocyanate is highly unstable in aqueous solutions, forming ρ-HBA, ρ-HBC and SCN^- depending on the environmental pH (Buskov et al., 2000). These hydrolysis products showed very weak (ρ-HBA and ρ-HBC) or no (SCN^-) inhibitory

activity against *E. coli* O157:H7 (Table 6.1). Therefore, hydrolysis of ρ-HBIT will result in reduction of its antimicrobial action against this pathogen.

Cold yellow mustard flour was added to dry fermented sausage (Graumann and Holley, 2008) and to Müller-Hinton broth (*in vitro*) in order to kill *E. coli* O157:H7 (Fig. 5.2). The bacteria *P. pentosaceus* UM116 and *S. carnosus* UM109 were also used in both studies. Levels of *S. carnosus* declined during the course of these studies (1-2 log CFU/g), while *E. coli* O157:H7 was reduced to undetectable levels (> 6 log CFU/g). In addition, the population of *P. pentosaceus* remained constant throughout these experiments. Although *E. coli* and *S. carnosus* showed similar resistance to isothiocyanates, staphylococci are more resistant to other hurdles found in the dry fermented sausage environment (high salt content and a_w) (Incze, 1998). This may partially explain why numbers of *S. carnosus* were not reduced to the same extent as those of *E. coli* O157:H7. In addition, *E. coli* O157:H7 was able to more extensively degrade the glucosinolates than the starter cultures (Fig. 5.1), and thus probably forms greater concentrations of isothiocyanates. These newly formed isothiocyantes are thought to quickly react upon the synthesizing organisms, causing bacterial death.

6.5 Conclusion

Cold yellow mustard flour was shown to decrease the levels of *E. coli* O157:H7 in dry fermented sausage (Graumann and Holley, 2008). It is assumed that this was at least partially the result of the conversion of sinalbin to ρ-HBIT by the bacteria present in the sausage, especially *E. coli* O157:H7 (Fig. 5.1 and Fig 5.2). In the present study, it was shown that *E. coli* O157:H7, *S. carnosus* and *P. pentosaceus* also have the ability to

degrade sinigrin. Comparison of MBC values showed that AIT was significantly more potent than ρ-HBIT against *E. coli* O157:H7, while the opposite was found for the starter cultures. Formation of isothiocyanates *in vitro* by myrosinase also demonstrated that AIT was more effective in killing *E. coli* than ρ-HBIT. These results suggest that cold black (*Brassica nigra*) or brown/oriental (*Brassica juncea*) ground mustard could be used in dry fermented sausage for the elimination of *E. coli* O157:H7. However, it should be noted that the levels of sinigrin in black (0.8%) (Rangkadilok, 2002a; Rangkadilok, 2002b) and brown/oriental mustard (0.8%) are usually significantly lower than the levels of sinalbin in yellow mustard (2.5%) (Zrybko et al., 1997).

It appears that when deodorized ground mustard (used as a binder/extender in cooked cured meats) is used in dry sausages it can be converted to an effective *E. coli* O157:H7 control agent. This pathogen itself acts as the switch to exert this control.

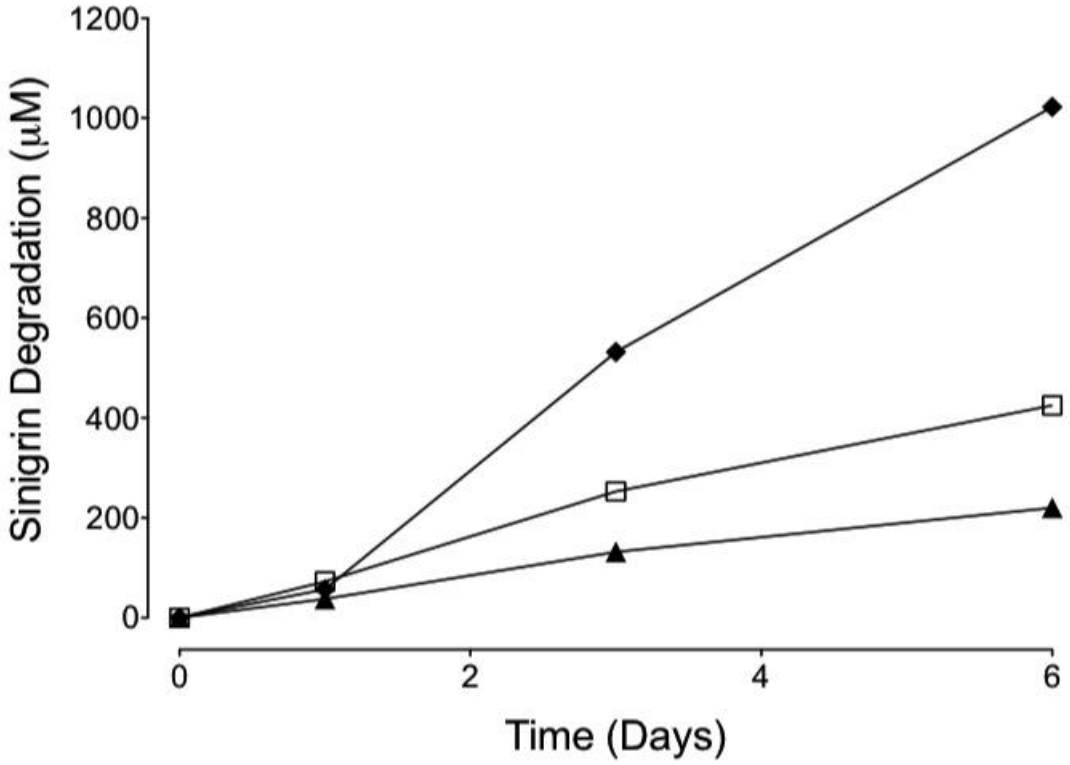


Fig. 6.1 - Degradation of sinigrin by *Staphylococcus carnosus* (□), *Pediococcus pentosaceus* (▲) and *E. coli* O157:H7 cocktail (◆). Reduction of sinigrin levels was analyzed by HPLC and quantified by comparison to a 2.5 mM sinigrin standard.

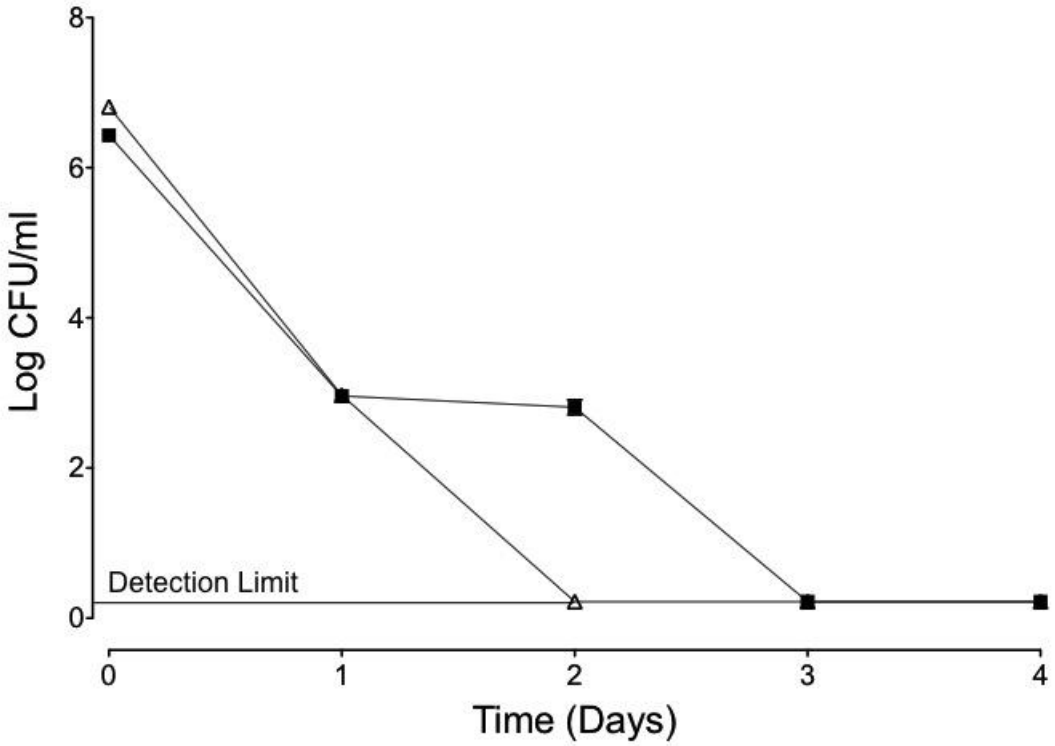


Fig. 6.2 - Population of *E. coli* O157:H7 in Miller-Hinton broth containing the dry ingredients used for the production of dry-fermented sausages. The bacteria were incubated (25^{o}C and 200 rpm) in the presence of myrosinase (0.2 U/tube) and either 2.5 mM of sinigrin (△) or 2.5 mM of sinalbin (■). Bacterial enumeration was done on VRB agar.

Table 6.1 - Minimum bactericidal concentration of AIT and ρ-HBIT against *E. coli* O157:H7, *S. carnosus* and *P. pentosaceus*. Hydrolysis products of ρ-HBIT were also tested against *E. coli* O157:H7.

Substance	Organism	MBC	Growth Inhibition ($p < 0.05$)
AIT	*E. coli* O157:H7	1.04 mM	0.26 mM
	Staphylococcus carnosus	1.04 mM	0.26 mM
	Pediococcus pentosaceus	20.80 mM	10.40 mM
ρ-HBIT	*E. coli* O157:H7	1.48 mM	0.59 mM
	Staphylococcus carnosus	0.59 mM	0.3 mM
	Pediococcus pentosaceus	5.92 mM	1.48 mM
ρ-HBA	*E. coli* O157:H7	NA*	20.14 mM
ρ-HBC	*E. coli* O157:H7	NA*	18.78 mM
SCN^-	*E. coli* O157:H7	NA*	NA*

*NA – concentration not achieved
Maximum concentration tested was 40 mM

Chapter 7

Multi-valent origin of the bactericidal activity of yellow mustard powder toward *Escherichia coli* O157:H7 during dry sausage ripening

7.1 Abstract

Bacteria were found to degrade glucosinolates and form isothiocyanates with antimicrobial activity towards *E. coli* O157:H7. In the present work, 24 cultures (mostly isolates from commercial mixtures) were screened for their capacity to decompose sinalbin. The most active pair of LAB + *Staphylococcus*, consisting of *Pediococcus pentosaceus* UM 121P and *Staphylococcus carnosus* UM 123M were selected and were used together for the production of dry fermented sausage contaminated with *E. coli* O157:H7 (~ 6.5 log CFU/g). They were compared to industrial starters used previously (*P. pentosaceus* UM 116P and *S. carnosus* UM 109M) and *E. coli* O157:H7 population was measured. Sausage batches containing 6% hot mustard powder (active myrosinase), 6% cold mustard powder (inactivated myrosinase), 6% autoclaved mustard powder (inactivated myrosinase) and no mustard flour (control) were prepared. Both pairs of starter cultures yielded similar results. Elimination of *E. coli* O157:H7 (>5 log CFU/g) occurred after 31 d in the presence of hot flour and in 38 d when the cold flour was added. Reductions > 5 log CFU/g of the pathogen did not occur (up to 38 d) in the control group. It was found that *E. coli* O157:H7 itself had a greater effect on sinalbin conversion than either pair of starter cultures, and glucosinolate degradation by the starter cultures did not play an important role in determining *E. coli* survival. The autoclaved powder

caused more rapid bactericidal action against *E. coli* O157:H7, yielding a > 5 log CFU/g reduction in 18 d. This may have been a result of the formation and/or release of antimicrobial substances by the autoclave process. Potentially, autoclaved mustard powder could solve an important safety issue of the meat industry within normal time of dry sausage production.

7.2 Introduction

Fermentation and drying are among some of the first methods used to preserve foods for extended storage. Dry fermented sausage has been traditionally produced using raw meat without thermal processing. The multiple hurdles present in this type of product are able to prevent or reduce the growth of spoilage bacteria, resulting in a shelf-stable food. These hurdles include: pH drop produced by the fermenting bacteria, commonly lactic acid bacteria (LAB); reduction of the water activity (a_w) during drying; and addition of antimicrobial compounds such as salt, nitrite and spices (Lucke, 1986). Unfortunately, some pathogenic organisms can overcome these barriers and survive in this type of product without causing sensory alteration. The most prominent of these pathogens and the focus of the present work is *E. coli* O157:H7. This organism has been shown to grow at low pH (4.0-4.5) (Buchanan and Bagi, 1994) and even survive in very acidic environments (pH 1.5-3.0) (Arnold and Kaspar, 1995). Moreover, *E. coli* O157:H7 has pronounced salt tolerance, being able to survive in sausage containing 4.4-4.8% NaCl (Hinkens et al., 1996; Riordan et al., 1998). Low a_w is another feature that prevents microbial spoilage in dry sausage, and *E. coli* requires a minimum a_w of 0.95 for proper growth (Sperber, 1983). A few studies demonstrated that the combination of low pH/low

a_w found in dry fermented sausage was able to significantly reduce the population of *E. coli* O157:H7 (1-2 log CFU/ml), but this was insufficient to eliminate the pathogen when present at high levels (Chacon et al., 2006b; Graumann and Holley, 2008).

The first reported outbreak involving *E. coli* O157:H7 in dry fermented sausage occurred in 1994, when this pathogen sickened 18 individuals after their consumption of pre-sliced dry salami (Tilden *et al.*, 1996). Other similar outbreaks have occurred where hundreds of people have been sickened and several deaths have been reported (Williams et al., 2000; MacDonald et al., 2004). These outbreaks led food regulatory agencies in both Canada (CFIA, 1999) and the US (Reed, 1995) to adopt very strict rules for the manufacture of dry fermented sausage products, requiring production processes to cause ≥ 5-log CFU/g reduction of the *E. coli* O157:H7 population. Presently, most of the processes used to assure the absence of this bacterium from dry fermented sausage rely on raw ingredient and final product testing, extension of the ripening period or cooking. However, cooking alters sensory properties and reduces the market value of these products. Extending storage increases inventory cost and is not an effective means for eliminating *E. coli* O157:H7 from dry sausage.

Mustard seeds and mustard powder have been used as common spices in fermented sausages. Both *Sinapis alba* (yellow or white mustard) and *Brassica juncea* (brown and oriental mustard), the main botanical species of mustard, contain high levels of glucosinolates. These are secondary metabolites that have their thioglucoside bond cleaved by an intrisic mustard enzyme, myrosinase (EC 3.2.1.147) in the presence of moisture, forming isothiocyanates plus thiocyanates, nitriles and some other minor

compounds. In yellow mustard, myrosinase forms ρ-hydroxybenzyl isothiocyanate (ρ-HBIT) from the principal glucosinolate present, sinalbin.

Recently, isothiocyanates and ground mustard have been tested in meat products for their ability to eliminate *E. coli* O157:H7 (Muthukumarasamy et al., 2003; Nadarajah et al., 2005; Chacon et al., 2006b). Both treatments significantly reduced the levels of the pathogen in meat products, including dry fermented sausage, but they can generate hot/pungent flavors in the final product (Chacon et al., 2006b). In the 90's, Canadian-based UFL Foods introduced a thermal process for inactivation of myrosinase in yellow mustard seeds. After grinding, the mustard powder (also known as cold or deodorized powder) yielded an ingredient with excellent emulsifying, bulking, stabilizing and thickening properties, but lacking the intense hot flavor normally caused in yellow mustard by ρ-HBIT (Cui and Eskin, 1998). This novel ingredient has been accepted and extensively used by the meat industry in cooked processed products because the powder improves water holding capacity, facilitates emulsion formation, has high protein content and is not expensive.

Very few studies have examined the potential for use of yellow mustard powder and/or ρ-HBIT to eliminate microbial pathogens from food products. Examination of the antimicrobial effects of purified ρ-HBIT has been difficult due to its significant instability (Kawakishi et al., 1967), and difficulties in the extraction of the essential oil (Buskov et al., 2000). In addition, very few commercial suppliers of sinalbin and ρ-HBIT are available, and usually the quantities sold are very limited. Ekanayake et al. (2006) showed that ρ-HBIT had significant bactericidal activity against several foodborne pathogens including *Escherichia coli*, *Staphylococcus aureus*, *Campylobacter jejuni*,

Pseudomonas aeruginosa, *Salmonella* Enteritidis, *Listeria monocytogenes*, *Shigella boydii* and *Clostridium perfringens* at concentrations between 0.35-2.13 mM.

Muthukumarasamy et al. (2004) tested the antimicrobial effect of hot (active myrosinase) and cold mustard powder on the growth of *E. coli* O157:H7. A 5-strain mixture of bacteria was inoculated in ground beef at 3 log CFU/ml and stored at 4^{o}C. The hot mustard powder eliminated the pathogen after 3 d when used at 20% (wt/wt), whereas 18 d were needed when it was added at 10%. The cold powder had no effect on the population of *E. coli* O157:H7. More recently, Graumann and Holley (2008) added yellow mustard powder to dry cured sausages in an attempt to kill *E. coli* O157:H7. When the hot and cold flour were tested at 6% (wt/wt), 24 d were required for the hot flour to reduce the population of the pathogen > 5 log CFU/g. Interestingly, 6% cold mustard powder showed an unexpected and significant reduction in *E. coli* O157:H7 viability to levels less than the detection limit (> 5 log CFU/ml reduction) in only 6 d. Since the cold powder contained no myrosinase activity, it could not have produced isothiocyanates, intrinsically. In a more recent study (Luciano and Holley, 2010a) it was hypothesized that bacterially-derived myrosinase activity was responsible for forming ρ-HBIT and perhaps killing *E. coli* (Fig. 5.1 and Fig 6.1). It was found that both the starter culture and the *E. coli* cocktail used (Graumann and Holley, 2008) degraded sinalbin, with decomposition capacity in the order of *E. coli* O157:H7 > *S. carnosus* UM 109M > *P. pentosaceus* UM 116P.

It is possible that *E. coli* degraded sinalbin in an attempt to acquire energy (glucose), and simultaneously formed ρ-HBIT. Another possibility is that the alterations in methabolism and protein synthesis at different stages of bacterial growth (stationary

phase) may produce enzymes with myrosinase-like activity. Observation of *in situ* formation of isothiocyanate by contaminating *E. coli* O157:H7 would represent discovery of a unique method for the targeted self-control of *E. coli* O157:H7 during the manufacture of dry fermented sausage. This could be an alternative to the utilization of hot mustard powder, minimizing organoleptic alterations caused by the formation of intense pungent flavor.

Apart from isothiocyanates, mustard seeds produce other metabolites with antimicrobial and antioxidant properties such as phenolic acids and phytic acid (Cui and Eskin, 1998). Extracts from deodorized yellow mustard powder were shown to have significant antioxidant activity, even in meat, and this was believed mainly due to the presence of phenolic compounds (Shahidi et al., 1992; Saleemi et al., 1993). These reports indicated that the major phenolics present in *Sinapis alba* were ρ-hydroxybenzoic acid and sinapic acid, which combined represented 36% of the total phenolics. Dabrowski and Sosulski (1984) reported even higher values. A variety of studies have reported that some processes applied to foods, such as cooking under pressure, can increase the levels of free phenolic acids in the final product (Gliszczynska-Swiglo et al., 2006; Rhandir et al., 2008; Ju et al., 2010). Gliszczynska-Swiglo et al. (2006) showed that steamed broccoli compared to raw not only increased the levels of phenolic acids, but also yielded higher amounts of vitamin E and β-carotene.

An objective of the present study was to screen two genera of lactic acid bacteria (LAB) and three *Staphylococcus carnosus* strains for myrosinase-like activity. From these, the best glucosinolate-degrading pair was selected for use together as a starter culture for the production of dry sausage. Yellow mustard powder was added to the

sausage batter as a source of the glucosinolate sinalbin, and the ability of the starter culture myrosinase-like activity to eliminate *E. coli* O157:H7 was investigated. For comparison, myrosinase in mustard powder was inactivated by autoclave (steam under pressure) treatment and the cold, deodorized product was used in parallel as a sausage ingredient. To better understand the origin of the bactericidal activity observed against *E. coli* O157:H7, the phenolic content and antioxidant properties of the mustard powders were examined.

7.3 Material and Methods

7.3.1 Bacterial strains

Dry fermented sausage was contaminated with a five-strain mixture of *E. coli* O157:H7, comprising 02-0628, 02-0627, 00-0351, 02-0304 and non-motile 02-1840 (non-pathogenic, human isolates). These strains were supplied by Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency, Canadian Centre for Human and Animal Health, Winnipeg, MB, Canada. *Staphylococcus carnosus* (UM 109M) and *Pediococcus pentosaceus* (UM 116P) were isolated (Holley and Blaszyk, 1998) from commercial lyophilized dry sausage starter culture preparations (Trumark LTII and Trumark LTIIM, respectively; Rector Foods Ltd., Mississauga, ON, Canada). In addition, *S. carnosus* UM 123M and *P. pentosaceus* UM 121P also used for dry sausage preparation were isolated from the commercial starter culture mixture Lactacel 115 (Microlife Technics, Sarasota, FL, USA). Sources of other bacteria used for screening of sinalbin degradation are listed in Table 7.1. Commercial mixtures RM53 and

Duploferment 66 were acquired from Rudolf Muller & Co., Giessen, Germany; Rosellac A was from Institute Rosell Inc., Montreal, QC, Canada; Fermentang BN-1005 was from B. Heller & Co., Bedford Park, IL, USA; Diversitech and Saga were also obtained from Rector Foods Ltd.; American Type Culture Collection (ATCC, Rockville, MD, USA), and the Centre for Food and Animal Research/Agriculture and Agri-Food Canada (CFAR/AAFC) Ottawa, ON strains of lactic acid bacteria were also used.

7.3.2 Chemicals

Myrosinase (EC 3.2.1.147), ferulic acid, tetrabutylammonium hydrogen sulfate (TBA), Trolox™, Folin-Ciocalteau reagent 2 M, 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); sinalbin was from C_2 Bioengineering (Copenhagen, DK); ρ-HBIT was from Toronto Research Chemicals (North York, ON, Canada); methanol and acetonitrile HPLC grade were from Fisher Scientific Co. (Fair Lawn, NJ, USA). Other chemicals used were of analytical grade.

7.3.3 Screening for degradation of sinalbin

LAB was grown in deMan, Rogosa and Sharpe (MRS) broth (Oxoid, Unipath, Nepean, ON, Canada) and *Staphylococcus carnosus* grew in tryptic soy broth (TSB) (Oxoid) prior to the experiments (18h at 35^{o}C). Then 0.1 ml of each individual strain was inoculated into 9.9 ml Muller-Hinton broth (MHB) (Oxoid) containing 2.5 mM sinalbin (the method was adapted from Brabban and Edwards, 1994). Glucosinolate degradation at 25^{o}C was evaluated by comparing the levels of sinalbin at day 0 and 6. Separation and quantification of sinalbin was performed using an HPLC equipped with a C18 column

(4.6 x 250 mm i.d. 5 μm; Waters Co., Milford, MA, USA). Elution was carried out isocratically for 20 min at a flow rate of 1 ml/min, using a solvent system containing 20% (v/v) acetonitrile and 80% water + 0.02M tetrabutylammonium hydrogen sulfate (pH 5.5). The injection volume used was 10 μL. A UV detector was used to measure the absorbance at 227 nm in order to verify and quantify the presence of sinigrin. The selected starter culture combination was identified as "Starter Culture B" (*P. pentosaceus* UM 121P and *S. carnosus* UM 123M) and the starter culture mixture used by Graumann and Holley (2008) was named "Starter Culture A" (*P. pentosaceus* UM 116P and *S. carnosus* UM 109M).

7.3.4 Preparation of starter cultures and *E. coli* O157:H7 for production of dry sausage

The dry fermented sausage was manufactured using the method of Graumann and Holley (2008) with some adaptations. Briefly, both *S. carnosus* and *E. coli* O157:H7 strains were grown in TSB, whereas *P. pentosaceus* was cultured in MRS broth. Overnight cultures were transferred to fresh broth and incubated at 35^{o}C for 16h in 500 ml flasks. The cultures were spun (Sorvall Instruments RC-5C; DuPont, Newton, CN, USA) for 20 min at 5,000 rpm/4225 x *g* (Sorvall Instrumements SLA-3000 rotor). The supernatant was discarded and the bacterial pellet washed with 0.1% (wt/vol) peptone (Fisher Scientific Co.), and spun again. The supernatant was discarded once again and cultures were resuspended in 50 ml of 0.1% peptone. Starter cultures were mixed resulting in 100 ml inoculum that was used for the production of the sausages. A 50 ml *E. coli* O157:H7 cocktail was used as the contaminant inoculum.

7.3.5 Dry fermented sausage manufacture

Batches of sausage containing "Starter culture A" or "Starter Culture B" were produced on alternate weeks. On each day of production 3 treatments were applied: Control (no mustard powder), 6% cold yellow mustard powder and 6% hot yellow mustard powder (*Sinapis alba* L., G.S. Dunn Ltd., Hamilton, ON, Canada). All treatments were repeated 3 times for each of the two starter culture mixtures. A total of 10 kg of salami batter was produced for each batch of sausage. Fresh lean pork trim, pork back fat and lean beef trim were purchased bi-weekly from a local butcher shop. They were cut or assembled in ~ 400g portions and frozen (-18^{o}C) until used. The pork and beef meats were tempered overnight at 5^{o}C prior to production. Fat (17.55%), pork trim (60.63%) and beef (17.55%) were added in decreasing order of fatness to a prechilled (1 to 2^{o}C) rotating bowl chopper (Titane 40, Dadaux, Bersaillin, France). The frozen pork fat was chopped to ≥ 3 mm particles and the bacteria were added. The *P. pentosaceus* and *S. carnosus* mixture and *E. coli* O157:H7 cocktail inocula were added to yield approximately 8, 6, and 6.5 log CFU/g, respectively. The beef and pork trim were added almost simultaneously, and were chopped until 3 mm granules were produced. Then, the dry ingredients were added: salt (2.91% wt/wt; HyGrade, Sifto Canada Corp., Mississauga, ON, Canada), D-glucose (0.60% wt/wt; Sigma Chemical Co.), Cervelat spice mixture code C719 (0.44% wt/wt; Wiberg Corp., Oakville, ON, Canada), pickle cure concentrate as a source of nitrate (0.31% wt/wt; Canada Compound Corp., Winnipeg, MB, Canada) and 0.05% (wt/wt) sodium erythorbate (Canada Compound Corp.). Yellow mustard powder was added near the end of the chopping process at levels

of 6% (wt/wt). The sausage batter was transferred to a pre-cooled vacuum stuffer (VF 608, Handtmann, Waterloo, ON, Canada) and mechanically stuffed into water-softened 55 mm diameter fibrous casings (Kalle GmbH, Wiesbaden, Germany), resulting in sausages of approximately 500 g each. These sausages were then hung on horizontal aluminum sticks and placed into a single rack automated smokehouse (ASR 1495 EL/ WA) with a programmable temperature, relative humidity (RH) and pH controller (Titan, Maurer AG, Reichenau, Germany). During fermentation sausages were periodically smoked (2h total). The temperature was initially set at 26^{o}C for a fast fermentation, and the temperature was programmed to drop by 2^{o}C every 24h until 20^{o}C was reached. Subsequent 2^{o}C decrements occurred every 12 h to 14^{o}C. The RH was initially set at 88% and was decreased to 80% during the first 24h. Another 2% RH decrease occurred after 24h and the RH was maintained between 75-78% for the remainder of ripening. Sausages were moved to a second temperature and RH-controlled single rack smokehouse (AFR-Fishmaster "Roundair"; Rauch und Warmetchnik GmbH & Corp., Reichenau, Germany) when the smoking cycles were finished and sausages were dried for 34 d at 14^{o}C and 75% RH. The total production time was 38 d.

7.3.6 Autoclaved yellow mustard powder

The autoclaved mustard powder was produced using commercial hot ground mustard (G.S. Dunn Ltd.), which was added to a metal tray to form a 3 cm layer and covered with aluminum foil. The powder was then autoclaved for 15 min at 115^{o}C. Stability of sinalbin levels when the flour was added to water (3h) confirmed lack of myrosinase activity (result not shown) and sustained levels of sinalbin (Appendix 7.1).

This flour was added to the salami batter at 6% (wt/wt). A powder containing 50% autoclaved powder and 50% hot powder (auto + hot) was also tested in sausage formulations at the same 6% (wt/wt) concentration in the batter. Sausages containing the autoclaved powder and the auto + hot powder were only formulated with "Starter Culture A".

7.3.7 Dry sausage sampling and analysis

Sausages were selected at different intervals during ripening for microbial and physiochemical analyses. The sausage batter was directly sampled after formulation at day 0 and sausages were tested on days 6, 12, 18, 24, 31 and 38. A 25 g sample was aseptically removed from the core of the sausage using sterilized utensils and placed into a stomacher bag (Filtra-bag, VWR, Edmonton, AB, Canada). The sample was homogenized in 225 ml 0.1% peptone (BagMixer 400, Intersciences Inc., Markham, ON, Canada) for 1.5 min. Serial dilutions from 10^{-2} to 10^{-5} were prepared by pipetting 1 ml of the stomached sample (10^{-1}) into glass tubes containing 9 ml 0.1% peptone (wt/vol). Samples were then plated with an Autoplate 4000 Spiral Plater (Spiral Biotech, Bethesda, MD, USA). *P. pentosaceus* was plated on MRS agar (Oxoid); *S. carnosus* on mannitol salt agar (MSA; Oxoid); and *E. coli* O157:H7 on sorbitol MacConkey agar (Oxoid) with cefixime and tellurite supplement added (ctSMAC; Oxoid). Inoculated plates were incubated at 35^{o}C for 24-48 h. A selective enrichment using immunomagnetic separation with Dynabeads (Dynal Biotech, Oslo, Norway) was used when *E. coli* O157:H7 populations < 0.62 log CFU/g (minimum detection level). The method for immunomagnetic separation was performed according to the manufacturer's instructions.

Water activity of sausages was measured using a Novasina AW-Sprint Machine (Axion AG, Pfaffikon, Switzerland). In addition, 20 g samples were homogenized with 180 ml sterilized distilled water in stomacher bags, and pH was analyzed (Accumet Basic pH meter; Denver Instrument Co., Denver, CO, USA).

7.3.8 Total phenolic content and antioxidant activity

Samples (200 mg) of cold, hot or autoclaved mustard powder were extracted in 4 mL methanol/water (50:50 v/v) for 30 min in an ultrasonic bath (B-3200R-2, Bransonic® Cleaning Equipment Company, Shelton, CT, USA) for determination of total phenolic content (adapted from Gliszczynska-Swiglo et al., 2006). The samples were spun at 3000 rpm/1643 x *g* (GLC-1 centrifuge, Sorvall, Newton, CT, USA) for 10 min. The supernatant was diluted 10 times before analysis due to the very high phenol content. Total phenol content was determined using the Folin–Ciocalteau method (Gao et al., 2002). Briefly, 0.2 ml aliquots of the extracts were added to 1.5 mL fresh 10-fold diluted Folin–Ciocalteau reagent. The mixture was held for 5 min before the addition of 1.5 ml sodium carbonate solution (60 g/L). The mixture was kept at room temperature for 90 min and the absorbance was read at 725 nm. The extraction solvent (methanol/water, 50:50 v/v) was used as a blank and samples were compared to a standard curve using ferulic acid. Results were expressed in mg ferulic acid equivalents per 100 g of sample. The linearity range of the calibration curve was 0–200 mg ($r = 0.99$).

Antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (modified from Chen and Ho, 1995). A 0.3 g mustard powder sample was extracted with methanol (3 ml) for 30 min in an ultrasonic bath. This solution was

centrifuged for 10 min at 3000 rpm/1643 x *g* (GLC-1 centrifuge, Sorvall). Then a 10-fold dilution of the supernatant was made prior to the analysis (necessary because of the high antioxidant levels found in the mustard powder). A 0.1 ml aliquot was added to 3.9 ml of a 6.34 x 10^{-5} M DPPH solution. The absorbance was recorded at 0 and 30 min at 515 nm (Ultraspec 200, Pharmacia Biotech Piscataway, NJ, USA). The results were expressed as a percent of discoloration according with the formula:

$$\text{Antioxidant activity} = [1 - (\text{Absorbance}_{515nm} \text{ sample t=30min})/(\text{Absorbance}_{515nm} \text{ sample t=0min})]$$

A standard curve using Trolox™ was prepared (25 to 800 μM in methanol) in parallel with the samples. Results were expressed as μM of Trolox equivalents per 100 g of sample.

7.3.9 Statistical analyses

The data reported were analyzed by one-way analysis of varience (ANOVA) and statistical differences among treatments were compared using Tukey's test. All values presented are averages from a minimum of 3 experiments conducted in triplicate (n=9). Data are represented by means ± SEM. A *P* value of 0.05 was used as the cut-off for statistical significance.

7.4 Results and Discussion

7.4.1 Bacterial screening

Recently it was found that *E. coli* O157:H7 and some starter cultures possessed myrosinase-like activity, and were able to degrade sinalbin and form ρ-HBIT (Fig. 5.1 and Fig. 5.3). In the present study, 24 different isolates mainly from commercial starter cultures with some from meat or from the ATCC were screened for their capacity to decompose sinalbin. Of these, 7 strains were *P. pentosaceus*, 6 were *P. acidilactici*, 4 were *Lactobacillus plantarum*, 4 were *L. curvatus* and 3 were *S. carnosus* (Table 7.1). Results showed that most of the bacteria examined were able to cause sinalbin degradation to some extent. However, no strain was able to cause as high an amount of sinalbin decomposition as found for the *E. coli* O157:H7 cocktail (507.9 μM) (Fig. 5.1). Coincidently, the LAB and *S. carnosus* strains with higher myrosinase-like activity were isolated from the same commercial starter culture marketed as Lactacel 115. When compared to the starter culture mixture used by Graumann and Holley (2008), the selected *S. carnosus* UM 123M had a slightly higher myrosinase-like activity (250.3 μM) than the *S. carnosus* UM 109M (220.5 μM) (Fig. 5.1), whereas the newly-chosen *P. pentosaceus* UM 121P caused much greater degradation of sinalbin (282.8 μM) than *P. pentosaceus* UM 116P (16.4 μM) (Fig. 5.1). Potentially the selected starter culture mixture (B) had a > 2-fold greater ability to decompose sinalbin than the starter culture mixture (A) used by Graumann and Holley (2008). Both paired starters were compared for their ability to eliminate ~ 6.5 log CFU/g viable *E. coli* O157:H7 during sausage ripening.

Degradation of glucosinolates by LAB has been previously reported (Krul et al., 2002; Cheng et al., 2004; Luciano and Holley, 2010a,b). Palop et al. (1995) have also shown that *Lactobacillus agilis* R16 was able to degrade sinigrin and produce glucose

plus allyl isothiocyanate as end products, suggesting that this organism had myrosinase-like activity. It was recently found that this reaction could be harmful to *E. coli* O157:H7 when the latter was grown under adverse conditions (Fig. 5.2). However, it was uncertain whether sufficient isothiocyanate was produced by the starter cultures to be fatal to *E. coli* O157:H7 or if the lethal effect was caused by myrosinase-like activity provided by *E. coli* itself.

7.4.2 Effect of starter cultures and different yellow mustard powders on the microbiological populations and physico-chemical characteristics of dry sausage

When sausage batches containing hot mustard powder (active myrosinase), cold mustard powder (inactivated myrosinase), autoclaved powder (inactivated myrosinase) and no mustard flour (control) were prepared, both pairs of starter cultures yielded similar results. The a_w values were quite similar among most treatments (Table 7.2), but with the autoclaved powder the a_w was significantly higher. Structural changes in this powder may have occurred leading to its higher water-binding capacity. Hampton et al. (1975) reported that the combination of steam + pressure was able to inactivate amylases of wheat, yielding a higher degree of integrity in the starch molecules, which led to better water-binding capacity. High temperatures are usually necessary to inactivate amylases, and this could be why the sausages containing autoclaved mustard powder had higher a_w during ripening. Since the supplier did not disclose the industrial process used for deheating mustard, it is possible that a temperature was used that deactivated myrosinase, but was not sufficient to destroy amylase activity. Van Eylen et al. (2006) found that 10 min at 75^{o}C or 30 min at 72.5^{o}C were needed to totally inactivate myrosinase in yellow

mustard, and amylases were usually resistant to these temperatures (Hampton et al., 1975).

Differences were found among the pH values of sausages, depending on the presence or absence of mustard powder (Table 7.3). These differences were not influenced by the type of starter culture and were probably a result of the carbohydrate content present in mustard. Graumann and Holley (2008) found a similar profile. In addition, the ultimate pH drop for all sausages was achieved within the first week and the pH difference between the "cold mustard powder" and control groups did not seem to have a significant effect on the bacterial population, at least until day 24.

Levels of *P. pentosaceus* were stable throughout the experiments (Table 7.4), ranging from about 7.5 to 8.7 log CFU/g. These numbers were maintained even in the presence of isothiocyanate produced by myrosinase in the mustard powder itself (hot and auto+hot powders). It has been shown that *P. pentosaceus* has a 10-fold greater resistance to allyl isothiocyanate and ρ-HBIT than *E. coli* O157:H7 and *S. carnosus* (Table 6.1). This provides an opportunity to use isothiocyanates as antimicrobial agents in dry fermented sausages, since they do not affect the performance of the acidifying bacteria at levels that are bactericidal to *E. coli* O157:H7. Unfortunately, the presence of isothiocyanate from the hot powder also affected the viability of both strains of *S. carnosus* (Table 7.5). The cold powder significantly decreased the population of *S. carnosus* UM 123M (Starter Culture B), whereas the viability of *S. carnosus* UM 109M (Starter Culture A) was maintained. This could have been a result of the greater degradation of glucosinolates by the UM 123M strain, which could compromise its survival. More interestingly, both auto and auto+hot powders caused a ~ 3.5 log CFU/g

drop in the *S. carnosus* UM 109M population (Table 7.5), suggesting that the autoclaved powder may contain other or higher levels of substances with antimicrobial activity against this bacterium.

A reduction of *E. coli* O157:H7 viability by > 5 log CFU/g occurred after 31 d in the presence of hot flour and 38 d when the cold flour was added (Table 7.6). The same extent of pathogen reduction in the control group did not occur within 38 d. The results for the hot powder addition were only slightly different from those observed by Graumann and Holley (2008), but the outcome of cold powder addition to the dry sausages was very different. The latter authors found a > 5 log CFU/g reduction in 6 d and the only major variance between their experiment and the present one was the source of mustard powder. Differences in mustard variety and/or processing to inactivate myrosinase could be the reasons for such a difference.

Sausages containing cold powder and the "Starter Culture B" showed a significant difference in the reduction of *E. coli* O157:H7 in comparison to the same treatment with "Starter Culture A" on day 38. Although this difference was statistically significant, it did not seem that the much higher degradation of sinalbin by "Starter Culture B" played a major role in causing *E. coli* O157:H7 death. It is very well known that ρ-HBIT is extremely reactive (Kawakishi et al., 1967; Choubdar et al., 2010), and therefore, could interact with different meat components (Fig. 3.1) or against the starter cultures themselves before it could influence *E. coli* O157:H7 survival. It has also been reported that *E. coli* O157:H7 has intracellular myrosinase-like activity (Fig. 5.3), which could lead to formation of the isothiocyanate within the cell and, consequently, cause rapid inhibition of metabolic activity (Kojima and Ogawa, 1971; Luciano and Holley, 2009)

and destruction of cellular components, i.e. cell membrane (Lin et al., 2000; Ahn et al., 2001).

The autoclaved powder and the combination of autoclaved plus hot powders showed a much more pronounced capacity for killing *E. coli* O157:H7 (Table 7.6). This bacterium was reduced to levels below the detection limit (0.62 log CFU/g) of this experiment within 18 d for the auto+hot powder and 24 d for the autoclaved powder alone (although complete elimination did not occur; *E. coli* O157:H7 presence was confirmed by immunomagnetic separation and enrichment). However, a 5 log CFU/g reduction was found for both powders after 18 d, and if used as a sausage ingredient, they would bring the process in compliance with Canadian and US regulations for the production of dry fermented sausage.

7.4.3 Total phenolics and antioxidant activity

Results suggested that the autoclave process was able to release and/or form compounds with antimicrobial activity that had a synergistic or additive effect on the bactericidal activity of ρ-HBIT. Levels of sinalbin were essentially identical for the cold and autoclaved mustard powders (Appendix 7.1), suggesting another agent(s) was involved in causing the death of *E. coli* O157:H7 and *S. carnosus*. Several studies have reported that steam treatment was able to release phenolic acids from biological material, yielding greater antioxidant activity; i.e. mushrooms (Ju et al., 2010), broccoli (Gliszczynska-Swiglo et al., 2006), wheat, buckwheat, corn and oats (Rhandir et al., 2008). The antimicrobial activity of phenolic acids against various bacteria, including *E. coli* O157:H7, has been previously reported (Lacombe et al., 2010; Cueva et al., 2010).

When the autoclaved, cold and hot mustard powders were tested for their total phenolic content, the autoclaved powder was found to have the highest level of phenols [25.31±1.41 mg Ferulic Acid Equivalent (FAE)/g of powder], followed by the hot powder (23.53±1.92 FAE/g) and lastly the cold powder (20.91±0.94 FAE/g). Although the autoclaved powder possessed the greatest amount of phenols, the level was only significantly higher than that in the cold mustard powder ($P < 0.05$). This could be one of the reasons for the stronger antimicrobial effect of the autoclaved powder towards *E. coli* O157:H7. In addition, the phenolic content could be the reason for the more intense antioxidant activity found in the autoclaved [1.26±0.13 mmol of Trolox Equivalent (TE)/100g of powder] and hot mustard powders (1.23±0.15 mmol TE/100g) in comparison to the cold powder (0.96±0.09 mmol TE/100g). Dobrowski and Sosulski (1984) showed that mustard contained one of the highest levels of phenolics among 10 different oilseeds, and mustard powder has also been used to retard the oxidation of meat products (Shahidi et al., 1992). The low pH found in the dry sausage environment could also influence the antimicrobial activity of the mustard powders. Results in Table 7.3 show that sausages containing mustard presented a significantly lower pH (~ pH 4.5-4.6) than the control (~ pH 4.8). Phenolic acids were shown to have higher antimicrobial activity when at pH values that were approximate to their pKa (Wen et al., 2003). Sinapic acid and ρ-hydroxybenzoic acid were found to be the major phenolic acids in mustard (Dabrowski and Sosulski, 1984) and present pKa values of 4.47 and 4.58, respectively. These values coincide very well with the pH achieved in the mustard-treated sausages and it could be another factor to explain the better antimicrobial activity of the autoclaved

powder against *E. coli* O157:H7 in comparison to the other powders (lower phenolic content) and control.

Some of the issues that might be predicted for sausages containing autoclaved flour are oxidation and discoloration due to the lower population of *S. carnosus* (Papamanoli et al., 2002). One of the main reasons for the addition of this bacterium to the sausage batter is its capacity to produce catalase, which degrades the hydrogen peroxide produced by catalase negative LAB (Mauriello et al., 2004). Besides causing rancidity, hydrogen peroxide can react with myoglobin and cause discoloration of meat (Hugas and Monfort, 1997). However, the autoclaved mustard powder was shown to contain high levels of antioxidants, which could probably inactivate the hydrogen peroxide, preventing alteration in color or rancidity.

7.5 Conclusion

The diverse LAB and *S. carnosus* screened had the ability to degrade sinalbin to some extent. The most active pair of LAB + *S. carnous* was selected and used to produce dry fermented sausage. However, it did not seem that starter culture myrosinase-like activity played a very significant role in the elimination of *E. coli* O157:H7. This pathogenic bacterium previously was found to decompose much higher amounts of glucosinolates than the starter cultures (Fig. 5.1 and Fig. 6.1), which could be the major factor contributing to its own destruction when cold yellow mustard powder was present. *E. coli* O157:H7 is faced with a variety of hurdles in the dry sausage environment, which make it a weak competitor for nutrients in comparison to *S. carnosus*, and especially *P.*

pentosaceus. The pathogen may then use sinalbin as a source of energy and inadvertently form ρ-HBIT, which is lethal (Luciano and Holley, 2010b).

The autoclaved mustard powder was found to be more effective in killing *E. coli* O157:H7 than the cold and hot powders alone. The autoclave process was found to sustain the levels of glucosinolate in the powder (Appendix 7.1), but it increased the levels of phenolic acids. The latter compounds also exhibit antimicrobial activity, and may have acted simultaneously with the bacterially-formed isothiocyanate to kill *E. coli* O157:H7. It is also possible that other substances were formed or released during the autoclave process which could have contributed to the anti-*E. coli* activity of the powder. Rufian-Henares and Morales (2006) reported that Maillard reaction products have antimicrobial activity. The autoclaved mustard powder definitely had a visually darker color than either cold or hot powders, and the autoclave process could also have formed Maillard reaction products. In summary, both autoclaved and mixed autoclaved+hot powders were able to cause a > 5 log CFU/g reduction in *E. coli* O157:H7 viability within a suitable period (30 d) for commercial dry sausage production (Graumman and Holley, 2008). However, further studies using one or more shiga-toxin producing *E. coli* O157:H7 strains that caused human illness from contaminated dry sausage are necessary for validation and before mustard powder can be used industrially for this purpose (Health Canada, 2000).

Table 7.1 – Differences in myrosinase-like activity of single LAB and *S. carnosus* strains monitored by sinalbin degradation (μM) at 25°C for 6d.

Bacterial Strain	Source	Sinalbin Degradation
L. curvatus #10	CFAR/AAFC – cured meat isolate	60.7
L. curvatus #15	CFAR/AAFC – meat isolate	38.6
L. curvatus ATCC 25601	ATCC – milk isolate	115.9
L. curvatus UM133	RM53*	135.8
L. plantarum UM131L	Rosellac A*	99.8
L. plantarum UM134L	Duploferment 66*	-
L. plantarum UM135L	Duploferment 66**	15.0
L. plantarum BN1005	Fermentang BN-1005*	-
P. acidilactici UM104P	Diversitech LPH*	-
P. acidilactici UM119P	Diversitech 4P1*	-
P. acidilactici UM129P	Rosellac A*	79.0
P. acidilactici 122P	Lactacel 115*	-
P. acidilactici ATCC 8081	ATCC – fermented milk isolate	-
P. acidilactici #1228	Unknown	73.0
P. pentosaceus 2 UM102	Diversitech LPH*	125.6
P. pentosaceus 2 UM105	Trumark LT II*	162.3
P. pentosaceus 2 UM108	Saga*	142.8
P. pentosaceus 2 UM113	Diversitech LP*	146.6
P. pentosaceus 2 UM114	Trumark LT II*	106.1
P. pentosaceus UM121P	Lactacel 115*	282.8
P. pentosaceus UM127P	Rosellac A*	15.4
S. carnosus UM123M	Lactacel 115*	250.3
S. carnosus UM135M	RM53*	151.5
S. carnosus UM136M	Duploferment 66*	175.1

*Commercial meat starter culture isolate
**Same as *L. plantarum* 134L, but isolated at a different period in time
- no activity detected

Table 7.2 – Changes in a_w values during fermentation and drying of raw dry sausage. Results represent control (no mustard added) and treatments with yellow mustard powder (cold, hot, auto and auto+hot) at 6% (wt/wt).

Time (d)	Starter Culture A					Starter Culture B		
	Control	Cold	Hot	Auto	Auto/Hot	Control	Cold	Hot
0	0.955±0.002^{A}	0.957±0.002^{A}	0.954±0.003^{A}	0.959±0.001^{A}	0.960±0.001^{A}	0.958±0.004^{A}	0.953±0.003^{A}	0.955±0.002^{A}
6	0.937±0.002^{B}	0.935±0.003^{B}	0.933±0.002^{B}	0.944±0.002^{AB}	0.940±0.001^{AB}	0.938±0.003^{AB}	0.948±0.003^{A}	0.936±0.001^{B}
12	0.924±0.005^{A}	0.924±0.005^{A}	0.922±0.005^{A}	0.928±0.002^{A}	0.926±0.002^{A}	0.908±0.003^{AB}	0.914±0.002^{AB}	0.902±0.006^{B}
18	0.890±0.005^{ABC}	0.893±0.002^{ABC}	0.882±0.001^{BC}	0.909±0.007^{A}	0.904±0.006^{AB}	0.879±0.004^{C}	0.894±0.005^{ABC}	0.885±0.003^{BC}
24	0.866±0.005^{B}	0.868±0.003^{B}	0.869±0.002^{B}	0.889±0.002^{A}	0.877±0.004^{AB}	0.860±0.004^{B}	0.873±0.004^{AB}	0.858±0.004^{B}
31	0.850±0.004^{B}	0.843±0.004^{BC}	0.836±0.004^{BC}	0.876±0.003^{A}	0.856±0.003^{AB}	0.830±0.006^{C}	0.839±0.007^{BC}	0.827±0.003^{C}
38	0.831±0.004^{B}	0.831±0.002^{B}	0.825±0.002^{BC}	0.857±0.002^{A}	0.839±0.002^{AB}	0.815±0.002^{C}	0.826±0.007^{BC}	0.815±0.004^{C}

Values represent mean ± standard error of three trials replicated three times (n=9).
Different letters represent a significant difference ($P < 0.05$) among treatments in the same row.
Cold contains commercial yellow mustard powder (YMP) with inactivated myrosinase; Hot contains commercial YMP with active myrosinase; Auto contains Hot commercial YMP with myrosinase inactivated by autoclave treatment; and Auto + Hot is a mixture of equal volumes of Auto and Hot powders.
"Starter Culture A" contained *P. pentosaceus* UM 116P and *S. carnosus* UM 109M; "Starter Culture B" contained *P. pentosaceus* UM 121P and *S. carnosus* UM 123M.

Table 7.3 – Changes in pH values during fermentation and drying of raw dry sausage. Results represent control (no mustard added) and treatments with yellow mustard powder (cold, hot, auto and auto+hot) at 6% (wt/wt).

Time (d)	Starter Culture A					Starter Culture B		
	Control	Cold	Hot	Auto	Auto/Hot	Control	Cold	Hot
0	5.90±0.03^{A}	5.95±0.03^{A}	5.82±0.04^{A}	5.90±0.05^{A}	5.90±0.02^{A}	5.98±0.05^{A}	5.95±0.05^{A}	5.99±0.04^{A}
6	4.84±0.02^{A}	4.66±0.01^{BC}	4.57±0.01^{C}	4.55±0.02^{C}	4.51±0.02^{C}	4.83±0.07^{AB}	4.60±0.02^{C}	4.51±0.02^{C}
12	4.92±0.05^{A}	4.58±0.01^{B}	4.53±0.01^{B}	4.53±0.05^{B}	4.50±0.04^{B}	4.90±0.07^{A}	4.65±0.01^{B}	4.59±0.02^{B}
18	4.90±0.01^{A}	4.65±0.03^{B}	4.60±0.02^{B}	4.56±0.03^{B}	4.52±0.02^{B}	4.96±0.10^{A}	4.65±0.03^{B}	4.59±0.03^{B}
24	5.00±0.03^{A}	4.71±0.01^{B}	4.69±0.01^{BC}	4.59±0.02^{C}	4.58±0.02^{C}	5.01±0.06^{A}	4.68±0.02^{BC}	4.62±0.01^{BC}
31	4.99±0.03^{A}	4.68±0.01^{B}	4.70±0.03^{B}	4.64±0.03^{B}	4.59±0.01^{B}	4.95±0.06^{A}	4.68±0.01^{B}	4.62±0.02^{B}
38	4.97±0.03^{A}	4.66±0.01^{B}	4.66±0.02^{B}	4.65±0.01^{B}	4.64±0.01^{B}	5.01±0.08^{A}	4.66±0.01^{B}	4.63±0.01^{B}

Values represent mean ± standard error of three trials replicated three times (n=9).
Different letters represent a significant difference ($P < 0.05$) among treatments in the same row.

Table 7.4 – Number (Log CFU/g ± SE) of *P. pentosaceus* recovered during the production of dry fermented sausage. Results represent control (no mustard added) and treatments with yellow mustard powder (cold, hot, auto and auto+hot) at 6% (wt/wt).

Time (d)	Starter Culture A					Starter Culture B		
	Control	Cold	Hot	Auto	Auto/Hot	Control	Cold	Hot
0	7.96 ± 0.02^{A}	7.93 ± 0.04^{AB}	7.89 ± 0.04^{AB}	7.90 ± 0.03^{AB}	7.85 ± 0.02^{AB}	7.80 ± 0.10^{AB}	7.62 ± 0.18^{B}	7.80 ± 0.11^{AB}
6	8.24 ± 0.04^{BC}	8.24 ± 0.05^{BC}	8.67 ± 0.03^{A}	8.40 ± 0.02^{AB}	8.41 ± 0.02^{AB}	8.07 ± 0.06^{BC}	7.99 ± 0.20^{C}	8.41 ± 0.09^{AB}
12	8.27 ± 0.03^{CD}	8.16 ± 0.03^{D}	8.48 ± 0.04^{AB}	8.38 ± 0.02^{BC}	8.44 ± 0.03^{ABC}	8.12 ± 0.07^{D}	7.94 ± 0.19^{D}	8.64 ± 0.07^{A}
18	8.12 ± 0.03^{BC}	8.13 ± 0.03^{BC}	8.56 ± 0.03^{A}	8.12 ± 0.03^{BC}	8.03 ± 0.05^{CD}	7.59 ± 0.10^{E}	7.77 ± 0.09^{DE}	8.32 ± 0.12^{AB}
24	7.94 ± 0.07^{BC}	7.80 ± 0.10^{C}	8.22 ± 0.08^{AB}	7.91 ± 0.04^{C}	8.24 ± 0.03^{AB}	7.93 ± 0.08^{BC}	7.82 ± 0.18^{C}	8.45 ± 0.07^{A}
31	7.97 ± 0.07^{BC}	8.18 ± 0.06^{BC}	8.67 ± 0.04^{A}	8.05 ± 0.05^{BC}	8.27 ± 0.08^{B}	7.87 ± 0.05^{C}	7.52 ± 0.25^{C}	8.01 ± 0.18^{BC}
38	8.11 ± 0.03^{BC}	8.08 ± 0.08^{BCD}	8.72 ± 0.10^{A}	7.74 ± 0.09^{DE}	8.35 ± 0.07^{B}	7.52 ± 0.09^{E}	8.16 ± 0.26^{BC}	7.91 ± 0.15^{CD}

Values represent mean ± standard error of three trials replicated three times (n=9).
Different letters represent a significant difference ($P < 0.05$) among treatments in the same row.

Table 7.5 - Number (Log CFU/g ± SE) of *S. carnosus* recovered during the production of dry fermented sausage. Results represent control (no mustard added) and treatments with yellow mustard powder (cold, hot, auto and auto+hot) at 6% (wt/wt).

Time (d)	Starter Culture A					Starter Culture B		
	Control	Cold	Hot	Auto	Auto/Hot	Control	Cold	Hot
0	6.16±0.11^{AB}	6.54±0.06^{A}	6.46±0.04^{A}	6.23±0.04^{AB}	6.20±0.03^{AB}	6.54±0.04^{A}	6.32±0.09^{AB}	5.87±0.20^{B}
6	5.94±0.10^{A}	5.92±0.08^{A}	5.21±0.20^{CD}	5.49±0.03^{BC}	4.92±0.10^{D}	6.14±0.05^{A}	5.97±0.06^{A}	5.95±0.03^{A}
12	6.08±0.05^{A}	5.89±0.06^{AB}	5.09±0.30^{CD}	5.01±0.12^{CD}	4.57±0.09^{D}	6.08±0.09^{A}	5.32±0.19^{BC}	5.96±0.04^{A}
18	6.12±0.06^{A}	5.97±0.08^{AB}	5.11±0.23^{C}	3.89±0.07^{D}	3.97±0.16^{D}	6.16±0.06^{A}	5.23±0.19^{C}	5.62±0.06^{BC}
24	5.50±0.19^{AB}	5.84±0.11^{A}	4.80±0.25^{B}	3.48±0.24^{C}	2.44±0.21^{D}	5.99±0.02^{A}	5.26±0.18^{AB}	5.50±0.08^{AB}
31	5.88±0.06^{A}	5.69±0.07^{A}	4.82±0.33^{C}	3.54±0.14^{C}	2.63±0.11^{D}	5.93±0.06^{A}	4.69±0.29^{B}	4.54±0.24^{B}
38	6.12±0.06^{A}	5.93±0.09^{A}	4.99±0.31^{BC}	2.65±0.11^{D}	2.80±0.08^{D}	5.41±0.10^{AB}	4.48±0.24^{C}	4.70±0.22^{BC}

Values represent mean ± standard error of three trials replicated three times (n=9).
Different letters represent a significant difference ($P < 0.05$) among treatments in the same row.
See table 1 for individual culture identity.

Table 7.6 – Number (Log CFU/g ± SE) of *E. coli* O157:H7 recovered during the production of dry fermented sausage. Results represent control (no mustard added) and treatments with yellow mustard powder (cold, hot, auto and auto+hot) at 6% (wt/wt).

Time (d)	Starter Culture A					Starter Culture B		
	Control	Cold	Hot	Auto	Auto/Hot	Control	Cold	Hot
0	6.12±0.04^{B}	6.34±0.09^{A}	6.63±0.06^{A}	6.50±0.05^{A}	6.06±0.07^{B}	6.40±0.06^{A}	6.43±0.07^{A}	6.51±0.04^{A}
6	5.01±0.04^{A}	4.86±0.07^{A}	3.67±0.20^{B}	3.16±0.12^{C}	2.39±0.36^{D}	5.21±0.08^{A}	5.12±0.06^{A}	3.93±0.08^{B}
12	4.70±0.06^{A}	4.86±0.06^{A}	3.38±0.09^{B}	2.35±0.13^{C}	1.75±0.11^{D}	4.67±0.10^{A}	4.67±0.14^{A}	3.00±0.14^{B}
18	4.32±0.06^{A}	4.05±0.10^{A}	2.39±0.16^{B}	1.40±0.01^{C}	< 0.62^{D+}	4.39±0.22^{A}	4.26±0.21^{A}	1.88±0.18^{BC}
24	3.64±0.14^{A}	2.92±0.10^{B}	1.81±0.19^{C}	< 0.62^{D+}	< 0.62^{D+}	3.39±0.11^{AB}	3.24±0.16^{AB}	1.65±0.01^{C}
31	3.21±0.19^{A}	1.60±0.13^{B}	< 0.62^{C+}	< 0.62^{C+}	< 0.62^{C+}	3.33±0.23^{A}	2.15±0.05^{B}	< 0.62^{C+}
38	2.51±0.10^{A}	1.37±0.09^{B}	< 0.62^{C+}	< 0.62^{C+}	< 0.62^{C+}	2.15±0.05^{A}	< 0.62^{C+}	< 0.62^{C+}

Values represent mean ± standard error of three trials replicated three times (n=9).
Different letters represent significant difference ($P < 0.05$) among treatments in the same row.
[+] Presence of presumptive *E. coli* O157:H7 was confirmed using immunomagnetic separation and enrichment.

Appendix 7.1

Levels of sinalbin in cold (industrial deheating) and autoclaved (115^{o}C x 15 min) mustard powder. 6 g of mustard sample was extracted using 100 ml of distilled water for 1 h.

Deheating Process	Concentration (g sinalbin/100g mustard)
Cold	2.71
Autoclaved	2.52

Chapter 8

Overall conclusion

Reactions between AIT and thiol-containing substances like glutathione and cysteine at room and refrigeration temperatures are described in chapter 3. This reaction forms a conjugate between AIT and the thiol-compound, which neutralizes the antimicrobial activity of AIT at levels that were otherwise bactericidal *in vitro*. Reaction between AIT and GSH was found when conditions of storage and pH of beef and fermented sausage were simulated. This could be one of the resons that a much higher concentration of AIT is needed when applied to a meat product (Chacon et al., 2006a, Muthukumarassamy et al., 2004) as compared to results obtained in broth media (Chapter 4). Mechanisms that could prevent or reduce this reaction will probably increase AIT effectiveness against *E. coli* O157:H7 when used meat and meat products.

It was also shown (Chapter 4) that AIT antimicrobial activity was affected by the environmental pH. Higher pH values promote higher levels of OH^-, which can serve as an electron donor to the highly reactive isothiocyanate group, reducing the ability of AIT to kill *E. coli* O157:H7. In addition, at pH 8.5 20 times more AIT was needed than at pH 4.5 to achieve the minimum inhibitory concentration against *E. coli* O157:H7. The isothiocyanate was more stable at acidic pH, which resulted in better antibacterial action (Pachacek et al., 1997; Tsao et al., 2000). The decomposition products of AIT in water were found to have minimal or no bactericidal activity against *E. coli* O157:H7.

Some elements of the mechanism of action by which AIT, and perhaps other isothiocyantes, kill *E. coli* O157:H7 were also explored. The mustard-derived essential

oil was able to inhibit the activity of thioredoxin reductase (TR) and acetate kinase (AK) at releatively low levels. These are important enzymes of bacterial metabolism, where TR is involved with DNA synthesis and AK with energy generation. The ability of AIT to inhibit other enzymes (Kojima and Ogawa, 1971) and to cause membrane damage has been previously reported (Ahn et al., 2001; Lin et al., 2000), which leads to the suggestion that isothiocyantes exhibit multi-targeted antimicrobial activity. Further studies are necessary to verify if AIT is able to inhibit other enzymes from the *E. coli* metabolic arsenal leading to microbial death.

Observation that yellow mustard powder could cause a > 5 log reduction of *E. coli* O157:H7 viability during fermented sausage processing was reported by Graumann and Holley (2008). Yellow mustard contains high levels of sinalbin, which can be converted to ρ-HBIT by the action of myrosinase. However, it was found that yellow mustard powder with no myrosinase activity was also able to kill *E. coli* O157:H7. In Chapter 5, it was shown that *P. pentosaceus* UM 116P, *Staphylococcus carnosus* UM 109 and, more importantly, *E. coli* O157:H7 were also able to degrade sinalbin and presumably convert significant amounts of the glucosinolate to ρ-HBIT. This may explain why deheated yellow mustard flour was able to kill *E. coli* O157:H7 in dry-cured sausages (Graumman and Holley, 2008). Isolation and characterization of the enzyme with myrosinase-like activity would be important to better understand the mechanisms that lead to *E. coli* death.

The starter cultures *P. pentosaceus* UM 116P and *S. carnosus* UM 109M together with the 5-strain cocktail of *E. coli* O157:H7 were also found to degrade significant levels of sinigrin in broth media (Chapter 6). In fact, all bacteria tested were more efficient in

decomposing sinigrin than sinalbin. *E. coli* O157:H7 was able to decompose concentrations of sinigrin that could potentially form quantities of AIT that are well above the measured MIC (Chapter 4). When the MBCs of ρ-HBIT and AIT were compared, *E. coli* O157:H7 was more susceptible to the latter, while the starter cultures were more strongly affected by ρ-HBIT. *In vitro* formation of isothiocyanates by the activity of industrially isolated myrosinase also showed that *E. coli* was more rapidly affected by the formation of AIT than ρ-HBIT. Therefore, the utilization of deodorized black (*Brassica nigra*) or brown/oriental (*Brassica juncea*) ground mustard may be also an alternative ingredient to help in the elimination of *E. coli* O157:H7 from dry fermented sausage.

A large number of starter cultures used for the manufacture of dry sausages were examined for their ability to degrade glucosinolates after it was observed that *S. carnosus* UM 109M and *P. pentosaceus* UM 116P had myrosinase-like activity (Chapter 7). *P. pentosaceus* UM 121P and *S. carnosus* UM 123M were selected as the pair with higher myrosinase-like activity. Dry sausages containing cold mustard powder were prepared in the presence of two different starter cultures pairs. However, it was found that myrosinase-like activity of the starter cultures did not play a very significant role in the elimination of *E. coli* O157:H7. The cold powder was able to decrease the levels of the pathogen > 5 log CFU/g after 38 d in sausages containing either pair of starter culture. Hot yellow mustard was significantly faster, causing a > 5 log reduction after 31 d, again, with no difference between starters pairs. The greater capacity of *E. coli* O157:H7 to decompose the glucosinolates sinigrin and sinalbin, may be the major reason for its elimination in the presence of cold mustard powder. This event might not occur in normal

circumstances, where sufficient substrate to satisfy energy requirements is available. In the dry sausage environment the pathogen used sinalbin as a source of energy and inadvertently formed ρ-HBIT, which was bactericidal.

Other studies have shown that the autoclave process is able to form/release substances from plant materials with antimicrobial activity (Gliszczynska-Swiglo et al., 2006; Ju et al., 2010; Rhandir et al., 2008; Rufian-Henares and Morales, 2006). Autoclaved yellow mustard powder was produced and used as a dry sausage ingredient in the presence of contaminating *E. coli* O157:H7. The two treatments containing autoclaved mustard powder (autoclaved powder alone and autoclaved + hot powder) showed significantly faster reduction (> 5 log in 18 d) of *E. coli* O157:H7 viability in comparison to cold and hot powders. This may be a result of increased levels of phenolic acids found in the autoclaved powder, although the formation/release of other unknown compounds may have also contributed. These substances may act synergistically or additively with the formation of isothiocyantes from mustard by the target bacteria.

The autoclaved powder is a promising industrial alternative to achieve reductions of *E. coli* O157:H7 during dry sausage manufacture required by Health Canada. However, the utilization of shiga-toxin producing *E. coli* O157:H7 strains implicated in causing human illness from the consumption of contaminated dry sausage is necessary for the validation of this approach (Health Canada, 2000). Furthermore, better understanding of the mechanisms involved in the bacterial glucosinolate-to-isothiocyanate conversion are of paramount importance in order to optimize the use of cold/autoclaved mustard powder for control of *E. coli* O157:H7 and perhaps other food pathogens.

Furthermore, it would be important to test lower concentrations of the autoclaved

yellow mustard powder and verify if this can also reduce the viability of *E. coli* O157:H7 within the same timeframe or within a time that is feasible for the industrial production of dry fermented sausage. Additionally, the autoclave process could be optimized since it is uncertain whether different temperatures could result in mustard powder with enhanced antimicrobial activity.

Although the autoclaved mustard powder contained higher levels of phenolic compounds in comparison to the cold mustard powder, it is also possible that other antimicrobials were formed or released. Future research is needed to identify and characterize these substances, as well as the identity of the phenolic acids released. In addition, the mechanism of interaction between the phenolic compounds and isothiocyanates still remains obscure and needs further explanation.

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