Genetic Basis for Glucosinolate Hydrolysis in *E. coli* O157:H7 by Glycoside Hydrolase Action and Nature of its Adaptation to Isothiocyanate Toxicity

By

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science

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Winnipeg, Canada

"Because of the littleness of
your faith; for truly I say to you, if
you have faith the size of a mustard
seed, you will say to this mountain,
'Move from here to there,' and it will
move; and nothing will be
impossible to you”

Matthew 17:20

Acknowledgements

First and foremost I would like to thank God for His sovereign guidance that allowed me to accomplish this work along many others in the past. I would like to offer my heartfelt gratitude to my supervisor, Dr. Rick Holley, who has supported me throughout this research with his patience and knowledge while allowing me to work in my own way. I attribute the level of my PhD degree to his expertise and vision. Without his encouragement and insightful thought this work would not have been completed. One could simply not wish for a friendlier and more inspiring supervisor. I also would like to extend my gratitude to my committee members Dr. Richard Sparling, Dr. George Zhanel, and the late Dr. Denis Krause for the insightful discussions that led to the preparation of this work. I also want to thank Dr. Juan C. Rodriguez-Lecompte and Dr. Ehsan Khafipour for accepting to be part of the committee after the passing of Dr. Krause and for helping during the progress of this research.

My warmest Brazilian thanks to all faculty members of the Food Science Department, with special thanks to Dr. Gary Fulcher, Dr. Susan Arntfield, and Dr. Claudia Narvaez for their friendly support and suggestions. My sincere appreciatiation goes to the support and technical assistance provided by Carola Lange, Allison Cranmer, Yang Qiu, Alison Ser Yang, Babak, Pat Kenyon, Namita Goswami, Michael Stringer, Joel Lamoureux, Xuan Shi, Yao Wu, Chenyuan Liu, Nana Marmah, and Huizi Zuo. Furthermore, I want to thank my fellow graduate students for their friendship, especially Mussarat Jahan, Jeyachchandran Visvalinhgam, Amin Olaimat, May-Fong, Melissa, and Juan Rodriguez.

Funding support from the Natural Sciences and Engineering Research Council of Canada (NSERC) through a Strategic Grant, Piller's Sausages & Delicatessens (Waterloo, ON, Canada), and, the University of Manitoba Graduate Fellowship is acknowledged. Authors would like to

thank Sakai spice and G.S. Dunn Ltd for providing the mustard powder. I also would like to thank Dr. Mario Tenuta (Department of Soil Science, University of Manitoba, Winnipeg, Canada) for allowing open access to his laboratory.

My deepest love and gratitude to my parents, Ueliton Peixoto and Marinete Lopes, for the encouragement and strength during all my life. Thanks to my sinblings Helio Neto, Romelia, and Antonio, for not giving up to be family even in the hard times. I also want to thank my parent-in-laws Vanda and Rubens Cordeiro for their motivation. Thanks to my sister-in-law Raquel, her husband Wayne, and my shine niece Rebecca, for making Winnipeg a bit warmer with the good times we have had so far. Thanks to my friends Anna and Bladimir Gonzalez for their support and prayers.

Finally, I want to thank the most important person in this journey, my husband Marcos Cordeiro, for his continued and unfailing love, support and understanding through the ups and downs of this journey. I am so blessed for having you in my life and for being a family with you. With your caring love you raise me up, so I could stand on mountains; you raise me up, to walk on stormy seas; I am strong, when I am on your shoulders; you raise me up... to more than I can be. Thanks for our baby girl!

Dedication

To my husband and my baby girl.

Thesis Format

This thesis is comprised of four manuscripts at different stages of publication in peer-reviewed scientific journals (Chapters 3-6), which are indicated below. The formatting of manuscripts was modified to satisfy the requirements for the thesis. Chapter 1 gives an overall introduction to the subject of study in the thesis. Chapter 2 presents a comprehensive literature review of all the work described in the following chapters. Chapter 7 provides an overall discussion of the results found in this work, Chapter 8 gives an overall conclusion, and Chapter 9 gives suggestions for future studies. Lastly, references are documented in alphabetic order.

Chapter 3 entitled "Evaluation of deodorized yellow mustard concentrations for control of *Escherichia coli* O157:H7 viability in dry fermented sausage" by Roniele P. Cordeiro, Fernando B. Luciano, and Richard A. Holley, 2013 was originally published in the journal Food Control, 33, 20-24.

Chapter 4 entitled "Contribution of endogenous plant myrosinase to the antimicrobial activity of deodorized mustard against *Escherichia coli* O157:H7 in fermented dry sausage" by Roniele P. Cordeiro, Chen Wu, and Richard A. Holley, 2014 was originally published in the International Journal of Food Microbiology, 189, 132-138. This study built upon the Chen Wu (2013) research on the "Use of completely and partially deodorized yellow and Oriental mustards to control *E. coli* O157:H7 in dry-fermented sausage" and some of Mr. Wu's work was used in that paper. Data used from Wu (2013) included the measurement of myrosinase activity by substrate decline in mustard treatments used for sausage (Figure 4.1), pH changes during sausage ripening with 4% (w/w) mustard powder (Table 4.1), water activity changes during sausage ripening with 4%

(w/w) mustard powder (Table 4.4), and viability of *E. coli* O157:H7 during sausage ripening with 4 % (w/w) mustards containing myrosinase (Figure 4.3).

Chapter 5 entitled “Role of glycoside hydrolase genes in sinigrin degradation by *Escherichia coli* O157:H7” by Roniele P. Cordeiro, Juan H. Doria, George G. Zhanel, Richard Sparling, and Richard A. Holley, 2014 is under review by the International Journal of Food Microbiology.

Chapter 6 entitled “Role of the BaeSR two-component regulatory system in resistance of *Escherichia coli* O157:H7 to allyl isothiocyanate” by Roniele P. Cordeiro, Denis O. Krause, Juan H. Doria, and Richard A. Holley, 2014 was originally published in the journal Food Microbiology, 42, 136-141.

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Abstract

Ready-to-eat meat products such as dry-fermented sausages have been associated with foodborne outbreaks despite the multiple hurdles used in the manufacturing process to prevent growth of pathogens. As a result, new strategies such as natural products with antimicrobial activity are being used to control pathogens of importance like *Escherichia coli* O157:H7. This study investigated how different concentrations and sources of mustard can influence its antimicrobial activity against *E. coli* O157:H7 in dry-fermented sausage, as well as the contribution of residual myrosinase enzyme in mustard to this process. The genetic basis for the degradation of mustard glucosinolate by *E. coli* O157:H7, which is associated with the antimicrobial action of mustard, was also characterized. The ability of *E. coli* O157:H7 to withstand inhibitory allyl isothiocyanate (AITC) concentrations and the role of the two-component BaeSR system as a defense mechanism against AITC was also investigated. Results showed that 4% (w/w) deodorized yellow mustard powder was effective to control *E. coli* O157:H7 in dry-fermented sausage at 28 d. The presence of endogenous plant myrosinase in the mustard powder or meal enhanced *E. coli* O157:H7 reduction rates. Fully-deodorized, deoiled, yellow mustard meal as low as 2% (w/w) containing either 0.1% or 0.2% of residual plant myrosinase achieved the same results as 4% (w/w) mustard powder also containing similar residual myrosinase. Regardless of the type of mustard, the antimicrobial activity of yellow mustard derivatives were more pronounced than those of Oriental mustard. The initial genetic assessment through *in silico* analysis found similarity between plant myrosinase and enzymes encoded by genes (*bglA*, *ascB*, and *chbF*) from *β*-glucosidase families in *E. coli* O157:H7 strains. After disruption of these genes using lambda-red replacement, single (ΔbglA, ΔascB, ΔchbF) and double (ΔbglAascB, ΔchbFascB, ΔchbFbglA) mutant strains were created and assessed for glucosinolate degradation.

The comparison of the gene expression profiles and changes in the extent of sinigrin degradation by different mutants suggested that *ascB* have a prominent role in the degradation of this *β*-glucoside by *E. coli* O157:H7. *E. coli* O157:H7 did not develop resistance to AITC, the essential oil formed from sinigrin degradation that is responsible for the antimicrobial activity of Oriental mustard.

Chapter 1

Introduction

Escherichia coli O157:H7 infection is a major public health concern in North America, Europe, and other parts of the world (Lim et al., 2010). Since the first reported outbreak in 1982 associated with the consumption of undercooked beef (Riley et al., 1983), this pathogen has emerged as a main cause of both outbreaks and sporadic cases of human infection. As a non-thermally treated ready-to-eat (RTE) meat product, dry-fermented sausages have been linked to illness outbreaks caused by *E. coli* O157:H7 (Sartz et al., 2008). The multiple hurdles of dry-fermented sausage are not capable of controlling *E. coli* O157:H7 viability in such products. The recognition of dry-fermented sausage as a potential source for *E. coli* O157:H7 infection has prompted initiatives to improve safety of this product during the manufacturing process. Stringent regulations for fermented meat manufacture in Canada and the United States require the dry-fermented sausage production process to be capable of causing $\geq$ 5 log $CFU.g^{-1}$ reduction in the viability of *E. coli* O157:H7 (Health Canada, 2000; USDA, 2001). Strategies such as low heat, freeze-thaw, extended storage, high pressure, and irradiation have been tested to achieve this goal but have not been capable of reaching the mandatory reduction level (Holck et al., 2011). Moreover, there has been an increasing demand for natural products by consumers, which has led to searches for new antimicrobial agents from plants to improve the safety of food products (Goni et al., 2009). Within this context, research has investigated natural alternatives to reduce or eliminate *E. coli* O157:H7 from dry-fermented sausage.

Essential oils from mustard have been proposed as a natural antimicrobial against *E. coli* O157:H7 in dry-fermented sausage. Essential oils including allyl isothiocyanate (AITC) in brown or Oriental mustard (*Brassica juncea*), and ρ-hydroxybenzyl isothiocyanate (ρHBIT) in yellow or white mustard (*Sinapis alba*) (Kissen et al., 2009) are formed as a result of the exposure of mustard glucosinolates to the plant enzyme, myrosinase (EC 3.2.1.147) (Rask et al., 2000). The hydrolysis of glucosinolates can also occur by the myrosinase activity present in microorganisms like *E. coli* O157:H7 (Luciano and Holley, 2010; Luciano et al., 2011). The use of 6% (w/w) mustard powder as a sausage ingredient was able to reduce *E. coli* O157:H7 viability in uncooked dry-fermented sausages to the North American mandatory levels. However, sensory evaluation showed that dry-fermented sausages containing yellow mustard concentrations lower than 6% were more acceptable to consumers (Li et al., 2013). Thus, there is a need to reduce the concentrations of mustard powder that is needed to achieve the required 5 log $CFU.g^{-1}$ reduction of *E. coli* O157:H7 during dry sausage manufacture.

The inactivation of myrosinase in mustard powder is a strategy used by the food industry to reduce the pungency of mustard. This inactivation is usually done through thermal treatment and prevents the formation of isothiocyanates, which are responsible for the spicy and pungent flavor of mustard, as well as its antimicrobial activity. The thermal treatment yields a bland deheated (cold) powder that can be used as a binder or filler in cooked processed meats. However, the thermal stability of some plant myrosinase(s) (Van Eylen et al., 2008) may render its inactivation incomplete depending on the thermal process applied, which in turn, might influence consistency in the antimicrobial effectiveness of mustard against *E. coli* O157:H7 during ripening of sausage. It was considered important to establish whether the thermal

treatment delivered with the autoclave was sufficient to completely inactivate plant myrosinase. Besides the thermal treatment used to inactivate myrosinase, the antimicrobial activity of different types of mustard available for use in dry-fermented sausage needs to be compared since their use may enable use of lower concentrations of mustard. For example, studies have shown that AITC from sinigrin in Oriental mustard had greater antimicrobial action against *E. coli* O157:H7 than ρ-HBITC from sinalbin in yellow mustard (Luciano and Holley, 2011). The use of another fraction of mustard such as deoiled meal which is known to contain higher glucosinolate levels might also enable use of reduced concentrations. Thus, there is a need to compare the difference in antimicrobial activity between different mustard products such as yellow and Oriental mustard powder, as well as the antimicrobial effectiveness of a different type of mustard fraction such as deoiled mustard meal.

Microorganisms possessing myrosinase activity have been responsible for the degradation of glucosinolates in deodorized mustard devoid of endogenous plant myrosinase to form isothiocyanates which are bactericidal. This activity has been reported for microorganisms such as *Enterobacter cloacae* (Tani et al., 1974), *Lactobacillus agilis* (Palop et al., 1995), and *Escherichia coli* (Oginsky et al., 1965), which showed myrosinase activity from *in vitro* experiments. There are also studies suggesting that the microflora in the human intestinal tract is able to hydrolyse glucosinolates, producing AITC from substrates where myrosinase has been completely inactivated by heat treatments (Getahun and Chung, 1999; Rouzaud et al., 2004). While the properties of plant and fungal myrosinase have long been documented (Tani et al., 1974), the nature of bacterial myrosinase is poorly known, and studies on this topic are very scarce in the literature. Although *E. coli* O157:H7 can hydrolyze sinigrin (the glucosinolate

present in Oriental mustard), the specific enzyme(s) responsible for this myrosinase activity have not been formally identified.

Allyl isothiocyanate (AITC), formed from the degradation of the glucosinolate found in Oriental mustard is among the isothiocyanates produced by the myrosinase(s) of cruciferous vegetables. AITC has great potential for applications in food preservation due to its strong antimicrobial activity against several foodborne pathogens, including *E. coli* O157:H7. Studies have demonstrated the useful applications of this compound as a component in packaging film used for meat products (Shin et al., 2010; Jin and Gurtler, 2011). Despite the antimicrobial activity of AITC, this compound is very pungent and volatile, which limits its direct application in food products. The instability of AITC and its decomposition to new products usually occurs at high temperature and alkaline pH, which in turn, decreases its antimicrobial effects (Pecháček et al., 1997). Although the mode of antimicrobial action of AITC has been widely investigated (Lin et al., 2000; Turgis et al., 2009; Ahn et al., 2001), the mechanism is not clearly understood. In addition, studies describing the potential of bacteria to develop resistance to AITC (Neudecker and Henschler, 1985) and the mechanisms behind this resistance (Dufour et al., 2012; Chan et al., 2013) are rare in the literature. A few studies have associated the presence of the BaeSR two-component regulatory system with the ability of bacteria to overcome inhibitory concentrations of plant secondary metabolites (Zoetendal et al., 2008; Cuaron et al., 2013). However, the ability of *E. coli* O157:H7 to withstand inhibitory AITC concentrations and the role of the BaeSR two-component system as a defense mechanism against AITC has never been exploited. Also, conditions to minimize AITC instability in aqueous media and increase the antimicrobial activity of AITC against *E. coli* O157:H7 requires investigation.

Therefore the objectives of the present work were:

1. To investigate whether reduced concentrations of 2% or 4% (w/w) deodorized yellow mustard powder would be sufficient to yield a 5 log CFU.g^{-1} reduction of *E. coli* O157:H7 during dry sausage manufacture.

2. To compare the antimicrobial activity of Oriental and yellow mustard powder and deoiled meal against *E. coli* O15:H7 during dry-fermented sausage ripening (since deoiled meal contains more glucosinolate than unextracted mustard powder). The relative antimicrobial activity of these two mustard products was assessed in the presence and absence of residual endogenous plant myrosinase.

3. To investigate the role of glycoside hydrolase genes in the hydrolysis of sinigrin by *E. coli* O157:H7, and to quantify their relative importance in the degradation of this mustard glucosinolate.

4. To investigate the potential of *E. coli* O157:H7 to overcome inhibitory AITC concentrations and assess the role of the BaeSR two-component system in this process. Optimized conditions for AITC stability in an aqueous medium were also determined to enable the assessment of the MIC and MBC of AITC against *E. coli* O157:H7.

Chapter 2

Literature Review

2.1 Dry fermented sausages

2.1.1 Dry fermented sausage manufacturing

The changes in consumer demands and lifestyle have increased the consumption of ready-to-eat (RTE) meat products including cooked ham, dry ham, and dry fermented sausages. In general, dry fermented sausages are shelf stable products with no requirement for storage at refrigerated temperatures or even thermal treatment before consumption (Vignolo et al., 1993). The shelf-stability of dry fermented sausages is achieved by the hurdles used in the manufacturing process, which are assumed to inactivate or prevent the growth of bacterial pathogens. Hurdles such as low pH, low water activity (a_w), and chemical preservatives, enable dry-fermented sausage to be an uncooked RTE meat product considered “traditionally safe” for consumption.

The manufacturing of dry-fermented sausage takes approximately 4 weeks. The first step in the process is chopping the meat and fat to make a sausage batter, which is mixed with salt, sugar, additives, spices, and bacterial starter before being stuffed into casings. The filled sausage casings are fermented, smoked, and dried at controlled humidity and temperature to ensure stability and safety. At the end of the process, these RTE sausages reach a final pH of typically 4.5-5.0 and aw below 0.90, which is assumed to make them shelf-stable and safe from foodborne pathogens (Pragalaki et al., 2013).

For the manufacturing of dry-fermented sausage, each ingredient added to sausage batter plays a specific role to provide the organoleptic characteristics and safety of the final product (Cocconcelli, 2007). Salt contributes to the sensory characteristics of the product, in the reduction of aw, and in the creation of a protein gel that promotes the cohesion of the mixture during ripening of the product. Sugar affects the acidification of the product by serving as a source of energy for the growth of starter cultures like lactic acid bacteria (LAB). Additives also include sodium nitrate which, after being reduced to nitrite, influences the color of the product as well as inhibits the growth of *Clostridium.botulinum*. Spices influence the aroma of sausages due to their high content of essential oils. Some of the spices used may also act as a fat emulsifier and water absorptive agent. For example, mustards are used as condiments or spices because of their very hot flavor that results from the formation of isothiocyanates (ITCs) after glucosinolate hydrolysis. However, mustard known as cold or deodorized powder (e.g. without an intense hot flavor) has emulsifying, binding, stabilizing, and thickening properties that enable mustard to be used as a binder in cooked, processed meats (Graumman and Holley, 2008). In addition, the antibacterial activity of mustard against foodborne pathogens in sausage has been reported in several studies (Sethi et al., 2013; Lee et al., 2012; and Luciano et al., 2011).

2.1.2 Starter cultures in dry-fermented sausage

During the fermentation of sausages, bacterial starter cultures play a significant role in the metabolic activities responsible for maturation, where they can be used as single or multiple-species combinations of LAB and staphylococci (Ricke et al., 2007). The starter cultures used in fermented sausage usually improve safety, flavor, and appearance of the final products (Toldrá et al., 2007). These starter cultures include LAB belonging to the genera *Lactobacillus* or

Pediococcus, coagulase-negative staphylococci (CNS), and members of *Micrococcaceae* family. LAB are mainly responsible for acidification through the production of lactic acid from sugars in sausage, which decrease the pH of the product. This process is important for the development of flavor, color, and texture, as well as for controlling undesirable microflora during fermentation (Cocconcelli, 2007). The CNS contribute to the development of color and flavor in fermented meat products. They reduce nitrate to nitrite leading to the formation of nitrosomyoglobin that promotes the desired red color of the product (Rantsiou and Cocolin, 2008). The proteolytic and lipolytic activities of staphylococci also play an important role in the sensory quality of fermented sausages by the release of peptides, amino acids, aldehydes, amines, and free fatty acids that influence the aromatic profile of the final product (Rantsiou and Cocolin, 2008).

2.1.3 Pathogens in dry-fermented sausage

Although the manufacturing of dry-fermented sausage involves multiple hurdles that can inactivate or prevent the growth of undesirable microorganisms, total pathogen elimination cannot be guaranteed. When contamination of dry fermented sausage with foodborne pathogens occurs, it is possible that the hurdles present during the manufacturing process are not sufficient to ensure the microbial safety of the product. While proper manufacturing of dry-fermented sausage is able to control pathogens of human health concern like *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus*, the process is unable to control *E. coli* O157:H7 in these RTE meat products. A brief description of some of the concerns associated with contamination of sausage by these pathogens is given below.

- *Listeria monocytogenes*

The hurdles present during dry-fermented sausage manufacture such as low aw, low pH and high salt concentration are able to control the growth of *L. monocytogenes* in dry fermented sausage (Bunčić et al., 1991). If contamination of dry-fermented sausage with *Listeria* occurs, it would be indicative of post-processing contamination (Cabeza et al., 2009). However, listeriosis in humans is unlikely to occur via the consumption of dry fermented sausages.

- *Salmonella enterica* subsp. *enterica*

The risk posed by *Salmonella* in dry-fermented sausage is associated with the use of contaminated pork meat. Relevant studies have reported the prevalence of *S. enterica* in pig carcasses, slaughtered pigs, and the abattoir environment (Arguello et al., 2012). In a study done by Botteldoorn et al. (2003), *S.* Typhimurium, *S.* Livingstone, and *S.* Derby were the serotypes isolated from the slaughterhouse environment and from the animal colon. Fortunately, *Salmonella* is competitively inhibited by the hurdles used in dry-fermented sausage manufacture. The levels of *S.* Typhimurium in a traditional sausage of Germanic origin, made from pork and beef, decreased significantly during storage for 30 d (Dourou et al., 2009). The findings suggested that the use of lactic acid acid bacterial starter culture(s) and controlled fermentation during manufacture enhanced both the quality and safety of the finished product. In another study, contamination by *Salmonella* spp. during the production of pork sausage revealed that although this pathogen was present in raw material during sausage batter preparation, meat trimmings and chopped trimmings, *Salmonella* was not

found in any sample of sausage (Ducic et al., 2014). The results emphasized that a low initial number of pathogens or even their elimination by the hurdle effects of starter cultures, nitrite, salt, low pH, and low aw were plausible reasons for the absence of *Salmonella* from the final product.

- *Staphylococcus aureus*

S. aureus in dry-fermented sausage may cause food poisoning. Usually, illness (intoxication) is caused by the consumption of foods containing heat-stable enterotoxins produced by *S. aureus*. This pathogen is easily controlled in the early stages of the dry-fermented sausage process by ensuring their absence from the meat plus other ingredients and use of proper hygiene. The combination of lower fermentation temperature and the prevalence of starter cultures that reduce the pH to <5.3, are measures used to restrict the growth of *S. aureus* and toxin production (Skandamis and Nychas, 2007).

- *E. coli* O157:H7

Different from the other foodborne pathogens described above, *E. coli* O157:H7 has demonstrated an ability to endure the hurdles present in the dry-fermented sausage process and infect people. *E. coli* O157:H7 has cattle as its main reservoir, which facilitates the contamination of the carcass during slaughter as well as the fermented sausage produced from such beef (Nastasijevic et al., 2009). Despite the unfavourable conditions during dry-fermented sausage manufacture such as storage at low temperatures, high NaCl

concentrations, low pH, and low aw, *E. coli* O157:H7 can still survive in properly manufactured dry-fermented sausages (Dalzini et al., 2014).

E. coli O157:H7 possesses a number of undesirable characteristics that make it one of the most serious threats associated with the consumption of dry-fermented sausage. Because of its tolerance to acid and low moisture, *E. coli* O157:H7 may be present in uncooked, RTE products without changing their flavor. Also, the presence of *E. coli* O157:H7 in the final product may be problematic in RTE processing environments because this pathogen has the ability to form biofilms on food-contact surfaces and to be transferred from contact surfaces to other RTE meat products (Silagyi et al., 2009). Therefore, the inability of the dry-fermented sausage manufacturing process to control *E. coli* O157:H7 has raised food safety concerns in the meat industry, public health agencies, and among consumers.

2.2 Significance of *Escherichia coli* O157:H7 as a foodborne pathogen

2.1.1 E. coli O157:H7 *reservoir*

The primary sources and vehicles of *E. coli* O157:H7 infections are ruminants, especially cattle in which the bacteria are not pathogenic (Ferens and Hovde, 2011). Generally, *E. coli* O157:H7 does not cause disease in weaned calves and adult cattle, making these animals asymptomatic sources of *E. coli* O157:H7 in the food chain (Meng et al., 2007). Contamination of humans with this pathogen may occur through different vehicles after direct or indirect fecal contamination, such as meat, unpasteurized dairy products, fresh vegetables, fruits, and drinking or recreational water (Piérard et al., 2012). Usually, there is an association between fecal shedding of *E. coli* O157:H7 and contamination of feed, food, and the environment due to the

fact that this pathogen is transiently carried in the gastrointestinal tract of these animals and is irregularly excreted for weeks or months (Meng et al., 2007). The shedding of *E. coli* O157:H7 by animals appears to be seasonal, where a more pronounced prevalence of shedding occurs during summer and late fall, which mirrors the major incidence of foodborne outbreaks caused by *E. coli* O157:H7 in the summer months (Farens and Hovde, 2011). The shedding of *E. coli* O157:H7 by animals poses a risk of contaminating food because *E. coli* O157:H7 in these animals will enter the processing plant, may contaminate the carcass, and subsequently the finished product (Arthur et al., 2010). Data from over 15 years (n=388,895) showed that the number of *E. coli* O157 infections in the USA were sharply seasonal with 49% of isolates collected during July-September and only 9% during January-March (Sodha et al., 2014). Besides the increased animal shedding during summer, it seems that the elevation of temperature during this season is an appropriate environmental condition for the growth of *E. coli* O157:H7 in soil, bedding material, feed and water, which in turn may result in a constant source of infection for cattle (Edrington et al., 2006).

Cattle hides have been implicated as the main source for contamination of beef carcasses with *E. coli* O157:H7 (Nou et al., 2003; Arthur et. al., 2007). Nastasijevic et al. (2009) reviewed studies on the incidence of *E. coli* O157:H7 on hides of cattle at slaughter in different countries and found that *E. coli* O157:H7 incidence was highly variable, ranging from 4.5% to 56%. In fact, the prevalence of *E. coli* O157:H7 on carcasses during slaughter has been found to vary widely in the United Kingdom (Omisakin et al., 2003; Low et al., 2005), the United States (Elder et al., 2000), and Canada (Donkersgoed et al., 2005; Stanford et al., 2013).

Besides direct contamination of meat, contamination of water and soil with *E. coli* O157:H7, and consequently fruits and vegetables can occur, especially if untreated manures are used as fertilizers. Solomon et al. (2002) showed that *E. coli* O157:H7 associated with contaminated manure or irrigation water was transmitted to lettuce plants. Islam et al. (2005) showed that contamination of carrots and onions with *E. coli* O157:H7 can occur for several months through both contaminated manure compost and irrigation water. Cattle feed has also been suggested as a source of *E. coli* O157:H7 infection. Lynn et al. (1998) indicated that feeds are a potential factor in the ecology of organisms that can be transmitted from feces to the animal mouth. The authors showed the widespread contamination of cattle feeds with *E. coli* O157:H7 and the ability of this pathogen to replicate in these media. Moreover, Davis et al. (2003) found that feed plays an important role in the transmission of *E. coli* O157:H7 to cattle. A comparison between isolates from feed samples and bovine fecal isolates from the same farm using pulsed-field gel electrophoresis (PFGE) showed a very similar PFGE profile of *E. coli* O157:H7 between feed samples and feces collected from the same farm.

2.1.2 E. coli O157:H7 *pathogenicity*

E. coli O157:H7 possesses specific virulence factors facilitating their interactions with the target host including colonization of epithelial surfaces, crossing of mucosal barriers, invasion of the blood stream and internal organs, and production of toxins (Piérard et al., 2012). The acid tolerance of *E. coli* O157:H7 allows this pathogen to survive exposure to gastric acid during passage through the stomach and use the stimulus of the low pH to induce expression of proteins that facilitates adhesion to epithelial cells, and results in diarrhea after the bacteria reach the lower gastrointestinal tract (Page and Liles, 2013). The virulence of *E. coli* O157:H7 strains

is attributed to several factors such as production of Shiga toxins (Stx1 and Stx2), the presence of a pathogenicity island referred to as the "locus of enterocyte effacement" (LEE), and possession of a large plasmid encoding enterohemolysin (Ogura et al., 2009). The ability of *E. coli* O157:H7 to attach and efface (A/E) the host cell surface comes from an outer membrane protein called intimin. The attachment is facilitated by the receptor protein Tir, which is inoculated into the host cell and links to intimin inserted into the membrane.

One of the main concerns about *E. coli* O157:H7 pathogenicity is its ability to produce two different types of verotoxin. The term verotoxin comes from its ability to kill Vero (African green monkey kidney) cells. Both verotoxins produced by *E. coli* O157:H7 are characterized as Shiga-toxin because they are related to the toxin produced by *Shigella dysenteriae* (Duffy et al., 2006). The Stx1 (VT1) toxin is virtually identical to Shiga toxin, while Stx2 (VT2) has a limited degree of similarity. These two toxins are the major reasons for the symptoms following infection. People infected with organisms producting only Stx2 are 6.8 times more likely to develop disease than those infected by orgaisms carrying only Stx1 or both Stx1 and Stx2 (Erickson and Doyle, 2007). The Stx2 toxin exhibits greater ability to damage the human intestinal tract and can cause hemolytic-uremic syndrome (HUS), which is defined by the triad: non-immune hemolytic anemia; thrombocytopenia; and acute kidney injury (Page and Liles, 2013).

2.1.3 E. coli O157:H7 *infection*

E. coli O157:H7 is a significant pathogen responsible for severe gastrointestinal disease in humans. The consumption of contaminated food of animal origin seems to be the main route

for the transmission of *E. coli* O157:H7 to humans (Meng et al., 2007). However, infection by *E. coli* O157:H7 may also occur after direct contact with animals, humans or from the environment (Fig 2.1). Common victims of *E. coli* O157:H7 infections are patients at a very young or old age, and those with immunocompromised health conditions. A survey over 15 years in the USA showed that the isolation rate of *E. coli* O157:H7 was higher in children aged 1-4 years. The rate declined with increasing age until 30-39 years, and then steadily increased until age 70-79 years with similar rates in patients aged≥80 years (Sodha, et al., 2014). Although the symptoms and severity of the infection may differ according to individual susceptibility to disease, a low infectious dose where 10 to 100 organisms are sufficient to develop clinical infection is of particular relevance (Peacock et al., 2001). This small dose is sufficient to initiate illness within 3 or 4 days after ingestion.

Symptoms of *E. coli* O157:H7 infection may be initially similar to other foodborne illnesses and include cramps and diarrhea; however, people may have additional severe reactions. Dehydration, pallor, sudden gain in weight, and oliguria are some of the additional symptoms, suggesting the beginning of HUS, which is caused by Stx2 (Mead and Griffin, 1998). Usually, the time to recovery is from 5 to 7 d with 95% of the cases suitably resolved (Callaway et al., 2004). However, 5% of patients die as a result of HUS.

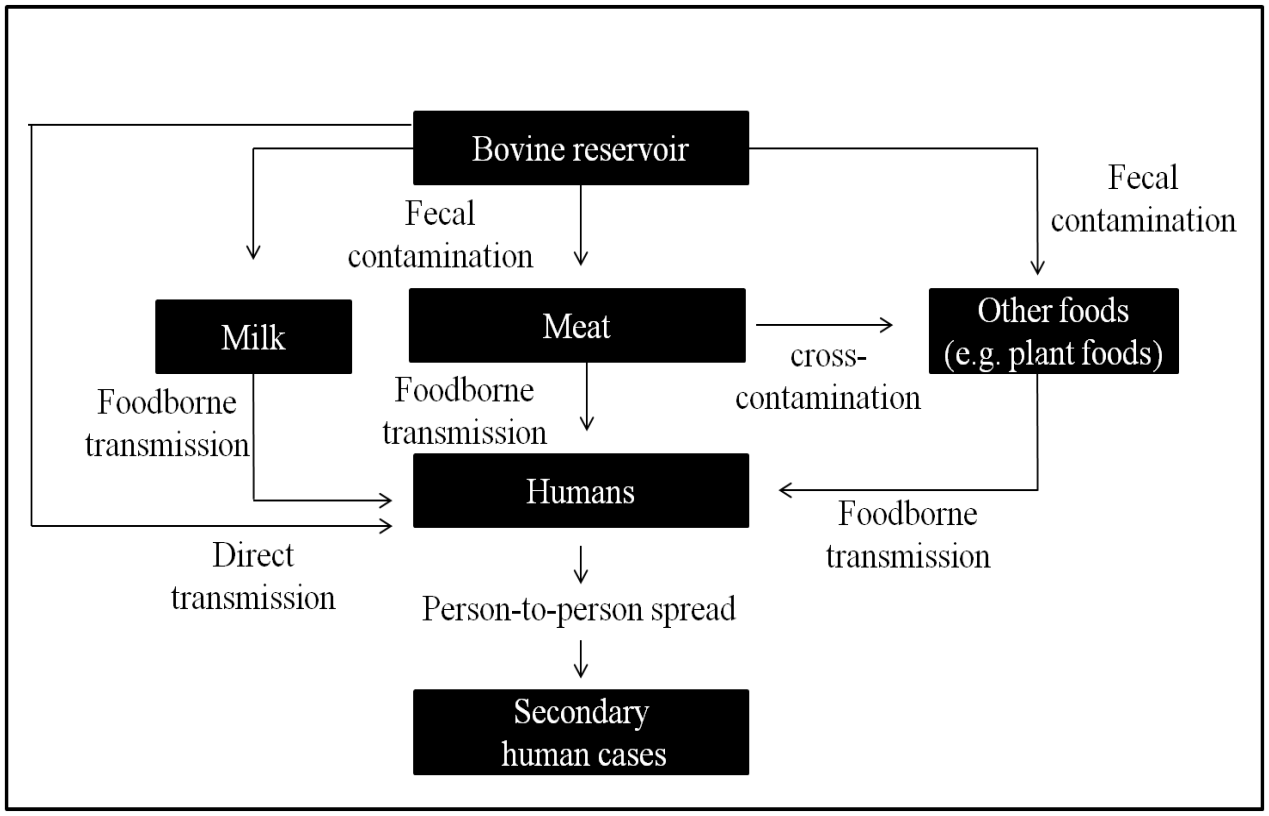

Figure 2.1. Reservoirs and transmission pathways of *E. coli* O157:H7 (adapted from Armstrong et al., 1996).

2.1.4 E. coli O157:H7 *foodborne illness outbreaks*

E. coli O157:H7 first emerged as a foodborne pathogen in the mid-1980s. While multiple sources and routes of transmission for this pathogen are recognized, meat products remain an important vehicle of pathogen transmission and continue to be linked to outbreaks (Table 2.1). The first outbreak of *E. coli* O157:H7 was reported in 1982, and was associated with the consumption of undercooked beef (Riley et al., 1983). In 1993, another *E. coli* O157:H7 outbreak was linked to home-cooked hamburger in California, causing the infection of 3 people (CDC, 1994). In 1998, 36 people became infected with *E. coli* O157:H7 after consuming dry-

fermented Genoa salami in southern Ontario, Canada (Williams et al., 2000). In 2002, a large outbreak in southern Sweden was reported with 30 confirmed cases of *E. coli* O157:H7 infection after the consumption of fermented sausage (Sartz et al., 2008).

Table 2.1 Selected outbreaks of *E. coli* O157:H7 attributed to contaminated meat products

Location	**Year**	**Product and mode of transmission**	**Reference**
USA	1992-1993	Undercooked beef burgers in fast-food outlets	Bell et al. (1994)
USA	1995	Roast beef at retail outlets	Rodrigue et al. (1995)
Australia	1995	Dry-fermented sausage	Patton et al. (1996)
Canada	1998	Dry-fermented Genoa salami	Williams et al. (2000)
Canada	1999	Salami	MacDonald et al. (2004)
Sweden	2002	Fermented sausage	Sartz et al. (2008)
Canada	2012	Beef and beef products	Lewis et al. (2013)
USA	2014	Ground beef	CDC (2014)

Since the first reported outbreak back in the 1980's, this pathogen has emerged as a main cause of both outbreaks and sporadic cases of human diarrhea in North America and all over the world. About 70% to 80% of sporadic cases of classic HUS reported in Canada, the United

Kingdom, Germany, Belgium, the Netherlands, and Japan have been associated with *E coli* O157:H7 infections (Boyce et al. 1995). The estimated annual number of community cases in Canada showed that for every case of *E. coli* O157:H7 infection reported in the National Notifiable Disease registry (NND), there were an estimated 10 to 47 unreported cases annually (Thomas et al. 2006). With this level of under-reporting of cases, the need to design appropriate interventions and better assess the impact of this pathogen on the population is evident. According to an estimate from the United States Centers for Disease Control and Prevention, this pathogen causes approximately 73,000 illnesses every year in the United States alone (Rangel et al., 2005). During 1982 to 2002, 49 states in the USA reported 350 outbreaks, with 8,598 cases, 1,493 (17%) hospitalizations, 354 (4%) HUS cases, and 40 (0.5%) deaths. The transmission route was foodborne in 183 cases (52%), unknown in 74 cases (21%), person-to-person contamination in 50 cases (14%), waterborne in 31 cases (9%), involved animal contact in 11 cases (3%), and was laboratory-related in 1 case (0.3%). The food vehicle was ground beef in 75 (41%) foodborne outbreaks, while 38 outbreaks (21%) were caused by produce. Over the past few years, the number of *E. coli* O157:H7 infections in the United States has declined as consequence of improved detection and investigation of *E. coli* O157:H7 outbreaks. Also, an increase in awareness in food service establishments and by consumers regarding the risk of consumption of undercooked ground beef has been observed (MMWR, 2011). In Canada, reported cases of verotoxigenic *E. coli* infection dropped from 1086 (3.41 per 100,000 people) in 2006 to 606 (1.82 per 100,000 people) in 2009, being the lowest number ever recorded through the National Enteric Surveillance Program (NESP, 2009). However, there is a difference in completeness of reports from province to province, where high rates of *E. coli* O157 isolation

may not mean high incidence of disease, but better sampling and reporting. The opposite might also be true, where a low incidence of this pathogen may reflect poor surveillance, limiting the accuracy of numbers of foodborne illnesses in Canada. Given the significance of *E. coli* O157:H7 as a pathogen and the outbreaks linked to meat products, it is essential to develop strategies for controlling this pathogen during the manufacture of RTE meat products such as dry-fermented sausage.

After many reported outbreaks of *E. coli* O157:H7 associated with fermented meat products (Sartz et al., 2008), some interventions were forced on sausage manufacturers to reduce the frequency of *E. coli* O157:H7 contamination and protect public health. As a result, several strategies have been studied to control *E. coli* O157:H7 in fermented sausages such as different fermentation temperatures, extended storage, high-pressure, and irradiation (Holck et al., 2011). However, the potential for pathogen reduction using these interventions is about 1-3 logs $CFU.g^{-1}$ which is below the 5 log $CFU.g^{-1}$ reduction required by the US and Canadian regulations (Health Canada, 2000; USDA, 2001). According to the North American regulations, the fermented sausage manufacturing processes are only considered effective against *E. coli* O157:H7 if it is shown that the process reduces the level of this pathogen from 100,000 CFU/g to less than 1 CFU/g, which is referred as a 5 log reduction. To validate the process, the enumeration of *E. coli* O157:H7 should use direct plating procedures, where an initial inoculum level in the meat batter should achieve about 7.0 log CFU/g. The initial inoculum level was chosen to allow direct enumeration of at least a 5 log reduction in the level of the inoculum between the initial count in the meat mixture and the finished product. Therefore, after fermentation and thermal processing or drying it is expectated that the pathogen enumeration

achieves a detection limit of < 1.0 log CFU/g (Health Canada, 2000). Therefore there is a need to find alternatives that are able to control *E. coli* O157:H7 in dry-fermented sausages by causing a 5 log CFU.g^{-1} reduction in its viability during dry-fermented sausage manufacture.

2.3. Using mustard for control of *E. coli* O157:H7 in dry-fermented sausage

2.3.1 Mustard in dry-fermented sausage

Contamination of RTE meat products like dry-fermented sausage by *E. coli* O157:H7 poses major challenges for food safety and quality. The control of this pathogen is essential for the safety of dry-fermented sausage, since these products are not given a lethal thermal treatment before consumption. In addition, there is a need to address consumer preferences for food with minimal preservative addition (Wójciak et al., 2014). As a result, natural products are in demand for the control of pathogens. In this context, mustard is a promising candidate to improve the safety and quality of RTE meat products including dry-fermented sausages because it can be antimicrobial.

The antimicrobial activity of mustard is mainly associated with its essential oil components, allyl isothiocyanate (AITC) in brown or Oriental mustard, and ρ-hydroxybenzyl isothiocyanate (ρHBIT) in yellow or white mustard. A series of studies have shown the effectiveness of the antimicrobial activity of mustard in different meat products. For example, yellow mustard powder was able to reduce *E. coli* O157:H7 viability by 5 log CFU.g^{-1} in dry-fermented sausages (Graumann and Holley, 2008; Luciano et al., 2011). Hams treated with yellow mustard powder had *E. coli* O157:H7 viability reduced by 3 log CFU.g^{-1} and > 5 log CFU.g^{-1} at 21 and 45 d, respectively (Nilson and Holley, 2011). An antimicrobial polyvinyl

polyethylene glycol graft copolymer (PPG) film containing Oriental mustard extract reduced viable *L. monocytogenes* in bologna sausages at 52 d of vacuum-packed storage (4°C) to below the detection limit (Lara-Lledó et al., 2012).

Although mustard powder was able to successfully reduce *E. coli* O157:H7 numbers in meat products, including dry-fermented sausage, the generation of flavors characterisitic of mustard was a concern that it might be a limiting factor for its application in food products (Chacon et al., 2006). A thermal process was developed for use with mustard powder to inactivate the enzyme myrosinase, responsible for the hydrolysis of glucosinolates and production of ITCs that are responsible for undesirable flavors. Thermal treatment yields a bland, deheated (cold) powder that can be used as a binder or filler in cooked processed meats. It was surprising that Graumann and Holley (2008) reported cold yellow mustard powder was able to kill *E. coli* O157:H7 in dry cured sausages. They found that application of 6% (w/w) cold (deheated, deodorized or non-spicy) powder with inactive myrosinase achieved a 5 log $CFU.g^{-1}$ reduction of *E. coli* O157:H7 in fermented sausages in only 6 d compared to 24 d using 6% (w/w) hot (untreated or spicy) powder with active myrosinase. Further, Luciano et al. (2011) showed that 6% (w/w) mustard powder with myrosinase inactivated by an autoclave treatment (15 min at 115°C, with mustard powder in a 2-cm thick layer) yielded > 5 log $CFU.g^{-1}$ reduction of *E. coli* O157:H7 in 18 d. While the plant myrosinase in untreated hot mustard was responsible for the degradation of glucosinolates to ITCs, it was hypothesized that myrosinase-like activity present in *E. coli* O157:H7 and starter cultures was likely responsible for the hydrolysis of glucosinolates in the thermally-treated (deodorized) mustard.

2.3.2 Glucosinolate-myrosinase system in mustard

Glucosinolates (GLS) are organic anions containing β-D-thioglucose and sulphonated oxime moieties, which are found in the seeds, roots, stems, and leaves of plants such as mustard, broccoli, and cabbage (Vig et al., 2009). GLS are thioglucosides with a common structure characterized by a side chain with aliphatic, aromatic, or hetero-aromatic carbon skeletons (Vig et al., 2009). It has been hypothsized that GLS are derived from amino acids through a chain lengthening process and hydroxylation or oxidation. Ishida et al. (2014) concluded that aliphatic glucosinolates were derived from alanine, leucine, isoleucine, valine, or methionine, while indole and aromatic glucosinolates were derived from tryptophan and phenylalanine or tyrosine, respectively. These compounds can be found in several Brassicaceae vegetables including *Brassica rapa* (Chinese cabbage, Chinese mustard, bok choy and turnip), *B. oleracea* (cabbage, broccoli, cauliflower, kale, Brussels sprouts and kohlrabi), *B. napus* (rapeseed and rutabaga), *B. juncea* (Oriental mustard), *Raphanus sativus* (radish), *Sinapis alba* (white mustard), and the model plant *Arabidopsis thaliana* (Kissen et al., 2009; Sønderby et al., 2010; Ishida et al., 2014; CSCA, 2014). There are over 150 known glucosinolates in plants (Agerbirk and Olsen, 2012). Each plant species might contain up to four different glucosinolates in significant amounts (Fahey et al., 2001). There are a number of studies in the literature describing the glucosinolate composition of different *Brassica* species. For example, sinigrin is the major glucosinolate found in *Brassica juncea* (Oriental mustard); however, gluconapin, glucobrassicanapin, and gluconapoleiferin may also be found in this species (Ishida et al., 2014). Usually, the content of glucosinolates in Brassicaceae is influenced by environmental factors such as soil, climate and cultivation conditions (Cartea and Velasco, 2007).

Plants possessing glucosinolates also contain enzymes that hydrolyse them such as myrosinase (β-thioglucoside glucohydrolase EC 3.2.3.1.147). When glucosinolate and myrosinase come in contact with each other in the presence of water, the plant enzyme causes the hydrolysis of the glucosinolate (Vig et al., 2009). The hydrolysis products of glucosinolates have played an important role in plant resistance to insects and pathogens, animal and human nutrition, agriculture, and can even generate compounds with cancer-preventive properties (Mithen, 2001). The hydrolysis products consist of an aglycone moiety, glucose, and sulphate. The aglycone moiety is unstable and rearranges to form isothiocyanates (ITCs), thiocyanates, nitriles, and epithionitriles, depending upon the glucosinolate structure, pH, and other reaction conditions (Fig. 2.2) (Ishida et al., 2014). At physiological pH, ITCs are the major products, while nitriles are formed at more acid pH (Halkier and Du, 1997; Shofran et al., 1998). Generally, glucosinolates and myrosinase coexist in plants, but are separated from each other. Myrosinase is located in myrosin cells, while glucosinolates are likely to reside in vacuoles separated from myrosinases (Kissen et al., 2009). Glucosinolates and myrosinase come into contact after a tissue destruction event and generate the 'mustard oil bomb' system, as proposed by Luthy and Matile (1984), where the degradation of glucosinolates by myrosinase takes place only in case of tissue disruption.

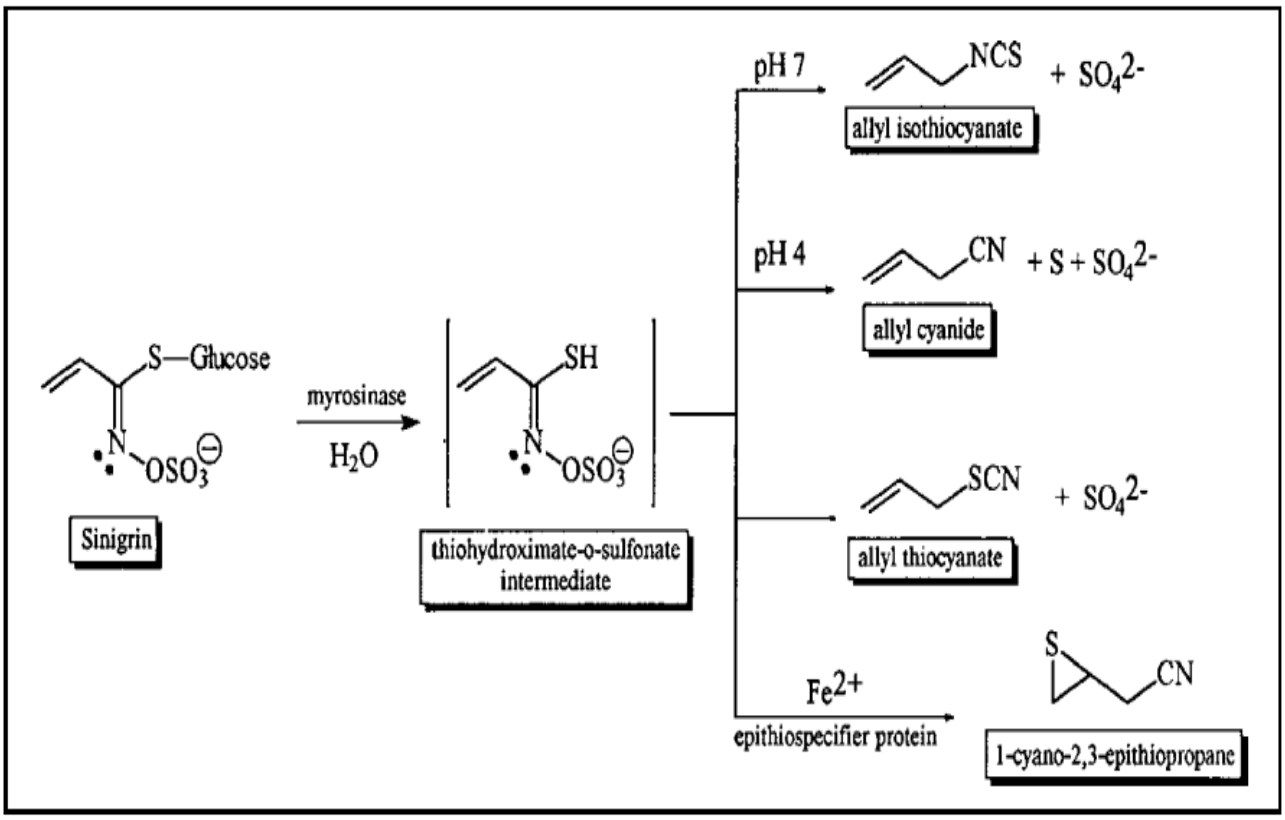

Figure 2.2. Sinigrin and its hydrolysis products (Bones and Rossiter, 1996).

2.3.3 Myrosinase activity in E. coli O157:H7

The glucosinolate-myrosinase system is believed to be part of the plant's defense against insects and pathogens. In addition to plant myrosinase, bacteria like *E. coli* (Tani et al., 1974) and *Lactobacillus agilis* (Palop et al., 1995), and fungi like *Aspergillus sp.* (Sakorn et al., 2002; Rakariyatham et al., 2006) contain myrosinase activity. Myrosinase activity is also present in many bacteria associated with the human microflora, which enables the conversion of glucosinolates to ITCs. Getahun and Chung (1999) showed that glucosinolates were converted to ITCs in humans after ingestion of cooked watercress, in which the myrosinase had been completely inactivated. Similarly, Shapiro et al. (2001) showed great bioavailability of

isothiocyanates after human ingestion of cooked broccoli sprouts. Bacteria possessing myrosinase activity have been useful in food applications where myrosinase has been inactivated, such as in the manufacture of dry-fermented sausage (Luciano et al., 2011). Myrosinase activity in starter cultures (*P. pentosaceus* and *S. carnosus*) and *E. coli* O157:H7 strain was mainly responsible for the formation of ITCs from mustard glucosinolates, which controlled the viability of this pathogen in dry-fermented sausage.

Glycoside hydrolases are enzymes that hydrolyze the glycosidic bond between carbohydrates, or between a carbohydrate and a non-carbohydrate moiety (Nong et al., 2010). Plant myrosinase is a unique class of glycosidases that catalyze the hydrolysis of *S*-linked glucosides like sinigrin present in Oriental mustard. While myrosinase is the only known *S*-glycosidase, the *O*-glycosidases including β-glucosidases, β-galactosidases, 6-phospho-*β*-glucosidases, and 6-phospho-*β*-galactosidases are extremely widespread in nature. Plant myrosinase belongs to the glycoside hydrolase (GH) family 1 along with *O*-glycosidases (Burmeister et al., 1997). There are approximately 133 glycoside hydrolase (GH) families listed in the frequently updated Carbohydrate Active enZYme (CAZY) database (Lombard et al., 2014). Each family member is assigned based on amino acid sequence similarity, which may cluster enzymes with different substrate specificity into a single family. In addition, the families can be grouped in “clans”, based on protein folding because the folding of proteins is better conserved than their sequences. In order to group GH families in clans, it is believed that the families must have a common ancestry, and be recognized by significant similarities in tertiary structure together with conservation of the catalytic residues and catalytic mechanism (Henrissat and Bairoch, 1996). There has been a significant increase in the application of glycoside

hydrolases enzymes in both agricultural and industrial settings. There has also been much progress towards developing a biotechnological understanding of these enzymes for more efficient use in animal feed (Jacobs and McAllan, 1991), in the textile industry (Miettinen-Oinonen et al., 1999), in food processing (Haarasilta et al., 1993; González-Pombo et al., 2014), and ethanol production from agricultural biomass substrates (Das et al., 2012).

Different mechanisms exist that enable bacterial uptake and metabolism of different sugars. Bacteria commonly use major facilitator superfamily (MFS) transporters, the phosphotransferase system (PTS), and ATP binding cassette (ABC) superfamily transporters (Saier, 2000). Regarding PTS, there are 7 PTS families that have been identified so far which transport different monosaccharides, disaccharides, glycosides, polyols, and other sugar derivatives (Michalska et al., 2013). The PTS system has been associated with the transport and metabolism of sugars such as glucose, sucrose, fructose in *E. coli* (Luo et al., 2014). The uptake of aromatic glycosides like arbutin and salicin in *Pectobacterium carotovorum* has been found mediated by PTS (An et al., 2005). Moreover, the uptake of salicin and disaccharides such as cellobiose and gentiobiose in the LAB and other bacteria is governed by the PTS system (Michalska et al., 2013). Considering the role played by the PTS system in the utilization of glycosides by bacteria as well as the similarity among arbutin, salicin, and sinigrin as glycosides, it is likely that the PTS system is involved in the uptake and hydrolysis of sinigrin by *E. coli* O157:H7.

The PTS system is comprised of two cytoplasmic components, enzyme I (EI) and a heat-stable phosphocarrier protein (HPr), which are common to all PTS carbohydrates. Carbohydrate

specificity resides in enzyme II (EII), and hence, bacteria usually contain many different EIIs. EII is responsible for selectively transporting sugar molecules across the inner bacterial membrane. This is accomplished in parallel with phosphorylation of the sugar, which prevents efflux of the sugar back across the membrane. This process is an important part of an extensive signaling network that allows bacteria to utilize preferred carbohydrate sources (Deutscher et al., 2006). The PTS system has been classified into four (super) families with distinct evolutionary origins based on the phylogenies of EIIs, as follows: (i) the glucose-fructose-lactose superfamily, which comprises the glucose family, the fructose-mannitol family, and the lactose family; (ii) the ascorbate-galactitol superfamily, which comprises the ascorbate family and the galactitol family; (iii) the mannose family; and (iv) the dihydroxyacetone family. Of these four superfamilies, the glucose superfamily of PTS transporters is the largest, containing five distinct subfamilies of proteins identified as the lactose family, the glucose family, the β-glucoside family, the mannitol family, and the fructose family (McCoy et al., 2015). Since sinigrin is a β-glucosidase, the relevance of superfamily 1 within the PTS system is highlighted.

Although myrosinase activity from bacterial strains derived from the human intestinal microflora or microorganisms used in food manufacturing (i.e starter cultures in dry-fermented sausage) have been reported, myrosinasehomologous genes have not been identified in microorganisms despite recent progress of genome projects (Suzuki et al., 2006). It is thought that an enzyme(s) other than myrosinase might, therefore, contribute to the bacterial degradation of glucosinolates. According to Vocadlo and Davies (2008), enzymes from the same family share similarities in their mechanism of action and, in most cases, at the level of their detailed catalytic mechanism. Thus, it possible that enzymes belonging to the glycoside hydrolase family

that share sequence similarities with plant myrosinase may be associated with the myrosinase activity of bacteria like *E. coli* O157:H7. So far, no study in the literature has investigated the role of genes from the glycoside hydrolase family in the myrosinase activity of *E. coli* O157:H7, which results in the formation of the antimicrobial compound allyl isothiocyanate.

2.4 Allyl isothiocyanate (AITC) as an antimicrobial essential oil

2.4.1 Antimicrobial activity of AITC

In recent years, there has been a growing interest in the use of natural antimicrobials, especially plant essential oils, for pathogen control. Allyl isothiocyanate (AITC) is an essential oil generated from its precursor sinigrin, which is the major glucosinolate found in Oriental mustard (Dufour et al., 2012; Lucera et al., 2012). The use of AITC has been permitted in Japan as a preservative and in the USA as a natural flavouring agent for foods (Isshiki et al., 1992; Delaquis and Mazza, 1995; Kim et al., 2002). Moreover, because of its food origin and relatively low toxicity, AITC acquired Generally Recognized as Safe (GRAS) status from the U.S. Food and Drug Administration (Isshiki et al., 1992). As a result, AITC has gained more attention as a natural preservative compared to synthetic antimicrobial agents, which elicit public concern when used in food (Li et al., 2015).

The antimicrobial activity of AITC against foodborne pathogens has been widely investigated using different approaches that have included its incorporation into packaging material and subsequent release into food, its application in a vapor form, or as a a liquid phase. Methods used to assess its antimicrobial activity commonly generate minimum inhibitory concentrations (MICs). The incorporation of AITC into antimicrobial bottle coatings was very

effective for control of *Salmonella* growth in liquid egg albumen (Jin and Gurtler, 2011). The interaction of AITC with modified atmosphere packaging (MAP) in the control of foodborne pathogens on fresh chicken breast during refrigerated storage was found to reduce growth of *L. monocytogenes* and *S.* Typhimurium by 0.77 log $CFU.g^{-1}$ and 1.3 log $CFU.g^{-1}$, respectively, when compared to controls (MAP without AITC) at 21 d (Shin et al., 2010). Microencapsulated AITC added to sausage batters reduced *E. coli* O157:H7 by 6.5 $\log_{10}$ $CFU.g^{-1}$ in sausages containing 750 and 1,000 ppm of AITC after 21 and 16 d of processing, respectively (Chacon et al., 2006). The latter also found that by adding 500 ppm AITC to the sausages, *E. coli* O157:H7 viability was reduced by 4.75 $\log_{10}$ $CFU.g^{-1}$ after 28 d of processing, with sausages being acceptable (taste, odour), despite being considered slightly spicy by sensory panelists in the sensory evaluation. Finally, concentrations of AITC at 1000 μg/ml demonstrated potential to inhibit bacterial motility and to prevent biofilm formation of several pathogenic bacteria including *E. coli* (Borgers et al., 2013).

In addition to the antimicrobial activity of AITC, this natural oil displays a synergy with conventional antibiotics that may be important for controlling or reducing antibiotic resistance in foodborne pathogens. The MIC of erythromycin and streptomycin against *Streptococcus pyogenes* (Palaniappan and Holley, 2010) and *E. coli* (Saavedra et al., 2010) was reduced by the synergistic effects of some antibiotics with AITC.

Despite the potent antimicrobial activity of AITC against foodborne pathogens, its application in food systems is restricted due to its high volatility, strong odor, poor water solubility and reactivity with natural food nucleophiles (Chacon et al., 2006; Kim et al., 2008).

While the pungency of AITC may be an undesirable sensory attribute for many consumers, its poor water solubility and decomposition in aqueous solutions decrease its antimicrobial activity (Delaquis and Mazza, 1995). AITC instability and its decomposition to new compounds occur more readily under alkaline conditions or at elevated temperatures (Pecháček et al., 1997). Strategies to reduce AITC instability and consequently increase its antimicrobial activity against foodborne pathogen have been investigated. Liu and Yang (2010) showed that an oil/water emulsion containing a medium chain triglyceride (MCT) increased AITC stability and was very effective in inhibiting *E. coli* O157:H7 during storage. Olaimat and Holley (2013) examined the interactive effects of pH and temperature on stability and antimicrobial activity of AITC against 5-strain cocktails of *Salmonella* and *L. monocytogenes*. AITC was more effective at combinations of 21°C and neutral pH against *L. monocytogenes,* while combinations of higher temperature (≥ 21°C) and acidic pH were more effective against *Salmonella*. However, studies examining the interactive effect of pH and temperature on the stability and antimicrobial activity of different concentrations AITC in aqueous media against *E. coli* O157:H7 is missing from the literature.

2.4.2 Mechanism of action of AITC

The antimicrobial activity of AITC against various foodborne pathogens including *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* has been reported (Rhee et al., 2003; Turgis et al., 2009; Luciano and Holley, 2011; Olaimat and Holley, 2013). However, the specific mechanism by which AITC inhibits microorganisms is not well understood yet. Most of the essential oils seem to exert their antimicrobial action by interacting with processes associated with the bacterial cell membrane, including electron transport, ion gradients, protein translocation,

phosphorylation, and other enzyme-dependent reactions (Burt, 2004). Based on that, several studies have investigated the mode of action of AITC in different microorganisms. Lin et al. (2000) compared the antimicrobial effect of conventional antibiotics with known mechanisms of action and AITC action against *E. coli* O157:H7, *Salmonella* Montevideo, and *L. monocytogenes*. Their data showed a great similarity between AITC and polymyxin B with respect to their antibacterial action resulting from effects on cell membranes and on leakage of cellular metabolites. Turgis et al. (2009) demonstrated that treatment with mustard essential oil (AITC) affected the membrane integrity of *E. coli* O157:H7 and *Salmonella* Typhi and caused a decrease in the intracellular ATP concentration. Also, an increase in extracellular ATP concentration and a reduction of the intracellular pH was observed in both microorganisms. Chan et al. (2013) demonstrated that AITC not only damaged cell membranes, but also disrupted cellular metabolism and energy production of microorganisms such as *Bacillus subtilis*, *E. coli*, *P. aeruginosa*, and *S. aureus*. In contrast, Ahn et al. (2001) found no leakage of ATP or damage in the cell wall when AITC was tested against *Listeria monocytogenes*, but internal levels of ATP were reduced, suggesting a reaction caused by the thiol groups of glutathione or redox-active proteins, which subsequently inhibited sulfhydryl enzyme activities and redox-based defenses. The inhibition of thioredoxine reductase and acetate kinase enzymatic activity of *E. coli* by AITC was reported by Luciano and Holley (2009).

Besides the specific studies conducted with AITC, the mode of action of other ITCs has also been described in the literature. Ganin et al. (2013) showed the ability of sulforaphane and erucin to inhibit quorum sensing (QS) by *P. aeruginosa*. Borges et al. (2013) confirmed that AITC, benzyl isothiocyanate (BITC), and 2-phenylethyl isothiocyanate (PEITC) had the capacity

to inhibit quorum sensing (QS) of *Chromobacterium violaceum* by modulation of N-acyl homoserine lactone (AHLs) activity and synthesis, interfering with the CviI/CviR QS systems, which are homologs to the LuxI/LuxR systems. Jakobsen et al. (2012) showed that the ITC, iberin, activated the expression of respiratory genes and heat shock proteins in *Pseudomonas aeruginosa*. However, the heat-shock response by ITC seems to be dependent on the conditions used. Dufour et al. (2012) performed a whole transcriptomic analysis of *Campylobacter jejuni* treated with subinhibitory concentration of BITC. They showed that heat-shock-like response in *C. jejuni* was clearly influenced by BITC with up-regulation of several heat-shock proteins including *clpB*, *dnaK*, *grpE*, *groEL*, *groES*, *cbpA*, and *hrcA* genes.

2.4.3 Mechanism of resistance to AITC

The rapid spread of antibiotic resistance has made it necessary to know whether foodborne pathogens like *E. coli* O157:H7 have the potential to develop resistance to natural antimicrobial agents and, if so, what the basis for this resistance mechanism would be. This understanding is essential to guarantee that these natural compounds will not lead to the appearance of drug resistance in bacteria. While the mode of action of several ITCs including AITC has been widely investigated, studies exploring the genetic basis for bacterial resistance to AITC are scarce in the literature. Neudecker and Henschler (1985) reported that AITC was mutagenic for *Salmonella* Typhimurium TA100. However, the mutagenicity was expressed only in the presence of a rat-liver homogenate metabolising system, suggesting an indirect process. Dufour et al. (2012) found that the GGT (Gamma Glutamyl Transpeptidase) enzyme may be involved in AITC detoxication in *C. jejuni* since a *ggt* mutant displayed decreased survival upon exposure to AITC compared to its isogenic wild-type. However, only a few *C. jejuni* isolates

encode a *ggt* gene, which suggests that the presence or absence of *ggt* does not correlate with ITC sensitivity. The resistance of *C. jejuni* to AITC probably depends on several factors, with the presence of *ggt* being only one of many genetic differences between *C. jejuni* isolates. Chan et al. (2013) postulated that the chances for *E. coli* to evolve antimicrobial resistance to AITC by mutagenesis are reduced since AITC did not induce expression of the universal DNA repair gene, *recA*. In fact, Říhová (1982) showed that AITC had no mutagenic effects on *E. coli* WP 67. However, there has been no study of the genetic basis for *E. coli* O157:H7 to overcome inhibitory concentrations of AITC.

Bacteria have a number of mechanisms that allow them to adapt to different environmental conditions. The molecular responses to environmental signals they use are complex and can depend on different two-component systems (TCS) of regulation (Tiganova et al., 2014). These TCS are essential for diverse adaptive responses in bacteria, including the expression of genes for nutrient acquisition, virulence, adaptation to stress, and antibiotic resistance (Tiganova et al., 2014). In order to adapt and survive complex environmental changes in nature, it is important that different TCS form regulatory networks and show dependencies and regulatory hierarchies (Oshima et al., 2002). An example of such regulation is the intensively studied *E. coli* acid response network. In this microorganism, at least 14 gene products are directly implicated in the biochemistry of acid resistance (AR) and at least 15 regulators govern the expression of one or more of the 14 AR genes (Foster, 2004). The TCS consists of a sensor, which is a histidine kinase (HK) capable of autophosphorylation on a conserved histidine residue, and a response regulator (RR) on which the phosphate is transferred (Sivaneson et al., 2011). The phosphate is loaded on to an aspartate residue in the conserved

receiver domain of the response regulator, and the phosphorylation event results in activation of the output domain of the regulator.

An analysis of the *E. coli* K-12 genome revealed the presence of 29 HKs and 32 RRs, but the functions of many of these systems remain unknown (Tiganova et al., 2014). However, many two-component regulatory systems (HK/RR) with known functions have been described, including the ArcB/ArcA system for modulation of the expression of numerous operons and regulons involved in respiratory and fermentative metabolism in response to oxygen deficiency or redox potential; the PhoR/PhoB system for controlling genes of the phosphate (Pho) regulon for assimilation of alternative P sources; the CpxA/CpxR system for controlling the expression of genes involved in relieving envelope protein stress, biofilm formation, motility, cell proliferation, adaptation to recovery from stationary phase, pathogenicity, and the BaeS/BaeR system for modulating genes for the efflux pump, *mdtABCD*, and a third envelope stress pathway (Zhou et al., 2003).

It is notable that the BaeSR two-component regulatory system has been found associated with the ability of *E. coli* to overcome the inhibitory effect of secondary plant metabolites. For example, Zoetendal et al. (2008) found that *E. coli* was also able to resist the inhibitory activity of tannins by a variety of mechanisms, which include genes that are under the control of the two-component BaeSR regulatory system, such as the multi-drug efflux pump genes (*mdtABCD*) and the outer membrane protein gene *spy* (spheroplast protein Y). Raffa and Raivio (2002) showed that the two-component BaeSR regulatory system was responsible for controlling the expression of *spy* in response to envelope stress. Although these studies have investigated the role of the

two-component BaeSR system in resistance and stress response of *E. coli* to tannins, there has been no study which has implicated the BaeSR regulatory system of *E. coli* O157:H7 in its response to AITC exposure.

Chapter 3

Evaluation of deodorized yellow mustard concentrations for control of *Escherichia coli* O157:H7 viability in dry-fermented sausage

3.1 Abstract

Although 6% (w/w) deodorized mustard powder can achieve the mandatory 5 Log CFU.g^{-1} reduction of *Escherichia coli* O157:H7 in 18 d during dry-fermented sausage manufacture, sensory analysis showed that mustard concentrations lower than 6% were more acceptable to consumers. In the present work the antimicrobial effectiveness of lower levels of deodorized (autoclave-treated) mustard was evaluated against *E. coli* O157:H7. Concentrations of 2% and 4% (w/w) mustard treated at 115°C for 15 min were first tested in Mueller-Hinton broth (14 d) and then during fermented sausage manufacture (42 d) against *E. coli* O157:H7. During tests in broth, 4% deodorized mustard yielded a significantly greater reduction in *E. coli* O157:H7 numbers than 2% at 14 d. Mixtures of autoclave-treated and hot mustard at 1% or 2% (w/w) each were equally or slightly less antimicrobial against *E. coli* O157:H7 in sausage than their corresponding concentrations (2% or 4%) of deodorized mustard. While 4% deodorized mustard in sausage reduced *E. coli* O157:H7 viability by 5 Log at 28 d, 2% deodorized mustard did not achieve the reduction target. Mustard addition influenced starter culture recovery by causing a reduction in *Staphylococcus carnosus* numbers, but did not change *Pediococcus pentosaceus* viability during tests. It is probable that a minimum threshold between 3 and 4% (w/w)

deodorized mustard may be required to assure control of *E. coli* O157:H7 viability in dry-fermented sausages.

3.2 Introduction

Dry-fermented sausages are non-thermally treated ready-to-eat (RTE) products which are processed and preserved through the development of low pH and water activity (aw), the use of nitrite, and the production of lactic acid by starter cultures (Incze, 1998). However, the unusual ability of *Escherichia coli* O157:H7 to survive during sausage manufacture and its low infectious dose of 10-100 cells (Feng and Weagant, 2009, Chapter 4), mean that its presence in fermented meat products is problematic. Illness outbreaks caused by *E. coli* O157:H7 contamination of dry-fermented sausage, which have been comprehensively summarized (Sartz et al., 2008; Luciano et al., 2011), resulted in the earlier adoption of strict guidelines for fermented meat manufacture in the United States (USDA, 2001) and Canada (Health Canada, 2000). One of the options proposed by the guidelines mandated the reduction of *E. coli* O157:H7 by 5 Log CFU.g^{-1}during product manufacture.

Recent studies have demonstrated that 6% (w/w) cold (deodorized, deheated or non-spicy) yellow mustard powder successfully reduced *E. coli* O157:H7 by 5 Log CFU.g^{-1}in dry-fermented sausages (Graumann and Holley, 2008; Luciano et al., 2011). The antimicrobial activity in mustard is mainly associated with essential oils including allyl isothiocyanate (AITC) in brown mustard (*Brassica juncea*), and ρ-hydroxybenzyl isothiocyanate (ρHBIT) in yellow or white mustard (*Sinapis alba*). These compounds are formed from glucosinolates by the action of endogenous myrosinase (EC 3.2.1.147) in plant tissue (Rask et al., 2000), or by myrosinase-like

activity in LAB starter cultures, *E. coli* O157:H7 (Luciano and Holley, 2010; Luciano et al., 2011) and some other bacteria (Herzallah et al., 2011). It was found that 6% (w/w) commercially deodorized mustard powder with inactive myrosinase achieved a 5 Log CFU.g^{-1}reduction of *E. coli* O157:H7 in fermented sausages in only 6 d compared to 24 d using 6% (w/w) hot (non-deheated or spicy) powder with active myrosinase. Further, when 2% or 4% hot yellow mustard powder were used, 48 d and 36 d, respectively, were required to achieve the mandatory 5 Log CFU.g^{-1}reduction (Graumann and Holley, 2008). Interestingly, 6% (w/w) mustard powder with myrosinase inactivated by an autoclave process (15 min at 115°C) was also antimicrobial and yielded >5 Log CFU.g^{-1}reduction of *E. coli* O157:H7 in 18 d (Luciano et al., 2011). The latter authors found that *E. coli* O157:H7 myrosinase-like activity was responsible for converting the glucosinolate in deodorized mustard into the antimicrobial isothiocyanate. Although hot mustard powder effectively eliminated *E. coli* O157:H7 from dry-fermented sausage, it is difficult to handle because of the odor when moist. Therefore, there is a need to determine the lowest threshold of deodorized (autoclaved) mustard powder which yields optimal reduction of *E. coli* O157:H7, and causes no adverse effect on the organoleptic characteristics of sausages. Sensory studies in this laboratory showed that overall, a consumer panel neither liked nor disliked salami containing 3% or 4% mustard (Li et al., 2013). To facilitate the use of mustard in salami by industry, the objective of the present work was to investigate whether concentrations of 2 % or 4% (w/w) deodorized yellow mustard powder would be sufficient to yield a 5 Log CFU.g^{-1}reduction of *E. coli* O157:H7 during dry sausage manufacture.

3. 3 Materials and Methods

3.3.1 Dry-fermented sausage simulation in broth

In order to assess the antimicrobial effect of lower concentrations of deodorized mustard against *E. coli* O157:H7, an *in vitro* test using broth was conducted to simulate the dry-fermented sausage environment. Mueller-Hinton broth (Oxoid, Unipath, Nepean, ON, Canada) containing ingredients present in dry-fermented sausages (Luciano et al., 2011) (2.91% salt; 0.31% pickle cure concentrate containing salt, sodium nitrite, sodium bicarbonate, and glycerol as manufacturing aids; 0.6% glucose; and 0.05% sodium erythorbate) was prepared in the absence or presence of deodorized yellow mustard powder (*Sinapis alba* L., G. S. Dunn Ltd., Hamilton, ON, Canada) at 2 and 4% (w/w). Overnight cultures of *Pediococcus pentosaceus* UM 116P plus *Staphylococcus carnosus* UM 109M isolated from commercial starter cultures (TrumarK LTII M and LTII, respectively; Rector Foods Ltd., Mississauga, ON, Canada), and a 5 strain cocktail of *E. coli* O157:H7 (00-3581, 02-0304, 02-0627, 02-0628 and non-motile 02-1840 from Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada) were used. The 5 *E. coli* O157:H7 strains included were positive for the *eae* and *hly* genes and all were $Stx_{1,\,2}$ negative, except for strain 02-1840 which was also negative for *hly* and was the only non-motile strain. These 5 strains were chosen because they demonstrated the ability to degrade sinigrin (Luciano and Holley, 2010; Luciano and Holley, 2011). Through this ability, these strains were controllable by deodorized mustard when they were used as a multi-strain contaminating cocktail during ripening of dry-fermented sausage (Luciano et al., 2011). While *P. pentosaceus* and *S. carnosus* were inoculated (0.1 mL each) in broth to achieve a final volume of 10 mL in screw-capped tubes containing 6 Log $CFU.mL^{-1}$ of each type of starter

culture, the 5 *E. coli* O157:H7 strains were inoculated to achieve 7-8 Log CFU.mL^{-1}. The inoculated tubes were maintained at 25°C and 200 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA). Numbers of viable *E. coli* O157:H7 were followed for 14 d using Sorbitol MacConkey Agar supplemented with cefixime and tellurite (CT-SMAC) (Oxoid).

3.3.2 Dry-fermented sausage manufacture

Dry-fermented sausage was manufactured as described elsewhere (Graumann and Holley, 2008) with slight modifications. *P. pentosaceus* (UM 116P) and *S. carnosus* (UM 109M) were added to 10 kg of chopped beef/pork batter along with the *E. coli* O157:H7 cocktail to yield approximately 6 Log CFU.g^{-1} of each genus. Prior to experimental use, each *E. coli* O157:H7 strain was grown in 10 mL Tryptic Soy Broth (TSB) (Oxoid, Basingstoke, England) and incubated at 35°C for 24 h. Then each strain was grown in 500 mL TSB and incubated at 35°C for 18 h. A mixture of equal numbers of the 5 *E. coli* O157:H7 strains was prepared and centrifuged for 10 min at 1650 x g (Sorvall Instruments RC-5, DuPont, Newton, CT, USA) at 4°C. Cultures were re-suspended in 0.1% (w/v) peptone water (Fisher, Fair Lawn, NJ, USA) and centrifuged as before. The supernatant was discarded, cell pellets were re-suspended in 50 mL of 0.1% (w/v) peptone water, and 50 mL of the *E. coli* O157:H7 cocktail was added to sausage batters. In this study, 2% or 4% (w/w) autoclaved mustard powder (deodorized by treatment at 115°C for 15 min) were used to assess mustard effectiveness against the *E. coli* O157:H7 cocktail during dry-fermented sausage manufacture. Two batches of sausage were produced using 3 treatments: control (no mustard powder); 2% (w/w) deodorized yellow mustard powder

lacking myrosinase activity, and a mixture of 1% (w/w) deodorized powder and 1% (w/w) hot yellow mustard (with normal myrosinase activity). A similar series of tests was done using 4% (w/w) deodorized mustard as well as with a mixture of 2% (w/w) deodorized plus 2% (w/w) hot mustard powder. The cure ingredients salt, nitrite and spices were added to the sausage batter and the fermentation/drying procedures used were as described elsewhere (Luciano et al., 2011). Briefly, sausages weighing 500 g each were fermented at 26°C for 3 d and dried at 14°C and 75% RH for 32 d. Sausages aged 35 d were placed in barrier bags (Deli*1, Winpak, Winnipeg, MB, Canada), vacuum packed (Bizerba Canada Inc., GM 2002; Mississauga, ON, Canada) and stored at 4°C.

3.3.3 Enumeration of E. coli O157:H7 and starter cultures from sausage

Inoculated sausage batter was analyzed immediately after formulation at day 0 and sausage samples were tested on days 7, 14, 21, 28, 35 and 42. For all microbial analyses, a 25 g sample was aseptically removed from the central part of the sausage and placed into a stomacher bag (Filtra-bag, VWR, Edmonton, AB, Canada) along with 225 mL of 0.1% peptone water. Then the samples were suspended by pummeling for 1 min (BagMixer 400, Intersciences Inc., Markham, ON, Canada). Serial dilutions were prepared and samples were plated using an Autoplate 4000 Spiral Plater (Spiral Biotech, Bethesda, MD, USA). Enumeration of *E. coli* O157:H7 was performed at 7 d intervals for 5 weeks during sausage ripening and then after one week storage at 4°C. Each sausage treatment was repeated twice on different occasions. Recovery of *E. coli* O157:H7 from fermented sausage containing 2% and 4% (w/w) mustard was done using CT-SMAC plates. *P. pentosaceus* and *S. carnosus* were plated on MRS agar (Oxoid)

and manitol salt agar (MSA; Oxoid), respectively. Plates were incubated at 35°C for 24-48 h. When no *E. coli* O157:H7 growth was detectable (numbers < 0.51 Log CFU.g^{-1}), selective enrichment using immunomagnetic separation with Dynabeads (Dynal Biotech, Oslo, Norway) was performed according to the manufacturer's instructions.

3.3.4 Water activity (aw) and pH of sausage

Sausage aw was analyzed with a Novasina AW-Sprint Machine (Axion AG, Pfaffikon, Switzerland). In addition, 20 g samples were suspended in 180 mL sterile distilled water in stomacher bags and pummeled for 1 min with the stomacher and then sausage pH was measured using an Accumet Basic pH meter (Fisher Scientific).

3.3.5 Statistical Analysis

Two trials with three replicates each (n = 6) were done to enumerate *E. coli* O157:H7 and starter cultures in dry sausage formulated with 2% or 4% (w/w) yellow mustard. The numbers of bacteria recovered through 42 d of testing were converted to Log10 CFU.g^{-1} and averages were calculated. The statistical significance of mean differences at $\alpha = 0.05$ were assessed by analysis of variance using JMP® 8.02 (SAS Institute Inc.) and statistical differences among treatments were compared using Tukey's test. A similar procedure was used to assess the mean differences of bacterial numbers in the broth tests.

3.4 Results and Discussion

3.4.1 Antimicrobial activity of 2% and 4% (w/w) autoclaved mustard in broth

The use of 2% and 4% (w/w) autoclaved mustard in a simulated dry sausage environment (in broth) showed that greater concentrations of autoclaved mustard caused more rapid reduction of *E. coli* O157:H7 viability (Fig.3.1). With 4% mustard, a significant reduction was noted at day 14, reaching about 2 Log CFU.ml^{-1}. At 2% mustard, the reduction became significant at day 14, but was <2 Log CFU.g^{-1}. *E. coli* O157:H7 numbers in the control without mustard increased slightly during the 14 d trials. Loss of *E. coli* O157:H7 viability upon exposure to autoclaved yellow mustard powder was due to bacterial hydrolysis of the glucosinolate sinalbin to form ρHBIT which is bactericidal (Luciano et al., 2011).

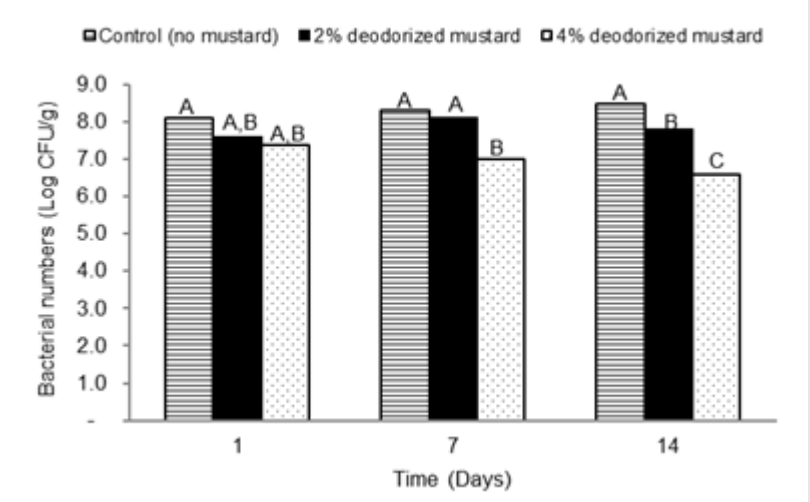

Figure 3.1: Numbers (Log CFU.ml^{-1}) of *E. coli* O157:H7 recovered from Mueller-Hinton broth containing the dry ingredients used for the production of dry-fermented sausages. Broth containing deodorized yellow mustard at 2% and 4% was compared to a control (no mustard using the same incubation conditions (25°C and 200 rpm). Treatments with different letters are significantly different.

3.4.2 Antimicrobial activity of 2% and 4% (w/w) yellow mustard in fermented sausage

The effect of mustard powder on *E. coli* O157:H7 survival in dry-fermented sausages is shown in Fig. 3.2. Although the 5 Log reduction required by the American and Canadian guidelines (USDA, 2001; Health Canada, 2000) was not achieved during the tests with 2% (w/w) autoclaved mustard powder, this concentration was antimicrobial to *E. coli* O157:H7. At day 42, the control treatment (no mustard) yielded ~ 2 Log reduction of this pathogen, while > 4 Log $CFU.g^{-1}$ reduction was achieved with addition of 2% autoclaved mustard during the same period. This additional 2 Log decrease in *E. coli* O157:H7 viability caused by 2% mustard was also observed when the mixed mustard preparation (1% deodorized + 1% hot) containing some myrosinase was used. The 4% deodorized mustard powder was able to reduce *E. coli* O157:H7 numbers by > 5 Log $CFU.g^{-1}$ at 28 d. Since glucosinolate degradation follows first-order kinetics (Oerlemans et al., 2006), it was expected that at low concentrations of mustard more time would be required to achieve the 5 Log reduction of *E. coli* O157:H7. Thus, the rate of glucosinolate degradation was dependent on mustard concentration, and reached its maximum during the first week when the glucosinolate concentration of 4% deodorized mustard was highest. This kinetic pattern explains in part the additional 2 Log reduction reached with 4% deodorized mustard when compared to the control treatment at 7 d (Fig. 3.2).

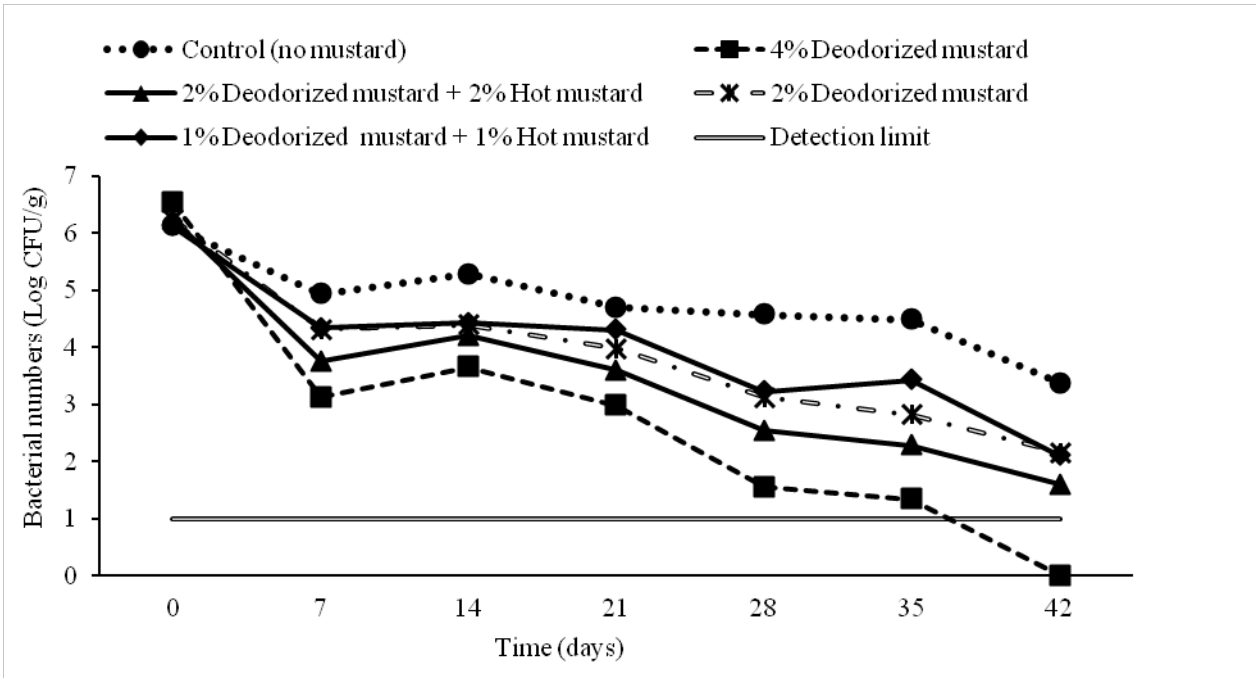

Figure 3.2: Comparison of the number (Log CFU.g^{-1}) of *E. coli* O157:H7 recovered during fermentation and drying of sausage.

As with glucosinolate degradation, the myrosinase-like activity of *E. coli* O157:H7 and starter cultures would likely be greater at higher mustard concentrations. Tani et al. (1974) showed that myrosinase from *Enterobacter cloacae* is an induced enzyme and its highest production occurred at 0.3% or 6% sinigrin and mustard extract, respectively. Glucosinolate (sinalbin) concentrations in sausages tested here would have been <0.1% based on earlier analyses (Lara-Lledó et al., 2012). Unfortunately, little is known about other factors governing the synthesis of bacterial myrosinase-like activity. It was unexpected that the 4% mixed mustard (2% deodorized + 2% hot) treatment would be less effective, requiring additional time to achieve

a 5 Log CFU.g^{-1}reduction of *E. coli* O157:H7 viability than 4% deodorized mustard (Fig. 3.2), since Luciano et al. (2011) observed the reverse effect. This might have been due to some thermal instability of the glucosinolate during autoclave treatment. However, the latter authors reported sinalbin was stable following treatment at 115°C for 15 min, but other work has shown that indole glucosinolates from cabbage were significantly degraded by treatment with boiling water for 3 min (Slominski and Campbell, 1989). Luciano et al. (2011) used a mixture of 3% (w/w) deodorized and 3% (w/w) hot mustard and suggested that autoclave treatment of mustard generated phenolic compounds and Maillard reaction products, which might enhance the antimicrobial action of isothiocyanates.

In the 2% deodorized/2% hot mustard mixture used here, a threshold inhibitory level of these compounds might not have been reached and this may explain its lower than expected antimicrobial effects. While 2% and 4% mustard powder had no effect on numbers of *P. pentosaceus* (which remained stable throughout the experiment, Table 3.1), a slight reduction in *S. carnosus* numbers was observed (Table 3.2).

Table 3.1: Comparison of the number (Log CFU.g^{-1}± SD)[a] of *P. pentosaceus* recovered during fermentation and drying of sausage

Day	Control (no mustard)	4% Deodorized mustard	2% Deodorized mustard + 2% Hot mustard	2% Deodorized mustard	1% Deodorized mustard +1% Hot mustard
0	7.84 ± 0.10a	8.07 ± 0.74a	7.81 ± 0.20a	7.88 ± 0.11a	7.97 ± 0.12a
7	8.26 ± 0.11a	8.00 ± 0.63a	8.00 ± 0.65a	8.31 ± 0.16a	8.27 ± 0.25a
14	8.14 ± 0.07c	7.39 ± 0.10d	8.47 ± 0.09a	8.09 ± 0.06c	8.27 ± 0.13b
21	8.20 ± 0.05bc	8.15 ± 0.32c	8.62 ± 0.42a	8.33 ± 0.20abc	8.49 ± 0.09ab
28	8.22 ± 0.08b	7.61 ± 0.26d	8.34 ± 0.15ab	8.02 ± 0.07c	8.44 ± 0.05a
35	8.12 ± 0.16b	7.89 ± 0.15c	8.41 ± 0.13a	7.91 ± 0.19c	8.26 ± 0.16ab
42	8.05 ± 0.21bc	7.94 ± 0.26c	8.65 ± 0.12a	7.60 ± 0.32d	8.25 ± 0.16b

[a]Values are the mean ± SD of two trials replicated three times (n=6). Within the same row, means with different letters are significantly different ($P< 0.05$).

Table 3.2: Comparison of the number (Log CFU.g^{-1}± SD)[a] of *S. carnosus* recovered during fermentation and drying of sausage

Day	Control (no mustard)	4% Deodorized mustard	2% Deodorized mustard + 2% Hot mustard	2% Deodorized mustard	1% Deodorized mustard +1% Hot mustard
0	6.35 ± 0.34a	6.24 ± 0.14ab	6.11 ± 0.16b	6.31 ± 0.14ab	6.31 ± 0.13ab
7	6.11 ± 0.25a	5.83 ± 0.56ab	5.29 ± 0.25c	5.70 ± 0.15b	5.70 ± 0.15b
14	6.02 ± 0.24a	5.05 ± 0.12c	5.14 ± 0.29c	5.55 ± 0.36b	5.77 ± 0.25ab
21	5.82 ± 0.53a	4.76 ± 0.24b	4.80 ± 0.62b	5.85 ± 0.31a	6.25 ± 0.26a
28	5.81 ± 0.52a	4.80 ± 0.37cd	4.50 ± 0.39d	5.13 ± 0.17bc	5.38 ± 0.18b
35	5.74 ± 0.53a	4.57 ± 0.33c	4.50 ± 0.39c	5.07 ± 0.18b	5.35 ± 0.11ab
42	6.17 ± 0.21a	4.64 ± 0.19d	5.11 ± 0.22c	5.06 ± 0.14c	5.54 ± 0.12b

[a]Values are the mean ± SD of two trials replicated three times (n=6). Within the same row, means with different letters are significantly different ($P< 0.05$).

This was consistent with previous work that showed *P. pentosaceus* and *S. carnosus* were more resistant to ρHBIT than *E. coli* O157:H7 (Luciano and Holley, 2010). In addition, both starter cultures are capable of degrading glucosinolates in mustard, enhancing its antimicrobial action against *E. coli* O157:H7. Storage of sausages under vacuum at 4°C was used after 35 d ripening for one week to prevent further drying of ripened products. This is normal commercial practice for≥ 6 months, and in the present tests had no measurable effect on starter culture viability. Storage under these conditions did allow for further reduction in *E. coli* O157:H7 viability.

At present the relative contributions to the overall antimicrobial activity of mustard made by phenolic compounds, Maillard reaction products, endogenous mustard or microbial myrosinase activities, as well as glucosinolate concentrations and stability in the mustard powder are not fully understood. The work reported is part of a systematic evaluation of these factors for their contribution. It is possible that inactivation of plant myrosinase by treatment at 115^{o}C for 15 min may be dependent upon the original moisture content of the mustard seed (normally 6%). This, as well as contributions to bacterial myrosinase synthesis and activity by intrinsic factors are under active study. The observation here that deodorized mustard powder alone was significantly more antimicrobial than an equal mixture of deodorized plus hot mustard powder is unexplained, but may lead to optimization of the antimicrobial system through better understanding of its important elements.

3.4.3 Water activity (a_w) and pH of fermented sausage

The initial pH values of the different sausage (salami) treatments ranged from 5.7 to 5.8 and these rapidly decreased during fermentation to <5.0 within 48 h. This change brought the process in compliance with degree•hour limits which require a reduction to pH 5.3 within 63.8 h at a fermentation temperature of 26ºC (Health Canada, 2000). The initial aw of the salami batter (0.97) decreased to < 0.96 at day 7 in all treatments, and reached values < 0.92 after day 14. Final product aw at day 42 was between 0.85 and 0.83, while pH values were between 4.9 and 4.6 for the treatments. These values are typical of those available commercially in North America.

3.5 Conclusion

Sausage manufacture with 2% (w/w) mustard formulations caused reductions in *E. coli* O157:H7 viability of about 3 Log CFU/g up to day 35 of ripening. Thus, this level was not adequate to satisfy the 5 Log *E. coli* O157:H7 reduction requirements in both the United States and Canada. However, a 5 Log reduction of *E. coli* O157:H7 viability was reached at day 28 with 4% (w/w) deodorized mustard powder. Mixtures of mustard containing active plant myrosinase were less antimicrobial than their fully deodorized counterpart at either 2 or 4% (w/w). Starter cultures numbers were not affected by these levels of mustard, nor were changes caused in product pH or aw. Since dry sausage containing >2% (w/w) mustard seems to be less acceptable to consumers (Li et al., 2013), additional work is planned to explore different starter culture ratios and ripening parameters (temperature, relative humidity) with deodorized mustard

fractions containing different levels and types of glucosinolates in order to achieve suitable reductions of *E. coli* O157:H7 in dry-fermented sausages.

Chapter 4

Contribution of endogenous plant myrosinase to the antimicrobial activity of deodorized mustard against *Escherichia coli* O157:H7 in fermented dry sausage

4.1 Abstract

This work investigated the antimicrobial activity of residual endogenous plant myrosinase in Oriental and yellow mustard powder and deoiled meal (which contained more glucosinolate than unextracted mustard powder of each type of mustard), against *Escherichia coli* O157:H7 during dry-fermented sausage ripening. When small amounts of "hot" mustard powder or meal containing endogenous plant myrosinase were added to fully-deodorized powders and meal of the same type, pathogen reduction rates were enhanced. The higher glucosinolate level in the deoiled mustard meal enabled the use of 50% less mustard in dry sausage to achieve the mandatory $\geq$ 5 Log CFU.g^{-1} reduction of *E. coli* O157:H7. The myrosinase-like activity present in *E. coli* O157:H7 contributed to glucosinolate hydrolysis in sausages with fully-deodorized, deoiled mustard meal, although the period necessary for a 5 Log pathogen reduction was 14 d longer. Yellow mustard derivatives were more potently antimicrobial than Oriental mustard.

4.2 Introduction

The inhibition of *Escherichia coli* O157:H7 in ripening dry-fermented sausage by addition of ground mustard has been reported in the literature for a range of conditions. Graumann and Holley (2008) reported that 6% commercially deodorized (devoid of myrosinase activity) yellow mustard caused a 5 log reduction of *E. coli* O157:H7 viability in dry-fermented sausage at 24 d of ripening, while hot mustard of the same cultivar with active myrosinase achieved the same reduction by 30 d. Luciano et al. (2011) showed that 6 % yellow mustard deodorized in the laboratory (a 2 cm layer heated at 115^{o}C for 15 min to inactivate plant myrosinase) and a 6% mixture of half-deodorized/half-hot (with active myrosinase) yellow mustard were able to eliminate the pathogen by 18 d of sausage ripening. This antimicrobial activity was attributed to the hydrolysis of glucosinolates by bacterial myrosinase-like activity found in *E. coli* O157:H7 and meat starter cultures (Luciano et al., 2011). It was concluded that the antimicrobial activity was generated from the organoleptically neutral glucosinolate precursor by its bacterial hydrolysis to the corresponding isothiocyanate (ITC) (Luciano and Holley, 2011). The ITC formed by myrosinase action in brown or Oriental mustard (*Brassica juncea*) is allyl isothiocyanate (AITC) and in yellow or white mustard (*Sinapis alba*) the ITC formed is ρ-hydroxybenzyl isothiocyanate (ρ-HBITC).

Legislation that requires fermented dry sausage manufacturing methods to achieve ≥ 5 Log CFU.g^{-1} reduction of *E. coli* O157:H7 is more stringent in Canada and the United States (Health Canada, 2000; USDA, 2001) than other countries (Holck et al., 2011). A variety of approaches have been used to maximize reduction of this pathogen in these uncooked products (low heat, freeze-thaw, extended storage, high pressure, and irradiation) with limited success (Holck et al., 2011), in part due to regional manufacturing and formulation differences in the

products, costs of the intervention, as well as the generation of undesirable sensory properties in treated products (Heir et al., 2013). Work has been done using various concentrations (2 to 6% w/w) of hot (spicy) and deodorized (organoleptically neutral) yellow mustard, and results have been promising but somewhat variable (Graumann and Holley 2008; Luciano et al., 2011; Cordeiro et al., 2013). With 4 to 6% deodorized mustard from 18 to 38 d was necessary to satisfy the North American *E. coli* O157:H7 reduction requirements, but the normal production period for commercial products of the size tested (0.5 kg) is 35 d. In addition, at deodorized mustard concentrations ≥ 3%, consumers were able to detect its presence in these products (Li et al., 2013). The inconsistency in the rate of *E. coli* O157:H7 elimination observed in the previous studies raised questions about reasons for variability in the antimicrobial action of mustard. However, the development of resistance to the ITC produced from mustard glucosinolate was unlikely (Cordeiro et al., 2014b). During the 6 year period over which these studies were conducted, deodorized yellow mustards from commercial sources and those created by laboratory methods were used to obtain material containing inactive plant myrosinase and minimize the effects of mustard on sensory quality. Since myrosinase(s) from mustard and related Brassicaceae are unusually resistant to thermal denaturation (Van Eylen et al., 2008), the present work was undertaken to determine whether the laboratory deodorization process used permitted some plant myrosinase to occasionally remain active in the deodorized mustard, and whether it contributed to the differences in rates of *E. coli* O157:H7 reduction noted in these products during ripening. Another source of variation in control of the pathogen by mustard could have been the concentration and type of glucosinolate present in the mustard. Some *in vitro* work has shown that AITC from sinigrin in Oriental mustard was more potently antimicrobial against *E. coli* O157:H7 than ρ-HBITC from sinalbin in yellow mustard (Luciano

and Holley, 2011), but data comparing the antimicrobial effects of the two types of mustard glucosinolates in sausage do not exist. An approach that could prove useful to reduce the concentration of mustard needed to control *E. coli* O157:H7 might be the use of cold-pressed mustard seeds after oil extraction (deoiled meal or cake) which has elevated glucosinolate levels.

The objectives of this work were to investigate the prospect that the antimicrobial activity of deodorized mustard noted in sausage had been influenced by the inadvertent presence of residual plant myrosinase in deodorized mustard, and to examine whether a small amount of hot mustard might reduce the variability in the antimicrobial effectiveness of fully-deodorized mustard powder against *E. coli* O157:H7 in dry sausages. To attempt to reduce the concentration of mustard needed to achieve ≥ 5 Log.g^{-1} reduction of *E. coli* O157:H7 in sausages, deoiled mustard meal was used. Further, in these tests powder and deoiled meal from yellow mustard were compared with equivalent preparations from Oriental mustard for the first time in ripening fermented dry sausage.

4.3 Materials and Methods

4.3.1 Mustard samples and thermal treatments

Two groups of trials were conducted with different varieties of mustard. For the first series, yellow (#106) and Oriental (#107F) mustard powders from G.S. Dunn (Hamilton, ON, Canada) were used. Mustard powders were fully-deodorized using a 1 cm thick layer of sample in a tray during treatment in an autoclave at 115°C for 15 min. After deodorization, mustard was chopped into a fine powder using a blender (Model 33BL37, Waring, New Hartford, CT, USA) to facilitate its more uniform distribution in the sausage batter. These fully-deodorized mustard powders were added to sausage batter after being mixed to contain either 2.5% or 5% (w/w) hot

(untreated) yellow and Oriental mustard powders (hot yellow to deodorized yellow and hot Oriental to deodorized Oriental) yielding mixtures of the two separate types of mustard with predictable amounts of myrosinase. When the 2.5% and 5% mixtures were added to sausages at 4% (w/w), there was 0.1% and 0.2% (w/w) of hot mustard in the meat batters, respectively. In addition, partially-deodorized mustard powders (P-d) were prepared by heating a 2 cm thick layer of sample at 115^{o}C for 15 min in an autoclave. Deodorized mustard powders (P-d) were also chopped to form a fine powder before being added to the sausage batter. Residual myrosinase activity was detected by monitoring changes for 120 h in glucosinolate content by RP-HPLC. Partially-deodorized yellow and Oriental mustards at 4 % (w/w) without the addition of hot mustard were used as two independent treatments.

In the second set of trials, deoiled yellow (DYM) and deoiled Oriental (DOM) mustard meal supplied by Sakai spice (Lethbridge, AB, Canada) were used. The DYM and DOM were also fully-deodorized by autoclave treatment of samples in 1 cm thick layers and chopped to a fine particle size using the blender. These mustard meals were added to dry sausage separately or after being mixed to contain 5% hot deoiled yellow and Oriental mustard meal before addition to the sausage batter, yielding mustard meal mixtures with predictable amounts of myrosinase. The mixtures were added to achieve a total concentration of 2% (w/w) of mustard in sausages. Thus, for the 5% mustard mixture, 0.2% hot mustard was present in the meat batter.

4.3.2 Verification of the absence or presence of active myrosinase in mustard treatments

In these tests, analytical standards and purified myrosinase (EC 3.2.1.147, thioglucoside glucohydrolase) were used as positive controls. Sinigrin hydrate and myrosinase were from Sigma-Aldrich (St Louis, MO, USA) and sinalbin hydrate was from AppliChem Inc. (St Louis, MO, USA). For all mustard treatments, the presence or absence of myrosinase was confirmed by

RP-HPLC before their use in sausage manufacture as described below. In the first experiments, the naturally occurring glucosinolates and myrosinase in mustard mixtures were separately extracted and used as reagents in assays instead of purified materials because of cost considerations. This was done by adding the myrosinase extracted from mustard to the extracted glucosinolate and detecting the presence of myrosinase activity in each mustard mixture by measuring changes in glucosinolate content, as well as the lack of its activity in an extracted glucosinolate control without added myrosinase.

The glucosinolate substrates sinalbin and sinigrin used for the tests were extracted from yellow and Oriental mustard powder, respectively. For glucosinolate extraction, mustards were defatted by hexane treatment before aqueous extraction. Mustard was then stirred with boiling sodium phosphate buffer (10 mM, pH 6.5) for 1 h at 300 rpm using a sample-water ratio of 2 % (w/w). After cooling to room temperature, sample mixtures were centrifuged for 20 min at 12000 xg and 4^{o}C (Avanti® J-26 XP, Beckman Coulter Inc., Mississauga, ON, Canada) and the collected supernatant was filtered through No. 40 ashless Whatman paper (Fisher Scientific, Whitby, ON, Canada) (Tsao et al., 2002). All mustard samples were extracted in triplicate. After extraction, and based on initial analyses by RP-HPLC, sinigrin and sinalbin concentrations in the extracts were adjusted to yield 8 mM by dilution in distilled water. Further analysis indicated that glucosinolate concentrations and pH were essentially identical among the substrates. All assays were conducted at 22^{o}C ± 1 in duplicate.

For myrosinase extraction, a 10 g sample of each mustard mixture to be tested after a deodorization treatment (115^{o}C for 15 min, arranged in either a 1 or 2 cm layer in a metal tray) was stored at -20^{o}C overnight and then extracted for 30 min with 50 ml ice-cold 0.1 M sodium phosphate buffer (pH 6.5) containing 0.01 M mercaptoethanol. Each sample mixture was

centrifuged at 12000×g, 20 min, 0^{o}C (Avanti® J-26 XP, Beckman Coulter) to separate the insoluble components and the supernatant was retained. Myrosinase was precipitated from the supernatant by the addition of ammonium sulfate to 30-80 % saturation and pelleted by centrifugation at 12000×g for 20 min at 0^{o}C (Ghawi et al., 2012) . The pellet was collected and re-suspended in 10-fold diluted sodium phosphate buffer as noted above. The procedure was conducted on ice as much as possible to prevent enzyme inactivation. The extracted myrosinase solution (50 µl) was then reacted individually with 1 ml extracted substrate (8 mM glucosinolate). Plant myrosinase in extracted mustard substrates had been inactivated by immersion in a boiling water bath for 1 min before myrosinase extracts were added. A negative control without the addition of myrosinase extract was included to examine the stability of the substrate after the boiling water bath treatment. No change in substrate concentration in the control (without hot mustard addition) over 120 h was used to indicate substrate stability. When the mixed (hot/deodorized) mustard treatments to be used for sausages were tested, samples were not further heated and enzyme activity was confirmed present by a decline in substrate levels over 120 h using the RP-HPLC.

For the next set of experiments, the absence of myrosinase in mustard meals was verified as described by Luciano et al. (2011) with slight modification. Briefly, 5 g of heat-treated mustard were mixed with 250 ml distilled water and held for 3 h at room temperature. The sample mixtures were centrifuged for 20 min at 12000 xg and 4^{o}C, and the collected supernatant was filtered through a 0.22 µm PES syringe filter (VWR Scientific, Toronto, ON, Canada). Analysis was conducted by RP-HPLC and the stability of glucosinolate levels was used to confirm inactivation of myrosinase in the deodorized mustard meal. In these trials, glucosinolate determination and separation was conducted by RP-HPLC using a C18 column (Gemini, 5 µm,

Phenomenex, Torrance, CA, USA). Isocratic elution was carried out for 10 min at a flow rate of 1 ml/min, using a solvent system containing 20 % (v/v) acetonitrile and 80 % water containing 0.02 M pH 5.5 tetrabutylammonium hydrogen sulfate (TBA) as an ion-pair agent. The injection volume used was 5 µl and the detector absorbance was set at 227 nm (Tsao et al., 2002; Luciano et al., 2011; Herzallah and Holley, 2012).

4.3.3 Dry-fermented sausage manufacture

The bacterial strains used in the manufacture of dry-fermented sausage were prepared as described by Luciano et al. (2011) with adaptation. Briefly, a 5 strain cocktail containing equal numbers of *E. coli* O157:H7 strains 00:3581, 02:0304, 02:0627, 02:0628 and non-motile 02:1840 from Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada, and *Staphylococcus carnosus* UM109M (commercial starter culture Trumark LTII, Rector Foods Ltd., Mississauga, ON, Canada) were used. These were cultured overnight twice in 10 ml Brain Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) at 35°C, then transferred and incubated in 500 ml BHI before use. *Pediococcus pentosaceus* UM116P (Trumark LTII M, Rector Foods Ltd.) was incubated at 35°C in de Man Rogosa Sharpe (MRS) broth (Oxoid). Bacterial cultures were centrifuged (Avanti® J-26 XP, Beckman Coulter) twice at 4225xg for 20 min at 4°C and washed with 0.1 % peptone water before being added to the meat batter. For each trial, two batches of dry-fermented sausage were manufactured as described elsewhere (Cordeiro et al., 2013) with slight modification. *P. pentosaceus* (UM 116P) and *S. carnosus* (UM 109M) were added to 10 kg of chopped beef/pork batter along with the *E. coli* O157:H7 cocktail to yield approximately 6-7 Log $CFU.g^{-1}$ of each genus. Formulation and manufacture of dry-fermented sausages were adapted from Luciano et al. (2011), but glucose content was reduced from 0.6 % to 0.4 % (w/w). The relative humidity of the smoke house

(AFR-Fishmaster, Rauch und Warmetechnik GmbH, Reichenau, Germany) during the drying stage was increased from 75 % to 85 % to compensate for increased internal airflow speeds and prevent rapid drying. Sausages aged 35 d were placed in oxygen barrier bags (Deli*1, Winpak, Winnipeg, MB, Canada), vacuum-packed (GM 2002, Bizerba Canada Inc., Mississauga, ON, Canada) and stored at 4°C.

4.3.4 Enumeration of E. coli O157:H7 and starter cultures from sausages

Bacterial numbers were analyzed according to Cordeiro et al. (2013). Inoculated sausage batter was analyzed immediately after formulation at day 0. Subsequent analyses were conducted at 6 and 7 d intervals for trial 1 and 2, respectively, until the end of ripening. For all microbial analyses, a 25 g sample was aseptically removed from the central part of the sausage and placed into a stomacher bag (Filtra-bag, VWR, Edmonton, AB, Canada) along with 225 mL 0.1% peptone water. Serial dilutions were prepared and samples were plated using an Autoplate 4000 Spiral Plater (Spiral Biotech, Bethesda, MD, USA). Recovery of *E. coli* O157:H7 from fermented sausage was done using CT-SMAC (Oxoid) plates. *P. pentosaceus* and *S. carnosus* were plated on MRS agar and Mannitol Salt agar (MSA, Oxoid), respectively. Plates were incubated at 35ºC for 48 h. When no *E. coli* O157:H7 growth was anticipated on plates (numbers $\leq$ 1 Log CFU.g^{-1} was the detection limit where 500 µl sample was spread on each of two plates), selective enrichment was done by adding 25g sausage to 225 ml BHI broth. After incubation at 35^{o}C for 48 h, 1 ml was used for immunomagnetic separation (detection limit $\leq$ 1 Log CFU.g^{-1}), which was performed with Dynabeads (Dynal Biotech, Oslo, Norway), according to the manufacturer's instructions.

4.3.5 Water activity (aw) and pH of sausages

In the first trial, two or 4 sausages were used for aw (n = 2) or pH (n = 4) analyses, respectively, at day 0 and at 6 d intervals until the end of ripening. For the second trial, 6 sausages were used (3/experiment) for aw and pH analyses (n = 6) at day 0 and every 7 d until the end of ripening. Sampling and analysis methods used followed the guidelines issued by Health Canada (2000) and were the same as in previous work (Graumann and Holley, 2008; Luciano et al., 2011; Nilson and Holley, 2012). The moisture content in different mustard samples was analyzed using a moisture meter (model: IR35, Denver Instruments, Bohemia, NY, USA). The method used followed the manufacturer's instruction.

4.3.6 Statistical analysis

Statistical analyses were conducted as described by Luciano et al. (2011). In the first and second trial containing mustard powders and meals, respectively, all values presented are averages from two individual experiments (i.e. two batches) conducted in triplicate (n = 6). Three individual sausage samples from each experiment were used for microbiological analyses. Treatments were analyzed for changes in bacterial numbers as a function of mustard concentration during sausage ripening. Changes in bacterial numbers, aw and pH were analyzed by one-way analysis of variance (ANOVA) and statistical differences among mustard treatments were compared using Tukey’s test in JMP 10.00 (SAS Institute Inc., Cary, NC, USA). Data used were represented by means ± SEM. A *P* value of 0.05 was used as the cut-off for statistical significance.

4.4. Results and discussion

4.4.1 Myrosinase activity in mustard powders after autoclave treatment

In the first trial with yellow (#106) and Oriental (#107F) mustard powders, the presence of myrosinase activity was indicated by continuous substrate decline during incubation for 120 h, whereas the absence of myrosinase activity was evident by no change in substrate concentration as noted in the control (Fig. 4.1). Where there was no change in substrate concentration, samples had been heat-treated in a 1 cm thick layer; however, the partially deodorized (P-d) treatments which showed evidence of myrosinase activity had been given the same thermal exposure but a 2 cm thick layer was used during heating. Thus, when a 1 cm thick layer of powdered mustard was treated at 115^{o}C for 15 min, myrosinase present was consistently inactivated. In some of the earlier work (Luciano et al., 2011), it is possible that residual endogenous plant myrosinase was present in deodorized yellow mustard and contributed to the inconsistent rates of *E. coli* O157:H7 reduction seen.

It is unlikely that differences in moisture content were responsible for variation in myrosinase inactivation because both mustard powder and meal samples had similar and relatively low moisture content (3.4 to 6.4 %, w/w); however, low moisture may have contributed to myrosinase thermal stability. Further, it was of interest that for the same amount of hot mustard in the mixed hot/deodorized samples, yellow mustard mixtures showed greater myrosinase activity and ability to hydrolyze substrate than Oriental mixtures (Fig. 4.1). This may have been related to differences in enzyme efficiency but there was about twice as much sinalbin (mean = 223 μmol.g^{-1}) in yellow than sinigrin (mean = 114 μmol/g) in Oriental mustard samples on a molar basis. Sinalbin was exclusively present in yellow mustard and only sinigrin was present in Oriental mustard as has been previously noted (Cui and Eskin, 1998).

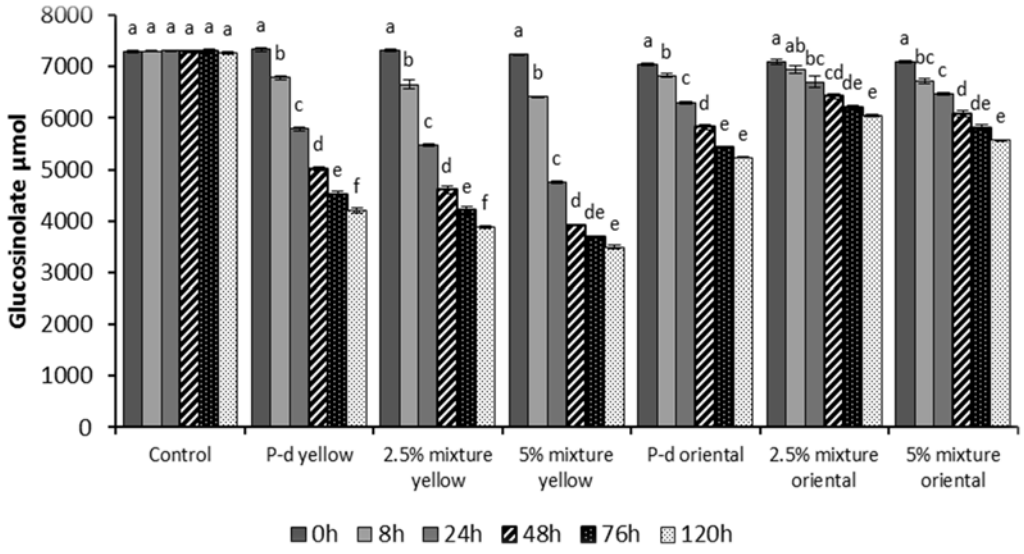

Figure 4.1: Measurement of the presence of myrosinase activity by substrate decline in mustard powder. Values represent the mean ± standard error of three separate trials (n = 3). Different letters on vertical bars in the same group indicate a significant difference ($P < 0.05$) from others within the group. Glucosinolate substrates sinalbin and sinigrin used were extracted from yellow and Oriental mustard powder, respectively. P-d = mustard powder partially deodorized by heating in a 2 cm thick layer.

4.4.2 Inactivation of myrosinase in mustard meal

In the second trial, complete inactivation of myrosinase in the mustard meal was achieved by autoclave treating 1 cm layers of material. This was confirmed by the stability of glucosinolate concentrations during 72 h measurement using RP-HPLC (Fig. 4.2). Concentrations of glucosinolates in deoiled yellow meal were substantially greater than those in deoiled Oriental meal. RP-HPLC assays also showed that both deoiled yellow and Oriental mustard meal contained greater glucosinolate concentrations (271 and 148 µmol/g, respectively) than mustard powders (yellow #106 had 246 µmol.g^{-1}and Oriental #107F had 82 µmol.g^{-1}). The results from both these trials demonstrated that when placed in layers of 1 cm thickness and treated at 115^{o}C for 15 min, myrosinase in both mustard powder and meal was inactivated.

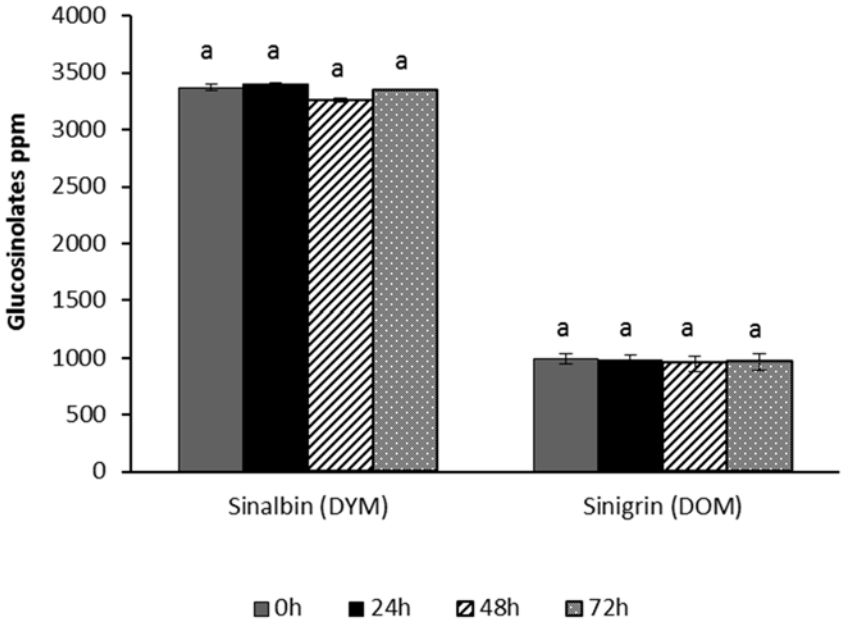

Figure 4.2: Measurement of the absence of myrosinase activity by substrate stability in mustard meal. Values represent the mean ± standard error of two separate trials (n = 2). Different letters on vertical bars in the same group indicate a significant difference ($P < 0.05$) from others within the group. The glucosinolate substrates sinalbin and sinigrin were extracted from deoiled yellow (DYM) and deoiled Oriental (DOM) mustard meals, respectively.

4.4.3 Antimicrobial acticity of mustard powders against E. coli O157:H7

It was found that a 5 log reduction of *E. coli* O157:H7 occurred within 18 d in sausages formulated with 4% (w/w) fully-deodorized yellow mustard powder (#106) containing 0.1 % or 0.2 % hot yellow mustard powder (Fig. 4.3). Similar results were obtained when the same yellow mustard powder (#106) was purposely partially-deodorized. For these three yellow mustard treatments, *E. coli* O157:H7 was not detectable after 24 d. However, the presence of *E. coli* O157:H7 was confirmed following enrichment and immunomagnetic separation at all intervals where plating gave ≤ 1 log CFU.ml^{-1}. In addition, when either 0.1 % and 0.2 % hot yellow or

Oriental mustard powders were used, there was no significant difference in the rates of *E. coli* O157:H7 elimination. Since the pathogen reduction target was achieved with yellow mustard powder treatments, the lower proportion of hot yellow mustard in the mixture (0.1%) might be successfully used for *E. coli* O157:H7 control. In contrast, the antimicrobial activity of Oriental mustard powder (#107F) given the same thermal treatment and with the same proportions of hot mustard powder in mixtures, failed to cause a 5 log reduction, even at 36 d of ripening (Fig. 4.3). The myrosinase activity of yellow mustards, based on a more rapid decline in glucosinolate content, was shown to be higher than that of Oriental samples (Fig. 4.1). This may mean that the antimicrobial potential of yellow mustard is greater than Oriental because of greater ITC (i.e. ρ-HBITC) formation following glucosinolate hydrolysis. The greater glucosinolate (sinalbin) content of yellow mustard than sinigrin in Oriental samples is likely another factor affecting the glucosinolate conversion rate because myrosinase activity follows first order kinetics (Ghawi et al., 2012; Van Eylen et al., 2007). Since the same amount of mustard was added to all sausages, the glucosinolate concentration in sausages with yellow mustard would have been higher. This would have enabled faster substrate hydrolysis in the presence of an adequate enzyme concentration. Although AITC derived from sinigrin hydrolysis has been widely reported to be an effective antimicrobial (Fujita et al., 1999; Suppakul et al., 2003; Nadarajah et al., 2005), it is likely that 4 % (w/w) Oriental mustard did not yield sufficient substrate to enable the AITC produced to reach threshold levels adequate to eliminate *E. coli* O157:H7 from the sausages.

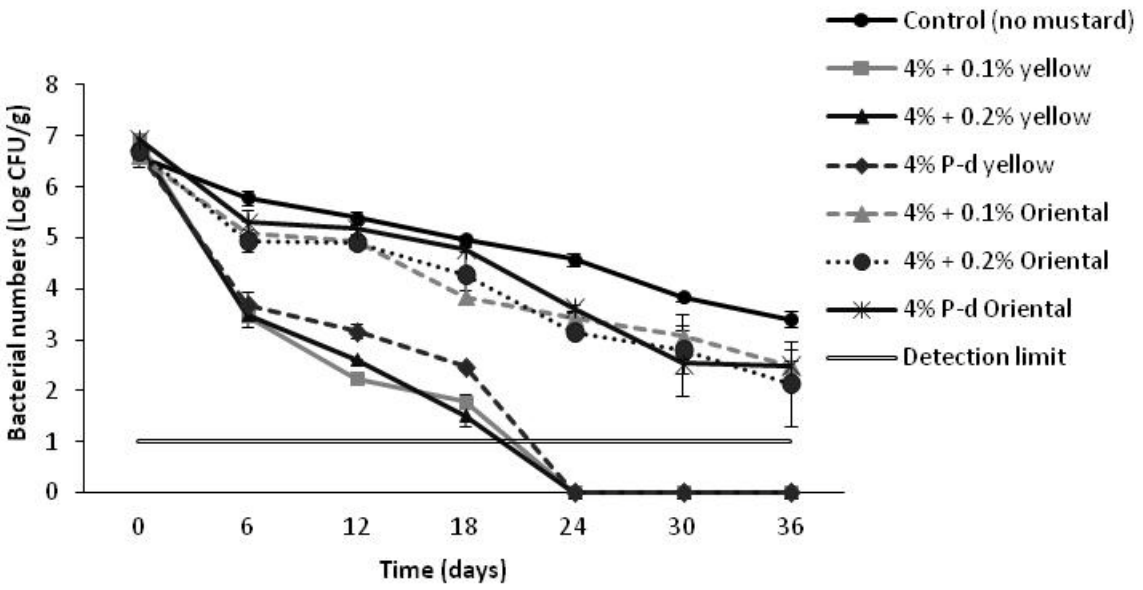

Figure 4.3: *E. coli* O157:H7 viability during sausage ripening with 4% (w/w) mustard powder containing myrosinase. Values represent the mean ± standard error of two separate experiments replicated three times (n = 6). When added to treatments, 0.1 % and 0.2 % represent the proportion of hot yellow or Oriental mustard powder in sausage. These were from mixtures of mustard powder containing 2.5 % and 5 % hot yellow or Oriental mustard, respectively; P-d = mustard partially deodorized by heating in a 2 cm thick layer. Where bacterial numbers were ≤ 1 Log CFU.g^{-1}, recovery of *E. coli* O157:H7 was after enrichment and immunomagnetic separation.

4.4.4 Antimicrobial activity of mustard meal against E. coli O157:H7

It was found that a 5 Log reduction of *E. coli* O157:H7 was achieved within 21 d in sausages treated with 2% deodorized, deoiled yellow mustard meal containing 0.2 % hot mustard (Fig. 4.4). Surprisingly, the treatment with 2% deodorized, deoiled Oriental mustard meal containing 0.2 % hot mustard showed a reduction in *E. coli* O157:H7 numbers to the mandatory level at 35 d. This is in contrast to the results where 4% Oriental mustard powder containing 0.2% hot mustard failed to cause the required reduction (Fig. 4.3). Again, this may have occurred

because the Oriental powder contained insufficient glucosinolate (82 μmol sinigrin.g^{-1}) to enable a threshold inhibitory concentration of ITC to be formed by myrosinase from the hot mustard and bacteria. Results also confirmed the greater antimicrobial activity of deoiled mustard meal compared to the mustard powders, which was likely associated with the higher amount of glucosinolates present in deoiled mustard meal. The greater glucosinolate content in the meal was believed responsible for enabling the mandated reduction in *E. coli* O157:H7 numbers at 21 d in spite of the use of only 2% deodorized meal instead of 4% deodorized mustard powder. In previous work, sausages with 2% deodorized yellow mustard powder were not able to achieve the required *E. coli* O157:H7 reduction even at 42 d ripening (Cordeiro et al., 2013). It was also found that a mixture of 1% deodorized plus 1% hot mustard powder was ineffective in reaching the mandatory goal.

Results from the present work showed that sausage treatments containing 2% fully-deodorized yellow or Oriental mustard meal without the addition of hot mustard were able to cause a 5 Log CFU.g^{-1} reduction in *E. coli* O157:H7 viability by 42 d. The present reduction is attributed to the myrosinase-like activity present in *E. coli* O157:H7 and the starter cultures, which has been shown in earlier work (Luciano et al., 2011; Luciano and Holley, 2011) to play a significant role in degrading the glucosinolates from both types of mustard to form antimicrobial ITCs. The more rapid reduction of *E. coli* O157:H7 in sausages with yellow powder plus 0.2% hot yellow powder when compared to Oriental powder with 0.2% hot Oriental powder was also shown for yellow and Oriental mustard meals without the addition of hot mustard. It appeared that the myrosinase-like activity of *E. coli* O157:H7 was affected more by the glucosinolate concentration than the type of glucosinolate. Cordeiro et al. (2013) showed that 4% deodorized yellow mustard had higher antimicrobial activity than 2% of the same type of mustard. Further,

in the present study, regardless of mustard type, when 2% deodorized meal was used there was no difference in the rate of *E. coli* O157:H7 reduction over 42 d ripening (Fig. 4.4). In the controls without mustard, the maximum reduction observed in sausages was < 4 Log CFU.g^{-1} by the end of ripening.

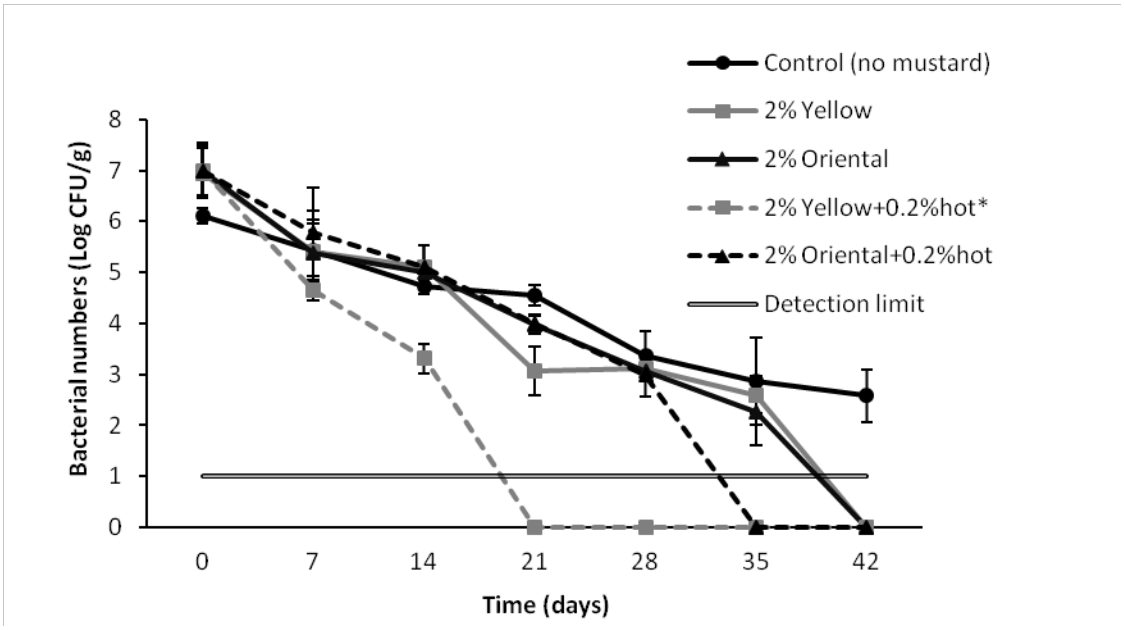

Figure 4.4: *E. coli* O157:H7 viability during sausage ripening with 2% (w/w) deodorized, deoiled mustard meal with or without hot mustard (plant myrosinase). Values represent the mean ± standard error of two separate experiments replicated three times (n = 6).Yellow and Oriental mustard meals were fully-deodorized and added to meat batters at 2% (w/w). When added to treatments, 0.2 % represents the proportion of hot yellow or Oriental mustard in the sausage batter. Where bacterial numbers were≤ 1 Log CFU.g^{-1}, recovery of *E. coli* O157:H7 was after enrichment and immunomagnetic separation.

4.4.5 Changes in pH, aw, and numbers of starter cultures

In separate repeated tests with mustard powder and meal, when glucose was present at 0.4 % (w/w), sausage pH was progressively reduced at the beginning and slightly increased at the end of ripening (Tables 4.1 and 4.2). This slight increase in pH did not occur in previous

experiments when the amount of glucose used was 0.6 % (w/w) (Luciano et al., 2011; Cordeiro et al. 2013). This reduction in carbohydrate available in the sausage formulation was undertaken to stimulate bacterial myrosinase-like enzyme synthesis and promote glucosinolate hydrolysis. Effects of glucose reduction on controlling pH were more evident in control sausages, but the amount of carbohydrate in mustard treatments still enabled reductions below pH 5.0 in samples (Tables 4.1 and 4.2) and sausages were compliant with the degree•hour requirements for fermented dry sausage manufacture in Canada (CFIA, 2013). It should be noted that the degree•hour requirement specifies the maximum period uncooked sausages may be safely held at $> 15.6^{o}C$ during ripening before reaching pH $\leq$ 5.3. This requirement was introduced to control *Staphylococcus aureus* growth and enterotoxin production. Although the glucose added was adequate from a safety perspective, the effects of glucose reduction on bacterial myrosinase-like activity could not be measured.

Water activity values were 0.93 to 0.95 at the beginning of ripening in all test replications. In the mustard meal experiments, aw values were reduced to$\leq$ 0.8 by the end of the tests (Table 4.3). For marketing purposes, an aw of about 0.85 is desirable and this was achieved within 21 d when mustard meal was used (Table 4.3). In contrast, sausage treatments containing mustard powder did not dependably reach this aw value until 30 d (Table 4.4). Thus there could be a commercial advantage in using mustard meal because it enabled sausages to dry faster than when mustard powder was used. Reduced air flow and better humidity control in the air conditioned drying room would have prevented further moisture loss, but it was difficult to achieve when the number of sausages in the chamber became small toward the end of the tests. Sausages containing yellow mustard powder tended to retain more moisture and had slightly higher aw values than controls at the end of ripening, but differences were not significant with

Oriental mustard powder (Table 4.4). However, sausages containing mustard meal of either type did not retain more moisture than the controls (Table 4.3). Where differences occurred, they may have been related to the fact that the meal, but not the powder, had been treated to remove mustard oil.

Table 4.1: pH changes during sausage ripening with 4% (w/w) mustard powders

Day	Control	0.1%[1] yellow #106	0.2%[1] yellow #106	P-d1 yellow #106	0.1% Oriental #107F	0.2% Oriental #107F	P-d Oriental #107F
0	5.74±0.01a	5.77±0.01 a	5.72±0.03 a	5.79±0.02 a	5.73±0.01 a	5.73±0.02 a	5.82±0.01 a
6	4.84±0.03 a	4.63±0.04 a	4.58±0.10a	4.58±0.04 a	4.58±0.02 a	4.57±0.09 a	4.64±0.03 a
12	4.79±0.03 a	4.61±0.03 b	4.61±0.04 b	4.59±0.02 b,c,d	4.56±0.01 c,d	4.54±0.01 d	4.61±0.02 b,c
18	4.81±0.01 a	4.59±0.02 b,c	4.57±0.01 c	4.58±0.03 c	4.55±0.01 c	4.56±0.02 c	4.63±0.02 b
24	4.77±0.03 a	4.64±0.01 b,c	4.63±0.04 b,c	4.61±0.01 c,d	4.55±0.01 e	4.58±0.01 d	4.66±0.01 b
30	4.84±0.02 a	4.63±0.01 b	4.60±0.01 c	4.59±0.01 c	4.54±0.01d	4.55±0.01 d	4.62±0.01 b
36	4.90±0.02a	4.72±0.06 b	4.65±0.05 b	4.63±0.03 b	4.63±0.08 b	4.62±0.06 b	4.67±0.02 b

Values represent the mean ± standard error of two separate batches replicated twice (n = 4). Different letters indicate a significant difference ($P < 0.05$) within the same row. [1] When added to treatments, 0.1% and 0.2% represent the proportion of hot yellow or Oriental mustard in sausage batter. These were from mixtures of mustard containing 2.5% and 5% yellow or Oriental hot mustard, respectively; P-d = partially deodorized mustard.

Table 4.2: pH changes during sausage ripening with 2% (w/w) deoiled mustard meal

Day	Control	Yellow	Oriental	Yellow + 0.2%[1]	Oriental + 0.2%[1]
0	5.55±0.07a	5.65±0.07 a	5.65±0.07 a	5.65±0.07 a	5.70±0.00 a
7	4.84±0.06 a	4.79±0.04 a,b	4.70±0.10c	4.73±0.05 b,c	4.69±0.04 c
14	4.81±0.05 a	4.75±0.03 b	4.73±0.01 b	4.71±0.01 b,c	4.68±0.01 c
21	4.78±0.06 a	4.67±0.09 a,b	4.64±0.13 b	4.61±0.10 b	4.60±0.11 b
28	4.76±0.06 a	4.60±0.11 b	4.60±0.08 b	4.57±0.07 b	4.57±0.07 b
35	4.81±0.19 a	4.74±0.06 a	4.74±0.06 a	4.73±0.05 a	4.68±0.10 a
42	4.81±0.08a	4.75±0.08 a,b	4.72±0.10 a,b	4.68±0.10 b	4.68±0.11 b

Values represent the mean ± standard error of two separate batches replicated three times (n = 6). Different letters indicate a significant difference ($P < 0.05$) within the same row. [1] When added to treatments, 0.2% represents the proportion of hot yellow or Oriental mustard in sausage batter.

Table 4.3: aw changes during sausage ripening with 2% (w/w) deoiled mustard meal

Day	Control	Yellow	Oriental	Yellow + 0.2%[1]	Oriental + 0.2%[1]
0	0.932±0.005a	0.930±0.004 a	0.926±0.003 a	0.929±0.004a	0.932±0.004 a
7	0.895±0.033 a	0.888±0.007 a	0.890±0.002a	0.886±0.004 a	0.891±0.005a
14	0.863±0.011 a	0.861±0.010 a	0.858±0.008 a	0.856±0.010 a	0.858±0.010 a
21	0.844±0.009 a	0.831±0.022 a,b	0.830±0.019 a,b	0.829±0.022 a,b	0.819±0.016 b
28	0.807±0.012a	0.790±0.025 a	0.811±0.046 a	0.794±0.026 a	0.792±0.015 a
35	0.780±0.025 a	0.764±0.025 a	0.759±0.034 a,b	0.751±0.023 a,b	0.727±0.034 b
42	0.740±0.029 a,b	0.745±0.030 a,b	0.762±0.032 a	0.747±0.027 a,b	0.719±0.030 b

Values represent the mean ± standard error of two separate batches replicated three times (n = 6). Different letters indicate a significant difference ($P < 0.05$) within the same row. [1] When added to treatments, 0.2% represents the proportion of hot yellow or Oriental mustard in sausage batter.

Table 4.4: aw changes during sausage ripening with 4% (w/w) mustard powder

Day	Control	0.1%[1] yellow #106	0.2%[1] yellow #106	P-d[1] yellow #106	0.1% Oriental #107F	0.2% Oriental #107F	P-d Oriental #107F
0	0.945±0.001[a]	0.944±0.000[a]	0.943±0.001[a]	0.943±0.000[a]	0.944±0.001[a]	0.945±0.002[a]	0.946±0.000[a]
6	0.920±0.002[a]	0.917±0.002[a]	0.920±0.001[a]	0.918±0.004[a]	0.919±0.001[a]	0.917±0.001[a]	0.916±0.000[a]
12	0.896±0.002[a]	0.900±0.003[a]	0.905±0.004[a]	0.900±0.006[a]	0.901±0.001[a]	0.899±0.005[a]	0.894±0.001[a]
18	0.873±0.004[a]	0.884±0.001[a]	0.877±0.000[a]	0.877±0.001[a]	0.884±0.002[a]	0.876±0.006[a]	0.973±0.006[a]
24	0.854±0.004[a,b]	0.866±0.008[a]	0.856±0.002[a,b]	0.851±0.006[a,b]	0.858±0.001[a,b]	0.841±0.003[b]	0.853±0.006[a,b]
30	0.828±0.003[a]	0.839±0.004[a]	0.834±0.005[a]	0.839±0.008[a]	0.838±0.007[a]	0.837±0.002[a]	0.830±0.010[b]
36	0.801±0.004[d]	0.821±0.006[a,b]	0.818±0.003[a,b,c]	0.823±0.004[a]	0.808±0.005[b,c,d]	0.806±0.000[c,d]	0.812±0.001[a,b,c,d]

Values represent the mean ± standard error of two separate batches (n = 2). Different letters indicate a significant difference ($P < 0.05$) within the same row.
[1]When added to treatments, 0.1% and 0.2% represent the proportion of hot yellow or Oriental mustard in sausage batter. These were from mixtures of mustard containing 2.5% and 5% yellow or Oriental hot mustard, respectively; P-d = partially deodorized mustard.

In sausage experiments, the numbers of starter cultures were affected to some extent by the antimicrobial activity of mustards. This was most evident with *S. carnosus* but a slight reduction in the final numbers of *P. pentosaceus* was also noted. It is known that *P. pentosaceus* has a10-fold greater resistance to ITCs found in yellow and Oriental mustards than *S. carnosus* (Luciano and Holley, 2011). While the addition of mustard powders caused a 1-2 Log CFU.g^{-1} reduction of *S. carnosus* by 36 d ripening, about a 1 Log CFU.g^{-1} reduction was found in one replicated trial, but only where deodorized mustard meals were mixed with 0.2% hot mustard meal. Thus, it seemed that the addition of mustard containing plant myrosinase to meat batter was more likely to affect the viability of *S. carnosus* than when mustard lacking myrosinase activity was used. These results were similar to those previously reported (Graumann and Holley, 2008; Luciano et al., 2011; Cordeiro et al., 2013).

4.5 Conclusion

The full inactivation of plant myrosinase was achieved by autoclave treatment (115^{o}C, 15 min) only when the mustard sample thickness during thermal treatment was reduced to 1 cm. It appeared that the thicker, 2 cm layer prevented thorough penetration of steam into the sample and enabled retention of some myrosinase activity. This residual enzyme may contribute to the high antimicrobial activity of mustard so treated when used in sausage against *E. coli* O157:H7 through its hydrolysis of glucosinolates. When 4% (w/w) deodorized yellow mustard powder containing myrosinase from hot mustard was added to the sausages, between 18 to 24 d ripening were required to reduce *E. coli* O157:H7 numbers by 5 Log CFU.g^{-1}. However, the 2 % (w/w) deoiled yellow mustard meal treatment containing myrosinase activity was as potently antimicrobial as 4 % yellow mustard powder and took 21 d to achieve the same reduction. In both powder and meal experiments, a significant difference in bactericidal activity was observed

between yellow and Oriental mustard treatments, where yellow mustard was more antimicrobial. This may have been because the yellow mustard contained higher glucosinolate levels than Oriental mustard. Additionally, it is possible, but uncertain whether the ρ-HBITC produced from sinalbin was more antimicrobial in sausage than AITC produced from sinigrin hydrolysis. However, Luciano and Holley (2011) found that *E. coli* O157:H7 was more susceptible to AITC than ρ-HBITC in broth. Enzyme kinetics may also have affected the results since the rate of enzyme action influences the amount of antimicrobial ITCs formed during ripening. It was evident that residual myrosinase in mustards contributed significantly to the early and substantial reduction of *E. coli* O157:H7 viability during dry sausage ripening. Yet, the myrosinase-like activity of *E. coli* O157:H7 was found to contribute to glucosinolate degradation where plant myrosinase was not present, regardless of mustard type. In further work, sensory studies should examine the consumer acceptability of dry sausages containing mustard with measurable residual myrosinase as well as those made with deoiled mustard meal. In previous work, consumer acceptability was negatively influenced by the detection of mustard flavor notes in sausages with ≥ 3% deodorized mustard powder (Li et al., 2013). Moreover, work is needed to explore the antimicrobial activity of deoiled mustard meal at concentrations < 2% (w/w). It may be possible to reduce levels of both hot and deodorized components to 0.1 and 1 % (w/w) in sausages, respectively, and still achieve ≥ 5 Log $CFU.g^{-1}$ reduction in *E. coli* O157:H7 viability.

Chapter 5

Role of glycoside hydrolase genes in sinigrin degradation by *Escherichia coli* O157:H7

5.1 Abstract

This work examined *Escherichia coli* O157:H7 strain 02-0304 for putative genes responsible for sinigrin hydrolysis. Sinigrin is a glucosinolate present in Oriental mustard (*Brassica juncea*), and its hydrolysis is mediated in plants by the enzyme myrosinase. Sinigrin hydrolysis by plant or bacterial myrosinase yields allyl isothiocyanate (AITC) which is bactericidal. *In silico* analysis using public databases found sequence similarity between plant myrosinase and enzymes encoded by genes from *β*-glucosidase families in *E. coli* O157:H7. Specifically, 6-phospho-*β*-glucosidase encoded by the genes *bglA* and *ascB* (family 1), and *chbF* (family 4) present in *E. coli* O157:H7 showed the highest similarity. Polymerase chain reaction (PCR) confirmed the presence of *bglA, ascB,* and *chbF* in the clinical *E. coli* strain tested. Disruption of these genes in wild-type *E. coli* O157:H7 strain 02-0304 using lambda-red replacement created single and double mutants. The relative importance of each gene in the hydrolysis of sinigrin by *E. coli* O157:H7 was also assessed by comparing gene expression and sinigrin degradation rates among the *E. coli* O157:H7 wild-type strain and its mutants. The results suggested that the *ascB* gene plays a substantial role in the degradation of sinigrin by *E. coli* O157:H7 strain 02-0304.

5.2 Introduction

The control of foodborne pathogen viability in dry-fermented sausages is crucial because these products are not given a thermal treatment before consumption. In spite of the unfavorable growth conditions for pathogens during the manufacture of dry-fermented sausage, such as high NaCl concentration, low pH and low water activity, without additional intervention (apart from heating) *Escherichia coli* O157:H7 can survive the process and are detectable in sausages (Luciano et al., 2011; Cordeiro et al., 2014a; Dalzini et al., 2014). Guidelines for fermented meat manufacture in Canada and the United States were revised to require that the dry-fermented sausage process achieve a 5 $\log_{10}$ CFU.g^{-1} reduction in the viability of *E. coli* O157:H7 (Health Canada, 2000; USDA, 2001). In this context, the use of mustard powder, as a natural antimicrobial ingredient in dry-fermented sausage production, was found capable of reducing *E. coli* O157:H7 numbers to the extent required by the North American regulations (Graumann and Holley, 2008; Luciano et al., 2011; Cordeiro et al., 2013).

The antimicrobial activity of mustard is a result of the conversion of glucosinolates (*β*-thioglucoside-*N*-hydroxysulfates) into bioactive isothiocyanates, and this is common among members of the genus *Brassica*. The hydrolytic conversion results from the action of the plant enzyme myrosinase (thioglucosidase glucohydrolase EC.3.2.1.147), which can be prevented by thermal inactivation of the enzyme. The bioactive compounds formed include allyl isothiocyanate (AITC) in brown or Oriental mustard (*Brassica juncea*), and ρ-hydroxybenzyl isothiocyanate (ρHBIT) in yellow or white mustard (*Sinapis alba*) (Kissen et al., 2009). Myrosinase is not unique to *Brassica* spp.; there are fungi and bacteria which exhibit myrosinase activity such as *Aspergillus niger* (Ohtsuru and Hata, 1973), *Enterobacter cloacae* (Tani et al., 1974), *Lactobacillus agilis* (Palop et al., 1995), and *Escherichia coli* (Oginsky et al., 1965).

There are also reports showing that the microflora in the human intestinal tract is able to hydrolyse glucosinolates, thus producing isothiocyanates from substrates where myrosinase had been inactivated by heat treatment (Getahun and Chung, 1999; Rouzaud et al., 2004; Mullaney et al., 2013). In food systems like dry-fermented sausage where myrosinase had also been inactivated by heat treatment, the degradation of mustard glucosinolates was attributed largely to the myrosinase-like activity of *E. coli* O157:H7 and meat starter cultures (*Pediococcus pentosaceus* and *Staphylococcus carnosus*) (Luciano et al., 2011; Cordeiro et al., 2014). The myrosinase activity in these bacteria played an essential role in the control of *E. coli* O157:H7 in dry-fermented sausage by its conversion of the Oriental mustard glucosinolate, sinigrin, into the bactericidal isothiocyanate AITC (Cordeiro et al., 2014). It is likely that *E. coli* O157:H7 degrade glucosinolates in order to acquire energy (glucose) and by doing so generate inhibitory AITC,

Myrosinase in mustard is a member of the glycoside hydrolase family 1 (GH1), which also includes the β-galactosidases, 6-phospho-β-galactosidases, and 6-phospho-*β*-glucosidases (Mian, 1998). However, myrosinase genes have not yet been found in microorganisms (Suzuki et al., 2006), even in *E. cloacae* from which purified myrosinase has been isolated (Tani et al., 1974). Although *E. coli* O157:H7 strains have shown myrosinase-like activity which causes the hydrolysis of sinigrin (Luciano and Holley, 2011), the specific enzyme(s) responsible for this activity have not been formally identified. It has been suggested that the bacterial equivalent of myrosinase may be a *β*-glucosidase with affinity for mustard oil glucosides (Tani et al., 1974). Therefore, it is possible that glycoside hydrolase enzymes may be responsible for the ability of *E. coli* O157:H7 strains to degrade mustard glucosinolates. The objective of this work was to investigate the role glycoside hydrolase genes play in the hydrolysis of sinigrin by *E. coli* O157:H7. Understanding the genetic basis of myrosinase activity expressed by *E. coli* O157:H7

will enable the optimization of conditions for its production and facilitate the use of naturally occurring glucosinolates to control this and other foodborne pathogens, not only in dry-fermented sausages but also in a broader range of foods. More specifically, greater understanding of factors influencing gene activation and expression can be used in setting boundaries for application of this antimicrobial system.

5.3 Material and methods

5.3.1 Bacterial strains and plasmids

E. coli O157:H7 strain 02-0304 used in this study was chosen from 5 strains shown to have the ability to degrade sinigrin (Luciano and Holley, 2011) because it alone was susceptible to ampicillin and chloramphenicol. *E. coli* O157:H7 strain 02-0304 was a human clinical isolate obtained from Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada (Table 5.1). This strain was positive for the *eae* and *hly* genes and was $Stx_{1,\ 2}$ negative. Its identity was confirmed by 16S rDNA sequencing using universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 342r (5'-CTGCTGCSYCCCGTAG-3') (Kotlowski et al., 2006). The sequencing was done by UCDNA Services, University of Calgary. The plasmids pkD46, pkD3, and pCP20 were purchased from the Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT, USA. *E. coli* O157:H7 strain 02-0304 was screened for antibiotic susceptibility using Luria-Bertani agar (LA) (Fisher Scientific, Fair Lawn, NJ, USA) plates containing 100 μg/ml of ampicillin (96-100.5% purity) and 25 μg/ml of chloramphenicol (≥ 98% purity) from Sigma-Aldrich (St. Louis, MO, USA). The *E. coli* O157:H7 strain was stored at -80°C and maintained on LA at 4°C with monthly transfer to new plates. Active *E. coli* culture was obtained by transfer of a single colony

from a plate to 10 ml Luria-Bertani broth (LB) (Fisher Scientific) with incubation at 37°C for 18 h.

Table 5.1: *E. coli* O157:H7 strains and their sources

Strain	Description	Sources
E. coli O157:H7 (02-0304)	Wild type strain from human/clinical source	Laboratory collection*
E. coli Δ*bglA*	Mutant with *bglA* gene disrupted	This study
E. coli Δ*ascB*	Mutant with *ascB* gene disrupted	This study
E. coli Δ*chbF*	Mutant with *chbF* gene disrupted	This study
E. coli Δb*glA*Δ*ascB*	Mutant with simultaneous disruption of *bglA* and *ascB* genes	This study
E. coli Δ*chbF*Δ*asc*B	Mutant with simultaneous disruption of *chbF* and *ascB* genes	This study
E. coli Δ*chbF*Δ*bglA*	Mutant with simultaneous disruption of *chbF* and *bglA* genes	This study

* Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada.

5.3.2 In silico analyses

The myrosinase protein sequence from *Brassica juncea* was retrieved from the National Center for Biotechnology Information (NCBI) protein sequence database, with accession number AAG54074.1. (available at http://www.ncbi.nlm.nih.gov/protein). This sequence was entered into the Basic Local Alignment Search Tool (BLAST) program tblastn, which searched translated nucleotide databases using a protein query in order to find homologous proteins in three *E. coli* O157:H7 genomes (strain Sakai Accession # BA000007.2, strain SS17 CP008805.1,

and strain TW14359 CP001368.1) that are also available at NCBI (http://www.ncbi.nlm.nih.gov). A BLAST search was also done using the genome of three strains of *Enterobacter cloacae* (Accession # CP003737.1, AEW75128.1, and CP008897.1), which had been identified as myrosinase-producing organisms (Tani et al., 1974). Default settings were used for tblastn. All data assessments were based upon sequence comparisons made using genomic data from the NCBI database. The EcoCyc database (Keseler et al., 2009) available at http://ecocyc.org/ was used in order to complement the *in silico* analysis done with BLAST.

5.3.3 Polymerase chain reaction (PCR)

Results from the *in silico* analysis showed that the enzyme 6-phospho-*β*-glucosidase, encoded by the genes *bglA*, *ascB*, and *chbF*, seemed to be associated with myrosinase-like activity in *E. coli* O157:H7 strain 02-0304. Thus, polymerase chain reaction (PCR) was used to assess the presence of 6-phospho-*β*-glucosidase genes in the *E. coli* O157:H7 strain. PCR reactions were conducted in volumes of 25 µL containing 12.5 µL Multiplex PCR Mastermix (Promega, Madison, WI, USA), 0.5 µL of primer, 2 µL bacterial DNA, and 9.5 µL water. All PCR amplifications were carried out using the following temperature program: initial denaturation at 94ºC for 5 min, 30 cycles at 94ºC for 1 min, annealing of primers at 58ºC, an extension at 72ºC for 1 min, and a final extension step at 72ºC for 5 min. The amplification products were subjected to gel electrophoresis in a 1.5% agarose gel at 120 V for 60 min. Specific primers for *bglA*, *ascB*, and *chbF* were designed using BioEdit sequence alignment editor software (Table 5.2). Primers were manufactured by UCDNA Services, Faculty of Medicine, University of Calgary (Calgary, AB, Canada).

5.3.4 Gene disruption

E. coli O157:H7 strain 02-0304 (sensitive to chloramphenicol and ampicillin) was used for disruption of 6-phospho-β-glucosidase genes using lambda red-mediated gene replacement described by Datsenko and Wanner (2000). Single and double mutants of *E. coli* O157:H7 strain 02-0304 were constructed by replacing 800, 684, and 498 bp of the conserved region of *bglA*, *ascB*, and *chbF* genes, respectively, with 1034 bp of the chloramphenicol marker (pkD3).

Table 5.2: Primers designed and used for mutant construction and validation

Primer names	**Sequence**
bglA_F	5’-*CGCGGAAGTGGTATTTGAAC*-3’
bglA_R	5’-*CACTGCCAGGGCGCTGGCG*-3’
bglApkD3_1	5’-CAGGTCGAAGGCGGCTGGAACAAAG**GTGTAGGCTGGAGCTGCTTC**-3’
bglApkD3_2	5’-TCCCACTCGTTCAACACATACGACG**CATATGAATATCCTCCTTAGT**-3’
ascB_F	5’-*ATGTCAGTATTTCCAGAAGGT*-3’
ascB_R	5’-*ATCTTCCCCATTACTGGCAATCAC*-3’
ascBpkD3_1	5’-ATCTTCCCCATTACTGGCAATCACTG**TGTAGGCTGGAGCTGCTTC**-3’
ascBpkD3_2	5’-TACAGTTGCAAGCCGGAAGATGTCTC**ATATGAATATCCTCCTTAGT**-3’
chbF_F	5’-*ATCGCGCCCCAGCGTACAGGTC*-3’
chbF_R	5’-*TCGGCCTCAACCATATGGTGTTCAT*-3’
chbFpkD3_1	5’-ATCGCGCCCCAGCGTACAGGTC**GTGTAGGCTGGAGCTGCTTC**-3’
chbFpkD3_2	5’-TCGGCCTCAACCATATGGTGTTCAT**CATATGAATATCCTCCTTAGT**-3’
bglB_F	5’-*ATTTGCCGAGATGGGCTTCA*-3’
bglB_R	5’-*ATGTTGCCCTGCGCATTTTT*-3’

Italicized letters represent primers that flank only the target gene. Bold letters represent primers that have internal overlap with the chloramphenicol resistance marker (pKD3), while non-bold letters represent primers having external overlap with the target knockout genes.

Briefly, a curable expression plasmid (i.e. pkD46) encoding both a Red recombinase and an ampicillin resistance gene was introduced into *E. coli* O157:H7 02-0304, and the expression of the Red system was induced with 1mM arabinose. Then, the chloramphenicol resistance gene (cat), flanked by target recognition sites, was amplified by PCR with primers that were

homologous to the beginning and end of the coding sequence of the gene to be disrupted (Table 5.2). The PCR reaction was conducted using a volume of 50 µL comprised of 25 µL Multiplex PCR Mastermix, 0.5 µL of each primer (25 pmol), 21 µL water, and 2 µL template plasmid pkD3 containing a chloramphenicol resistance (*cat*) gene. The PCR product was gel-purified, digested with *Dpn*I, repurified, and suspended in elution buffer (10 mM Tris, pH 8.0). Electroporation was done using a micropulser (Bio-Rad, Mississauga, ON, Canada), where 50 µL of the recipient 02-0304 strain expressing Red recombinase and 4 µL of the purified PCR product were mixed and placed into a cuvette. Shocked cells were added to 1 mL Super Optimal Catabolite-repression (S.O.C) medium (Hanahan, 1983), incubated for 3 h at 39°C, and then plated on LB agar containing 25 $\mu L.mL^{-1}$ of chloramphenicol to select chloramphenicol-resistant transformants. The elimination of the pkD46 vector was confirmed by the loss of ampicillin resistance after its exposure to the non-permissive temperature of 39°C. DNA was isolated from the mutants obtained, and the deletion of the targeted loci was confirmed by sequencing and PCRs with primers complementary to *cat* and adjacent regions. Single mutants Δ *bglA*, Δ*ascB*, and Δ*chbF* were obtained by individual disruption of the *bglA, ascB* and *chbF* genes. Before proceeding with the double disruption, the pCP20 helper plasmid was used for the elimination of the chloramphenicol resistance gene present in the single mutant. Briefly, electroporation was done by adding 4 µL of pCP20 to 50 µL of electro-competent cells of each single mutant. Then, ampicillin-resistant transformants were selected at 30°C and tested for the loss of chloramphenicol resistance by PCR. The transformed single mutant strain was then used for the re-introduction of the chloramphenicol resistance gene (*cat*), flanked by target recognition sites of the second gene to be disrupted. As a result, double mutants Δ*bglA*Δ*ascB*, Δc*hbF*Δ*bglA*, and Δ*chbF*Δ*ascB*, were constructed by disruption of the second target gene in the single mutant

(Table 5.1). *E. coli* O157:H7 mutants were confirmed by DNA sequencing at UCDNA Services, University of Calgary. PCR reactions using a total volume of 25 µL containing 12.5 µL Multiplex PCR Mastermix, 0.5 µL of each primer (1 µL total), 9.5 µL water, and 2 µL bacterial DNA were also conducted to verify the success of the mutations. The PCR amplifications were carried out using the following temperature program: initial denaturation at 94ºC for 5 min, 30 cycles at 94ºC for 1 min, annealing of primers at 58ºC, an extension at 72ºC for 1 min, and a final extension step at 72ºC for 5 min. The amplification products were subjected to gel electrophoresis in a 1.5% agarose gel at 120 V for 60 min. To further determine whether the disruption of 6-phospho-*β*-glucosidase genes would compromise bacterial viability, wild-type strain 02-0304 and mutants were grown in Mueller-Hinton broth (MHB; Oxoid Ltd., Basingstoke, England) with and without 0.1% sinigrin (2.407 mM, ≥ 99.0% purity, Sigma-Aldrich) and incubated at 25°C for 5 d. MHB was selected as the growth medium because it contained beef extract, casein, and starch which closely resembled the fermented sausage environment. After, strain 02-0304 and its mutants were plated on Violet Red Bile (VRB) agar (Oxoid) and enumerated for comparison of the viability between wild-type and mutant strains. The viability of *E. coli* O157H7 mutant strains was not compromised by either the single or double gene disruption, either in the presence or absence of 0.1% sinigrin (data not shown).

5.3.5 RNA extraction

Total RNA was isolated from wild-type 02-0304 and mutant strains grown for 5 d in MHB at 25°C with and without 0.1% sinigrin. RNA was extracted from three replicates using an RNeasy Mini Kit as described in the manufacturer's protocol (Qiagen, Valencia, CA, USA). The RNA concentration was determined spectrophotometrically based on absorbance A260/A280 (Nanodrop 2000, Thermo Fisher Scientific). Real-time reverse transcription-PCR (RT-PCR) was

performed using a high-capacity cDNA Reverse Transcription Kit (Life Technologies Inc., Burlington, ON, Canada) according to the manufacturer's protocol. According to bacterial numbers in both treatments, cultures with and without 0.1% sinigrin were taken at the stationary growth phase for RNA extraction and further gene expression analysis.

5.3.6 RT-PCR

RT-PCR was performed to analyze the change in expression of *bglA, ascB,* and *chbF* genes in relation to a reference gene (*rpoA*). Specific primer pairs for *bglA*, *ascB*, *chbF*, and the *rpoA* internal control were designed using Bioedit software (Table 5.3), and manufactured by UCDNA Services, University of Calgary. Relative expression levels of target transcripts were determined using the Power SYBR Green Master Mix (Bio-Rad) following the manufacturer's protocol. Each reaction contained a final volume of 20 µL and was run in 96-well multiplates (Bio-Rad) sealed with adhesive film (Bio-Rad). Individual reactions contained 3.4 µL of water, 10 µL of Power SYBR Green PCR Master Mix, 0.8 µL of forward primer, 0.8 µL of reverse primer, and 5 µL of sample DNA. Amplifications were carried out using a real-time PCR detection system (Bio-Rad CFX96), where the conditions were 95°C for 10 min, 95°C for 15 sec, and 61°C for 60 sec for a total of 40 cycles. Quantitative real-time PCR expression data from target genes *bglA, ascB*, and *chbF* at the threshold cycle (Ct) were compared with normalized levels of the reference gene or internal control gene, *rpoA.* The effect of sinigrin and mutation in the expression of the target genes was quantified using Equation (1), which compares gene expression in two different samples A and B (Shmittgen and Livak, 2008):

$$2^{-\Delta\Delta Ct} = [(Ct_{gene\,of\,\mathrm{int}erest} - Ct_{\mathrm{int}ernal\,control})_{Sample\,A} - (Ct_{gene\,of\,\mathrm{int}erest} - Ct_{\mathrm{int}ernal\,control})_{Sample\,B} \quad (1)$$

In quantifying the effect of sinigrin, Sample A Ct values were from the treated sample (i.e. wild type strain with 0.1% sinigrin), and Sample B Ct values were from the calibrator (i.e. wild type strain without 0.1% sinigrin). In quantifying the relative changes in gene expression caused by mutation, Sample A Ct values were from the mutant strain grown with 0.1% sinigrin, while Sample B Ct values were from the wild-type strain grown with 0.1% sinigrin (i.e. the calibrator). The results of the $2^{-\Delta\Delta Ct}$ method are presented as the fold change in gene expression normalized to a reference gene and relative to the calibrator. For the calibrator sample, $\Delta\Delta Ct$ equals zero and 2^0 equals one, so that the fold change in gene expression relative to the calibrator equals one (Appendix I of the thesis presents the Ct values used in the calculation of relative expression of all the genes in all experiments). For the treated samples, evaluation of $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the calibrator (Shmittgen and Livak, 2008). Since the fold change in gene expression relative to the calibrator equals one, values of treated samples that equal 1 or -1 are not significant changes in expression. A value of $2^{-\Delta\Delta Ct} < 1$ implies that there was a reduction in the expression due to the treatment. The negative inverse of $2^{-\Delta\Delta Ct}$ gives the fold change reduction in expression (Appendix I).

To support the validation of the $2^{-\Delta\Delta Ct}$ method, it is noted that Shmittgen and Livak (2008) stated that the amplification efficiency of the target and reference genes must be approximately equal in order for the $2^{-\Delta\Delta Ct}$ calculation to be valid. If the absolute value of the slope of the efficiency of the amplification curve is close to zero, and the efficiencies of the target and reference genes are similar, the $2^{-\Delta\Delta Ct}$ calculation for the relative quantification of the target gene may be used. The slopes of the curves for all the genes used in all experiments were close to zero only when *rpoA* was used as the internal control. The required slope was not achieved when other reference genes (i.e. *rpoS*, *arcA*, and *ompA*) were tested. The efficiency of

amplification curves used for validation of the $2^{-\Delta\Delta Ct}$ calculations of *rpoA* as internal controls is presented in Appendix II.

Table 5.3: Primers designed and used for RT-PCR

Primer names	**Sequence**
*bglA*_F	5'-GTGCCGCTCTATCCCTACTC-3'
*bglA*_R	5'-ACTCCTGAGCGAACATCACA-3'
*ascB*_F	5'-AGTGGATCAATTCCCCAGCC-3'
*ascB*_R	5'-CGCTGCGTAACCCGTATCTA-3'
*chbF*_F	5'-ACGGACTAAGGTTTAGCGCC-3'
*chbF*_R	5'-GCCGCACTTAGCGGAGAAT-3'
*rpoA*_F	5'-CGCGGTCGTGGTTATGTG-3'
*rpoA*_R	5'-GCGCTCATCTTCTTCCGAAT-3'

5.3.7 RP-HPLC analysis

Reversed phase high performance liquid chromatography (RP-HPLC) was conducted to compare glucosinolate degradation by strain 02-0304 and its mutants. Five replicates of all bacterial strains were grown in MHB at 25°C containing 0.1% sinigrin, and the concentration of sinigrin was examined in samples collected at days 0, 1, and 5. Separation and quantification of sinigrin was performed according to Herzallah and Holley (2012). Briefly, samples of 10 ml were injected and passed through an analytical Gemini-NX C18 reversed phase column (150 x 4.6 mm, 5 μm) protected by a security guard Gemini C18, 4 x 3.0 mm column (Phenomenex, Torrance, CA, USA). The HPLC system (Waters Corporation, Milford, MA, USA) consisted of a Waters model 486 detector (wavelength set at 227 nm), Waters 600E system controller and

Waters LC-module 1 Millennium software (version 32) was used to process the data. 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 (Fisher Scientific, Whitby, ON, Canada) and 80% water + 0.02M tetrabutylammonium hydrogen sulfate at pH 5.5 (Sigma). Before use, the solvents (ACN and TBA) were filtered through a 0.45 mm Millipore nylon filter (Fisher Scientific, Nepean, ON, Canada), and degassed for 5 min in a Bransonic bath (Branson, 5510, Grass Valley, CA, USA), and degassing was continued during the run using the built-in helium (He) degassing unit of the HPLC. The amount of sinigrin in samples was calculated from the curves of sinigrin standards injected at different concentrations and after peak areas were integrated. The standard curve which was prepared with MHB and concentrations of sinigrin ranging from 100 to 1250 mg/L, without *E. coli* O157:H7. The retention times on the selected column were used to characterize the peaks of the pure standards (Herzallah and Holley, 2012). The linearity of the curves and the respective correlation coefficients were computed from the peak area at each reference standard concentration, and the concentrations of the injected reference standards were calculated as mg/L.

5.3.8 Statistical data analysis

Data obtained from RP-HPLC and RT-PCR experiments were averaged and subjected to analysis of variance (ANOVA) using Student's test in JMP 10 (SAS Institute Inc., Cary, NC, USA). Data used were represented by means ± SEM. A p value of 0.05 was used as the cut-off for statistical significance.

5.4 Results and Discussion

Myrosinase from Oriental mustard (*Brassica juncea*) is 550 amino acids in length. *In silico* analysis using tblastn showed that between 45% (e-value 1e-43) and 43% (e-value 3e-33) of the amino acid sequence of 6-phospho-*β*-glucosidase encoded by the genes *bglA* and *ascB*, respectively, from the *E. coli* O157:H7 wild-type strain was substantially similar to the myrosinase from *B. juncea*. However, the EcoCyc database (Keseler et al., 2009) showed that 6-phospho-*β*-glucosidase activity may also be encoded by the *chbF* gene in *E. coli* O157:H7 strains different from those used in the present study. For *E. cloacae* strains, the similarity was about 46% (e-value 8e-41) to 6-phospho-*β*-glucosidase and about 50% (e-value 3e-54) to *β*-glucosidase. Similarities between plant myrosinase and 6-phospho-*β*-glucosidase or myrosinase isolated and identified in *E. cloacae* (Tani et al., 1974) were also found. In addition, *in silico* analysis found similarities between the 6-phospho-*β*-glucosidase from *E. coli* O157:H7 and the 6-phospho-*β*-glucosidases from other organisms possessing myrosinase-like activity such as *Salmonella enterica subsp.* Typhimurium, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Pediococcus pentosaceus*, but no similarity was found with bacterial species lacking myrosinase-like activity such as *P. fluorescens* (Herzallah et al., 2011) (data not shown). A comparable level of similarity between plant and bacterial protein sequences has been shown by others. Michalska et al. (2013) found 33% sequence identity between the 6-phospho-*β*-glucosidase from *Lactobacillus plantarum* and the enzyme dhurrinase, which is a glycoside hydrolase from the plant *Sorghum bicolour*.

When the sequence region was used as the signature pattern for the glycoside hydrolase family (Table 5.4), there was more than 52% identity between plant myrosinase and 6-phospho-*β*-glucosidase in *E. coli* O157:H7 and *E. cloacae* encoded by *bglA*. Similarly, 45% identity was found between plant myrosinase and 6-phospho-*β*-glucosidase in *E. coli* O157:H7 and *E. cloacae*

encoded by *ascB.* Comparing the genes encoding 6-phospho-*β*-glucosidase in *E. coli* O157:H7 and *E. cloacae* in the present study, a similarity over 70% was found. Using the signature pattern provided by the Prosite database (Bairoch, 1992), plant myrosinase and the 6-phospho-*β*-glucosidase genes *bglA* and *ascB* were assigned to glycoside hydrolase family 1, as described in the Cazy database (Cantarel et al., 2008). Mullaney et al. (2013) found that 6-phospho-*β*-glucosidase and *β*-glucosidase genes, present in several *Lactobacillus* and *Lactococcus* strains exhibiting myrosinase activity as well as in the known myrosinase-producing microorganism *E. cloacae*, were also assigned to the glycoside hydrolase family 1. In contrast, only 13% identity was found between plant myrosinase and 6-phospho-*β*-glucosidase encoded by the *chbF* gene, which was assigned to family 4 according to the Prosite database. In fact, a comparative alignment of amino acid sequences showed little homology between the sequence deduced for *chbF* and those deduced for *bglA* (Thompson et al., 1999).

PCR and sequencing analyses confirmed the presence of genes encoding 6-phospho-*β*-glucosidase (*bglA*, *ascB*, and *chbF*) in the *E. coli* O157:H7 strain 02-0304 tested in this study (Fig. 5.1). Considering that 6-phospho-*β*-glycosides in glycoside hydrolase family 1 are generally conserved in bacteria (Yu et al., 2013), and taking into account that the 6-phospho-*β*-glucosidase produced the best sequence match for plant myrosinase, that of *E. coli* O157:H7 and those of other myrosinase-producing microorganisms, it is likely that 6-phospho-*β*-glucosidase is associated with the degradation of sinigrin by *E. coli* O157:H7.

Table 5.4: Percent identity of myrosinase, *bglA*, *ascB*, and *chbF*

Organism	Protein/gene	Signature sequence pattern[a]	% identity[b]	GH Family
Brassica juncea	Myrosinase/*bglB*	FIFGVASSAYQIEGG	100	1
E coli O157:H7 str. TW14359	6-phospho-*β*-glucosidase/*bglA*	FLWGGAVAAHQVEGG	53.3	1
E. coli O157:H7 str. TW14359	6-phospho-*β*-glucosidase/*ascB*	FLWGGALAANQSEGA	46.7	1
E. coli O157:H7 str. TW14359	6-phospho-*β*-glucosidase/*chbF*	PNAWVINFTNPAGMVTEAVYR HTGFKRFIGVC	13.3	4
E.cloacae EcWSU1	6-phospho-*β*-glucosidase/*bglA*	FLWGGAVAAHQVEGG	53.3	1
E.cloacae EcWSU1	6-phospho-*β*-glucosidase/*ascB*	FLWGGALAANQSEGA	46.7	1

[a]The signature pattern was provided by Prosite database available at www.expasy.org/tools/scanprosite/. The signature sequence patterns are from myrosinase (Accession # AAG54074.1), *E. coli* O157:H7 TW14359 (Accession # ACT73612.1), and *E. cloacae* EcWSU1 (Accession # AEW75128.1) retrieved from the NCBI database at http://www.ncbi.nlm.nih.gov/. [b] The identity was calculated with ClustalOmega available at http://www.ebi.ac.uk/Tools/msa/clustalo/.

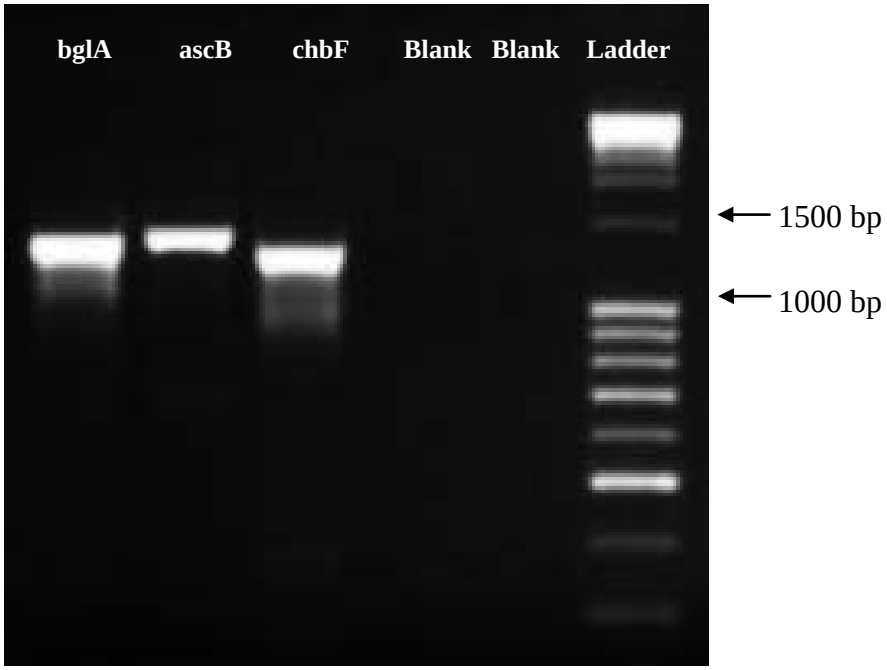

Figure 5.1: Gel-electrophoresis of PCR amplification products confirming the presence of 6-phospho-*β*-glucosidase genes in *E. coli* O157:H7 strain 02-0304. *bglA* = 1440bp, *ascB* = 1425bp, and *chbF* = 1353bp. Blank. A DNA ladder measuring 1kb was used.

The effect of sinigrin treatment was assessed by comparing the expression of the target genes identified in the *in silico* analysis in the wild-type strain grown with and without sinigrin. Results showed that the expression of the *bglA*, *ascB*, and *chbF* genes was increased 2.3-, 3.0-, and 6.4-fold in the presence of sinigrin, respectively (Fig. 5.2). Induction of gene expression in the presence of sinigrin suggested that the genes encoding 6-phospho-*β*-glucosidase are likely involved in the degradation of sinigrin by *E. coli* O157:H7. Due to the novelty of this work, there is no similar study in the literature with which to compare the activation of these genes in *E. coli* O157:H7 by sinigrin. However, previous studies at the phenotypic level demonstrated that myrosinase activity in *E. cloacae* (Tani et al., 1974) and *Lactobacillus agilis* R16 (Palop et al., 1995) was induced by the presence of sinigrin and mustard extract. While the genes associated

with myrosinase activity were not investigated in those studies, recent research has shown that the expression of 6-phospho-*β*-glucosidase genes can be induced by the presence of *β*-glucosides. Work has shown that the enzyme 6-phospho-*β*-glucosidase encoded by *bglA* and *ascB* genes is responsible for the hydrolysis of aromatic *β*-glucosides such as salicin and arbutin (Raghunand and Mahadevan, 2004). While the constitutively expressed *bglA* gene encodes an arbutin-specific phospho-*β*-glucosidase, the *ascB* gene can hydrolyze substrates such as salicin, arbutin and to a lesser extent, the disaccharide cellobiose (Neelakanta et al., 2009). Desai et al. (2010) showed that the expression of a 6-phospho-*β*-glucosidase encoded by the *ascB* gene in *E. coli* was induced with 7 mM salicin. Arbutin and salicin are *β*-glucosides similar to sinigrin in which a glucose residue is joined to a non-sugar residue. Considering the similarity among arbutin, salicin, and sinigrin as *β*-glucosides, it is likely that the role played by the *ascB* gene in the metabolism of sinigrin is similar to that for salicin and arbutin. So far, sinigrin is known to be a substrate for myrosinase (Palmieri et al., 1982; Bones and Rossiter, 1996; Kelly et al., 1998; Li and Kushad, 2004), but the authors are unaware of studies identifying sinigrin as a substrate for 6-phospho-*β*-glucosidase.

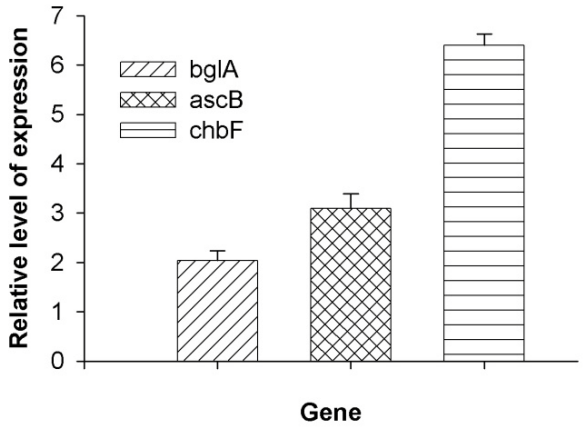

Figure 5.2: Relative expression (fold-change) compared to the parental strain in the level of the 6-phospho--glucosidase genes in *E. coli* O157:H7 strain 02-0304 in response to the presence of *β*sinigrin. The expression level of wild-type *E. coli* O157:H7 strain 02-0304 grown in the presence of 0.1% sinigrin was compared to that of the same strain grown in the absence of sinigrin at day 5. Each data point represents the mean of three independent biological replicates per treatment (mean ± SE).

Construction of single and double mutants of *E. coli* O157:H7 was confirmed through PCR assays (Fig. 5.3) and sequencing analysis. For the ΔbglA mutant, *ascB* gene expression was increased 2-fold due to the disruption of the constitutive *bglA* gene, with no change in the expression of the *chbF* gene (Fig. 5.4). For the ΔchbF mutant, expression of the *ascB* gene was reduced 8-fold, with no change in the expression of the *bglA* gene. No significant change in expression was observed for the remaining genes *bglA* and *chbF* in the ΔascB mutant. Results for the double mutants showed that the expression of the *ascB* gene was increased 4-fold after the disruption of both *chbF* and *bglA* genes (Fig. 5.5). Double disruption of the *chbFascB* and *bglAascB* genes resulted in no significant change in the expression of the remaining genes, *bglA* and *chbF*, respectively. Overall, *ascB* was the only gene to show > 2-fold changes in expression after single (*chbF* or *bglA*) and double (*chbFbglA*) gene disruption.

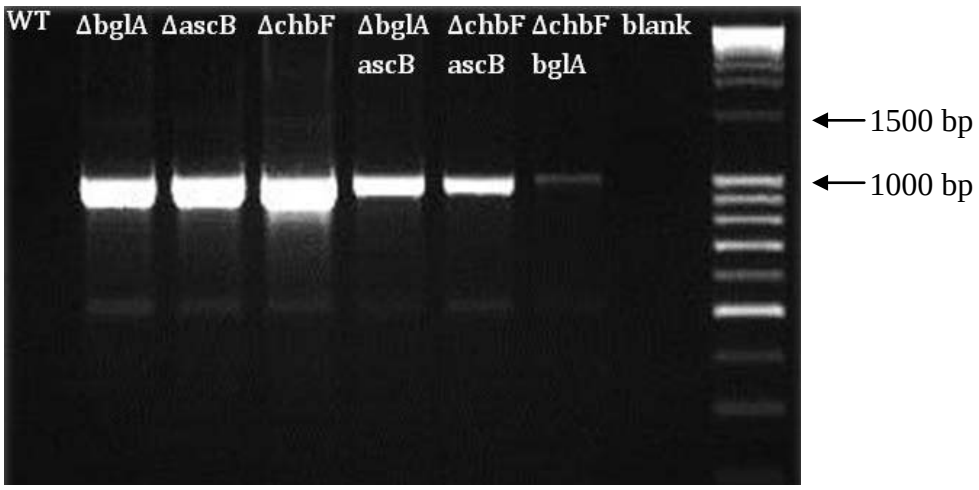

Figure 5.3: Gel-eletrophoresis of PCR amplification products confirming the construction of mutants by the presence of the chloramphenicol resistance marker (1034bp) in single (ΔbglA, ΔascB, ΔchbF) and double (ΔbglAΔascB, ΔchbFΔascB, ΔchbFΔbglA) mutants. The absence of pkD3 from the wild-type strain (WT) was confirmed. Blank. A DNA ladder measuring 1kb was used. *E. coli* O157:H7 strain 02-0304 was the WT used for all mutations.

When the ability of the single and double mutants to degrade sinigrin was compared to that of the wild-type strain 02-0304 (Fig. 5.6) there was a 28% increase in sinigrin degradation observed for the ΔbglA mutant compared to the wild-type strain. The disruption of *ascB* had no significant impact on the extent of sinigrin degradation, while the disruption of the *chbF* gene decreased sinigrin degradation by 6% when compared to the wild-type strain. The simultaneous disruption of *chbFbglA* genes caused an increase of 33% in sinigrin degradation, while the simultaneously disruption of *bglAascB* and *chbFascB* genes caused reductions of 21% and 13% in sinigrin degradation, respectively. Statistically significant differences ($p < 0.05$) in sinigrin degradation were detected among the double mutants ΔchbFbglA, ΔbglAascB, and ΔchbFascB.

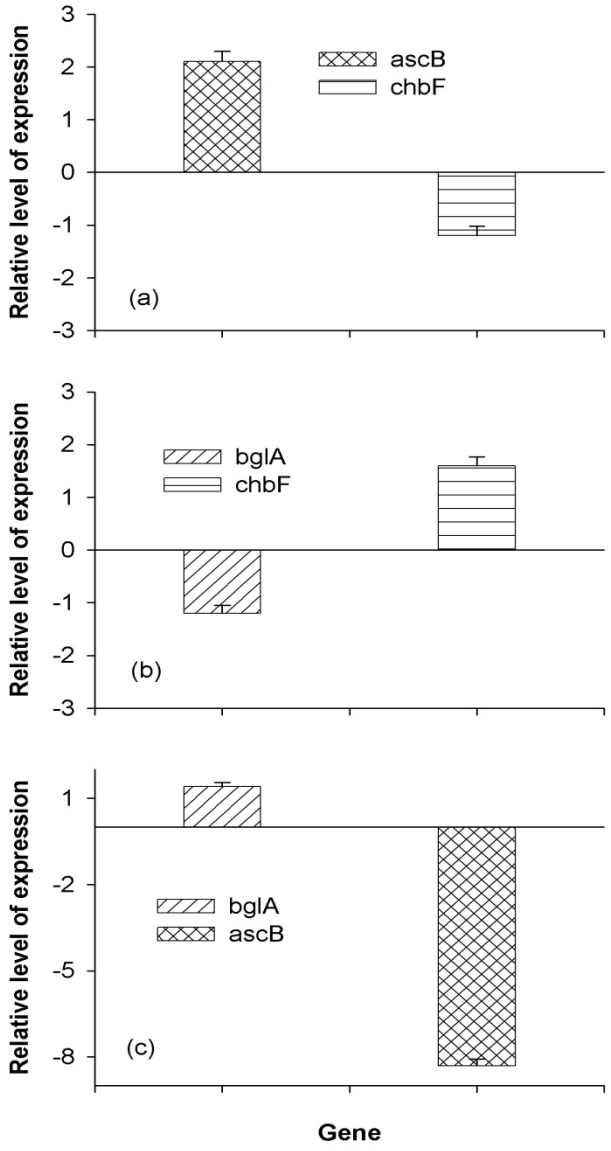

Figure 5.4: The effect of single mutation (a) ΔbglA mutant, (b) ΔascB mutant, and (c) ΔchbF mutant on the relative expression level (fold change) of the 6-phospho-*β*-glucosidase genes.The expression level was measured in single mutants of *E. coli* O157:H7 grown in the presence of 0.1% sinigrin compared to the wild-type *E. coli* O157:H7 strain 02-0304 also grown in the presence of 0.1% sinigrin at day 5. Each data point represents the mean of three independent biological replicates per treatment (mean ± SE).

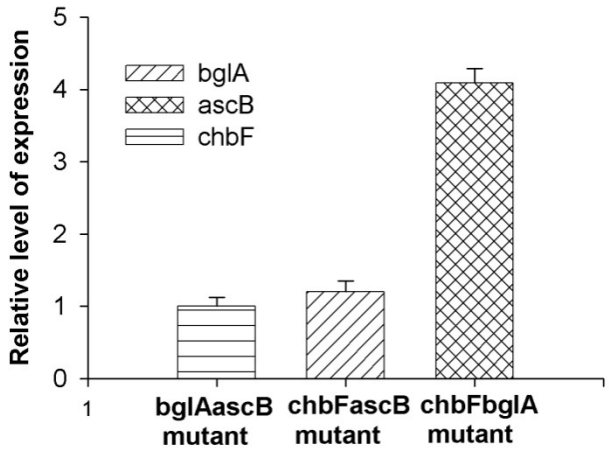

Figure 5.5: The effect of double mutation on the relative expression level (fold change) of the 6-phospho-*β*-glucosidase genes. The expression level was measured in double mutants of *E. coli* O157:H7 grown in the presence of 0.1% sinigrin compared to wild-type *E. coli* O157:H7 strain 02-0304 also grown in the presence of 0.1% sinigrin at day 5. Each data point represents the mean of three independent biological replicates per treatment (mean ± SE).

The expression profiles of mutants may be better understood if considered with data on the extent of their sinigrin degradation. The increase in degradation observed in the ΔbglA mutant could be related to the presence of both the ascB and chbF genes, but considering that ascB was the only gene upregulated after the disruption of bglA, it is more likely that the increased degradation by ΔbglA was due to the presence of the ascB gene. The lack of a significant change in degradation of sinigrin by the ΔascB mutant may have been due to the presence of the constitutive gene bglA, which in turn supports the importance of ascB in sinigrin hydrolysis.

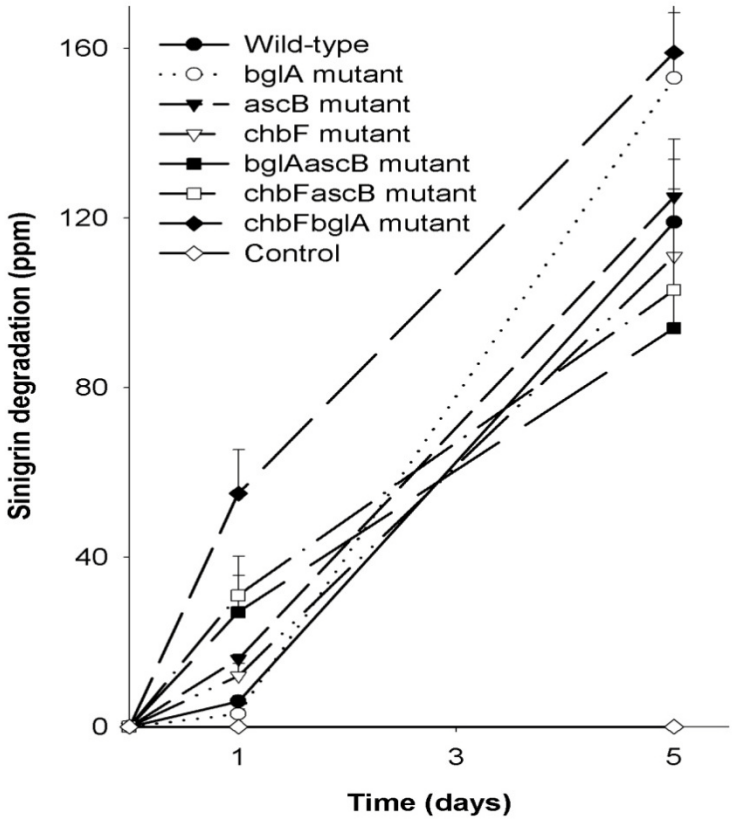

Figure 5.6: Differences in sinigrin degradation between *E. coli* O157:H7 wild-type strain 02-0304, single, and double mutants grown in Muller-Hinton broth containing 0.1% sinigrin. Changes in sinigrin concentration were measured by RP-HPLC for up to 5 d at 25°C. Values represent the mean ± standard error; n = 5.

Although the *bglA* gene was still present in the ΔchbF mutant, the noticeable decrease in degradation by ΔchbF may have been associated with the downregulation of the *ascB* gene. Taking into account that the expression of the *ascB* gene was the only case affected by the individual disruption of *bglA* and *chbF* genes, it is possible that the presence of the *ascB* gene in ΔbglA and ΔchbF contributed to the increase (*ascB* upregulation) and decrease (*ascB* downregulation) in sinigrin degradation observed in the respective mutants (Fig. 5.4). Interestingly, the HPLC results showed that the ΔchbFbglA mutant was better able to degrade sinigrin than the other two double

mutants in which *ascB* had been disrupted. The close similarity in degradation between ΔbglA and ΔchbFΔbglA, where the *ascB* gene was still present, also reinforces the important role that the *ascB* gene may play in sinigrin degradation by *E. coli* O157:H7. In addition, the expression of the *ascB* gene was upregulated in both ΔbglA and ΔchbFΔbglA mutants. Combined, these results suggest that the 6-phospho-*β*-glucosidase encoded by the *ascB* gene is associated with sinigrin degradation in *E. coli* O157:H7. A pattern of interdependence among genes, at least those identified in this study as belonging to the glycoside hydrolase family 1, emerges from the gene expression profile observed. The results showed that the regulation of the *ascB* gene seems to be associated with the presence of the *bglA* gene because the increased expression of the former was only observed when the latter was knocked out. It is known that mutations, recombination, or insertion elements may increase the rate of expression of cryptic genes (Hall et al., 1983; Kachroo et al., 2007). While *bglA* is a constitutively expressed gene that is not induced by any factor, *ascB* is part of a cryptic operon that is influenced by transcriptional factors that can either activate or repress its transcription (An et al., 2005). However, whether the metabolism of sinigrin by *ascB* in *E. coli* O157:H7 is influenced by other activation or repression factors, or even by mutation of other genes, requires further investigation. Moreover, it is also possible that other genes not considered in this study could be related to sinigrin degradation by *E. coli* O157:H7. However, the results found here represent an important step towards identification of the enzyme(s) involved in the degradation of sinigrin by *E. coli* O157:H7.

5.5 Conclusion

The results of the present study suggest that the three genes identified in the *in silico* analysis affect the degradation of sinigrin by *E. coli* O157:H7 to different extents, which enables this pathogen to convert sinigrin into bactericidal AITC. The role of these genes in degradation of

sinigrin as well as their relative importance in this process had not been previously documented in the literature. The comparison of gene expression profiles and changes in the extent of sinigrin degradation by different mutants seem to indicate that *bglA* and *ascB* have a prominent role in the degradation of this *β*-glucoside by *E. coli* O157:H7. However, further research is needed to determine if this importance is due to a greater affinity of sinigrin to the enzyme encoded by *bglA* and *ascB* or if it is due to the influence of the gene on the expression of *chbF*. Work is also needed to determine whether there is involvement of other genes comprising the *asc* operon in sinigrin degradation by *E. coli* O157:H7, and whether gene expression is conditioned by any activation or repression factors.

Chapter 6

Role of the BaeSR two-component regulatory system in adaptation of *Escherichia coli* O157:H7 to allyl isothiocyanate

6.1 Abstract

Allyl isothiocyanate (AITC) is an essential oil with antimicrobial activity against *E. coli* O157:H7. The ability of *Escherichia coli* O157:H7 to withstand inhibitory AITC concentrations and the role of the two-component BaeSR system as a defense mechanism against AITC was studied. Optimal conditions for AITC stability in an aqueous medium were 25°C and pH 5. The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations of AITC against wild-type *E. coli* O157:H7 were 51 and 412 ppm, respectively. After growing *E. coli* O157:H7 in stepwise increased concentrations of AITC, the strain withstood concentrations beyond its MIC (206 ppm), but resistance was reversed when AITC exposure was interrupted. Deletion of either the sensor or regulator genes, *baeS* or *baeR*, yielded cells only as resistant as the wild-type, but the complete deletion of the BaeSR system decreased AITC resistance of *E. coli* O157:H7 to half that of wild-type cells. This is the first demonstration that the ability of *E. coli* O157:H7 to withstand AITC challenge is compromised by the deletion of the BaeSR system. It also indicates that temporary adaptation of bacteria to repeated incremental AITC exposure may occur, but it is unlikely to restrict the importance of AITC as an antimicrobial against *E. coli* O157:H7.

6.2 Introduction

Escherichia coli O157:H7 has been responsible for illness outbreaks associated with uncooked, dry-fermented meat products (Sartz et al., 2008). In an effort to prevent this pathogen from causing foodborne illness, guidelines for fermented meat manufacture in Canada and the United States now require the fermented sausage process to be capable of causing a reduction in *E. coli* O157:H7 viability ≥ 5 Log CFU.g^{-1} (USDA, 2001; Health Canada, 2000). Unfortunately, the antimicrobial hurdles inherent in fermented sausage manufacture are not able to reduce *E. coli* O157:H7 viability to the mandatory level. For instance, the combined effect of low pH and low water activity (aw) present in dry-fermented sausages is only able to reduce the viability of *E. coli* O157:H7 by 1-2 Log CFU.g^{-1} even when starter cultures are used (Erkkila et al., 2000; Chacon et al., 2006; Graumann and Holley, 2008). As a result, new strategies have been sought to reduce *E. coli* O157:H7 numbers in dry-fermented meat products including storage at ambient temperatures, specific heat treatments, high pressure processing or irradiation (Holck et al., 2011).

Recent studies have shown that glucosinolate-containing mustard powder can be used as an ingredient to reduce *E. coli* O157:H7 viability in uncooked dry-fermented sausages and dry cured ham to mandatory levels (Luciano et al., 2011; Nilson and Holley, 2012). Glucosinolates in deodorized mustard (autoclave-treated), devoid of endogenous plant myrosinase, can be hydrolyzed by myrosinase-like activity in *E. coli* O157:H7 to form antimicrobial compounds such as allyl isothiocyanate (AITC) from brown or Oriental mustard (*Brassica juncea*), and ρ-hydroxybenzyl isothiocyanate (ρHBITC) from yellow or white mustard (*Sinapis alba*) (Luciano et al., 2011), which exert microbial control. It is also possible that the formation of phenolic

acids, following autoclave treatment of mustard have antimicrobial activity towards *E. coli* O157:H7 (Luciano et al., 2011).

Allyl isothiocyanate is a plant essential oil (EO) with potent antimicrobial activity against *E. coli* O157:H7. However, determination of important antimicrobial characteristics of AITC, such as its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values has been difficult because of its instability in aqueous media and the lack of a standard method for its analysis. The instability of AITC, which has been attributed to its rapid decomposition in aqueous solution (Ohta et al., 1995; Liu and Yang, 2010), is also a barrier to find whether there is a genetic basis for resistance to AITC sometimes observed with *E. coli* O157:H7 (unpublished, this lab).

The two-component BaeSR regulatory system is comprised of a signaling enzyme, histidine kinase (BaeS), and a cytoplasmic response regulator (BaeR), which are involved in i) modulating the expression of genes encoding multidrug efflux systems (i.e. *mdtABC* and *acrD*), and ii) regulating a third envelope stress pathway (*spy*) that allows organisms to sense and respond to changes during environmental stress (Nishino et al., 2005). The resistance of *E. coli* to compounds such as myricetin, gallic acid, nickel chloride, and sodium tungstate, has been associated with the regulation of the efflux pump genes by the BaeSR system (Zhou et al., 2003). Studies have reported that the BaeSR system in *E. coli* has been responsible for controlling the expression of *spy* in response to the addition of indole (Raffa and Raivio, 2002; Nishino et al., 2005). Moreover, the BaeSR system has been reported to give *E. coli* the ability to overcome inhibitory concentrations of secondary metabolites of plants by the upregulation of *spy* and *mdtABCD* (Zoetendal et al., 2008).

While the BaeSR system has been associated with bacterial response to a wide range of compounds as described above, its involvement with the reponse of *E. coli* O157:H7 to plant essential oils like AITC has not been described in the literature. Thus, the objectives of this study were: i) to investigate the potential of *E. coli* O157:H7 to overcome inhibitory AITC concentrations; and ii) to assess the role of the BaeSR two-component system as a survival response mechanism by *E. coli* O157:H7 to AITC. For these purposes, optimized conditions for AITC stability in an aqueous medium were determined to enable the assessment of the MIC and MBC of AITC against *E. coli* O157:H7.

6. 3 Materials and Methods

6.3.1 Stability of Allyl isothiocyanate

Screw-capped tubes containing Mueller-Hinton broth (MHB) (Oxoid, Basingstoke, England) were used to dilute AITC (Sigma-Aldrich Canada Ltd., Oakville, ON) to concentrations of 50, 100, 200, and 300 ppm. The pH of the broth was adjusted to 5 and 7 using 1 M HCl and 1M NaOH, respectively. The tubes were incubated at 25°C and 35°C for 5 h. Every hour, three samples from each concentration were analyzed for AITC using an HPLC equipped with a C18 column (4.6 × 250 mm, i.d. 5 µm; Waters Co., Milford, MA, USA). The solvent system used was a mixture of acetonitrile (Fisher Scientific Co., Fair Lawn, NJ, USA) and water (60:40, v/v). Isocratic elution was employed with a column flow rate of 1 $mL.min^{-1}$ and an injection volume of 10 µL. A UV detector was used to measure the absorbance at 227 nm in order to verify the presence of AITC. A calibration curve established with an AITC standard under the sample analytical conditions was used for AITC quantification.

6.3.2 Adaptation of E. coli O157:H7 *to increasing AITC concentrations*

Six strains of *E. coli* O157:H7 were used in this study. Strain 02-0304 was provided by Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada. Strains 1934, 1931, 16184, CO283, and EO122 were supplied by the Public Health Agency of Canada, Winnipeg, MB, Canada. The MIC and MBC of AITC against the 6 *E. coli* O157:H7 strains were determined using capped glass tubes. *E. coli* O157:H7 strains grew in Mueller-Hinton broth (MHB) with pH and temperature adjusted to the optimized conditions found in the AITC stability test (i.e. pH 5 and 25°C). The AITC concentrations ranged from 26 to 824 ppm. Turbidity was used to monitor growth of *E. coli* O157:H7 and the MIC was measured as the lowest concentration of AITC that inhibited visible growth of *E. coli* O157:H7. Bacterial cells were plated on Violet Red Bile (VRB) agar (Oxoid, Basingstoke, England) for determination of the MBC. To determine the MBC, the dilution representing the MIC and the three next higher concentrations were plated on VRB agar and enumerated to determine viable CFU/mL. The residual effect of AITC transferred from the dilutions to VRB plates was negligible even for the highest AITC concentration plated ($\leq$ 2 ppm). The MBC was measured as the lowest concentration that showed a reduction of 99.9% in CFU/mL when compared to the MIC dilution.

E. coli O157:H7 strain 02-0304 was exposed to concentrations below its MIC and challenged with progressively increased concentrations of AITC in order to allow its adaptation to higher AITC levels. *E. coli* O157:H7 was grown in MHB at the optimized pH and temperature found in the AITC stability test (pH 5 at 25°C), with sub-MIC AITC concentrations ($\geq$ 26 ppm). After incubation, bacterial turbidity was monitored at 600 nm (A600). When A600 was $\geq$ 0.5, 1 mL of bacterial suspension was transferred to a fresh tube of MHB containing a two-fold higher

concentration of AITC. This process was repeated at three-day intervals over a period of several weeks up to 206 ppm AITC. Upon reaching this concentration, the bacterial suspensions were stored at -20°C in MHB containing 20% glycerol.

6.3.3 Involvement of the BaeSR two-component system in the adaptation of E. coli O157:H7 *to AITC*

The role of the BaeSR two-component regulatory system in the adaptation of *E. coli* toward AITC was investigated by the development of BaeSR mutants. The BaeSR mutants were obtained by individual disruption of the sensor (*baeS*) and regulator (*baeR*) genes. Also, a dual mutation of BaeSR was developed by the simultaneous disruption of both sensor and regulator genes. For gene disruption, the lambda red-mediated gene replacement method described by Datsenko and Wanner (2000) was used. Briefly, a curable expression plasmid (i.e. pkD46) encoding Red recombinase and an ampicillin resistance gene was introduced into *E. coli* O157:H7 02-0304, and the expression of the Red system was induced with 1mM arabinose. The plasmid pkD46 was from the collection of the Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada. Then, the chloramphenicol resistance gene (*cat*), flanked by target recognition sites, was amplified by polymerase chain reaction (PCR) with primers that were homologous to the beginning and end of the coding sequence of the gene to be disrupted (Table 6.1). The PCR reaction was conducted using a volume of 50 µL comprised of 25 µL of Multiplex PCR Mastermix, 0.5 µL of each primer (25 pmol), 21 µL of water, and 2 µL of the template plasmid pkD3 containing a chloramphenicol resistance (*cat*) gene, which was purchased from the Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT, USA. The PCR product was gel-purified, digested with DpnI, repurified, and

suspended in elution buffer (10 mM Tris, pH 8.0). Electroporation was done using 50 μL of the recipient 02-0304 strain expressing Red recombinase and 4 μL of the purified PCR product. Shocked cells were added to 1 mL Super Optimal Catabolite-repression (S.O.C) medium (Hanahan, 1983), incubated for 3 h at 39°C, and then plated on Luria Bertani (LB) agar (Difco Laboratories, Sparks, MD, USA) containing 25 $\mu L.mL^{-1}$ of chloramphenicol to select chloramphenicol resistant transformants. The elimination of the pkD46 vector was confirmed by the loss of ampicillin resistance after its exposure to the nonpermissive temperature of 39°C. DNA was isolated from the mutants obtained, and the deletion of the targeted loci was confirmed by performing a series of PCRs with primers complementary to cat and adjacent regions. The baeS mutant was then selected for the double mutation of the BaeSR system.

Table 6.1: Primers used for mutant construction and validation.

Primers	**Sequence primer 5' → 3'**	**Source**
baeR_F	*ATG ACC GAG TTA CCA ATC GA*	This study
baeR_R	*CTA AAC GAT GCG GCA GGC GT*	This study
baeR_cat_F	CTAAACGATGCGGCAGGCGTCGGCTTCCCAGCGGTAACCGACGCCGTAAA**GTGTAG GGCTGGAGCTGCTTC**	This study
baeR_cat_R	TATGACCGAGTTACCAATCGACGAAAACACACCGCGTATTTTGATCGTGGAA**CATA T GAATATCCTCCTTAGT**	This study
baeS_F	*ATGAAGTTCTGGCGACCT*	This study
baeS_R	*TCTGTAAATCCCGTTCCAGC*	This study
baeS_cat_F	GTACGCCATTAGCGGTGCTGCGCGGT**GTGTAGGCTGGAGCTGCTTC**	This study
baeS_cat_R	TGCTTACCCCGCCAAAAGGCGAAT**CATATGAATATCCTCCTTAGT**	This study

Italicized letters represent primers that flank only the target gene. Bold letters represent primers that have internal overlap with the resistance marker (pKD3), while non-bold letters represent primers having external overlap with the target knockout genes. Primers were designed using BioEdit Sequence Alignment Editor software and manufactured by UCDNA Services, Faculty of Medicine, University of Calgary (Calgary, AB, Canada).

The pCP20 helper plasmid purchased from the Department of Molecular, Cellular, and Developmental Biology, Yale University, was used for the elimination of the chloramphenicol resistance gene present in the *baeS* mutant. After electroporation of 50 µL of the *baeS* mutant with 4 µL of pCP20, ampicillin-resistant transformants were selected at 30°C and tested for the loss of chloramphenicol resistance. The transformed *baeS* mutant strain was then used for the re-introduction of the chloramphenicol resistance gene (*cat*), flanked by target recognition sites of the second gene to be disrupted (i.e. *baeR*). The PCR assays were conducted using volumes of 25 µL comprised of 12.5 µL of Multiplex PCR Mastermix, 0.5 µL of each primer (25 pmol), 2 µL of bacterial DNA (100 ng.μL^{-1}), and 9.5 µL of water. All PCR amplifications were carried out using the following temperature program: initial denaturation at 94ºC for 5 min, 36 cycles at 94ºC for 1 min, annealing temperature for primers at 58ºC, an extension at 72ºC for 1 min, and a final extension step at 72ºC for 10 min. Amplification products were subjected to gel electrophoresis in a 1% agarose gel at 120 V for 60 min. The construction of *E. coli* O157:H7 mutants was verified by DNA sequencing at UCDNA Services, Faculty of Medicine, University of Calgary (Calgary, AB, Canada).

6.4 Results and Discussion

6.4.1 AITC stability

The stability of AITC was tested using four concentrations during 5 h incubation at different pH and temperatures (Fig. 6.1). Of all four conditions tested, AITC stability in broth adjusted to pH 5 and incubated at 25°C was significantly greater than at the other conditions tested ≤ 5 h (Figs. 6.1a-d). Thus, the more acidic pH and lower temperature maintained AITC stability best.

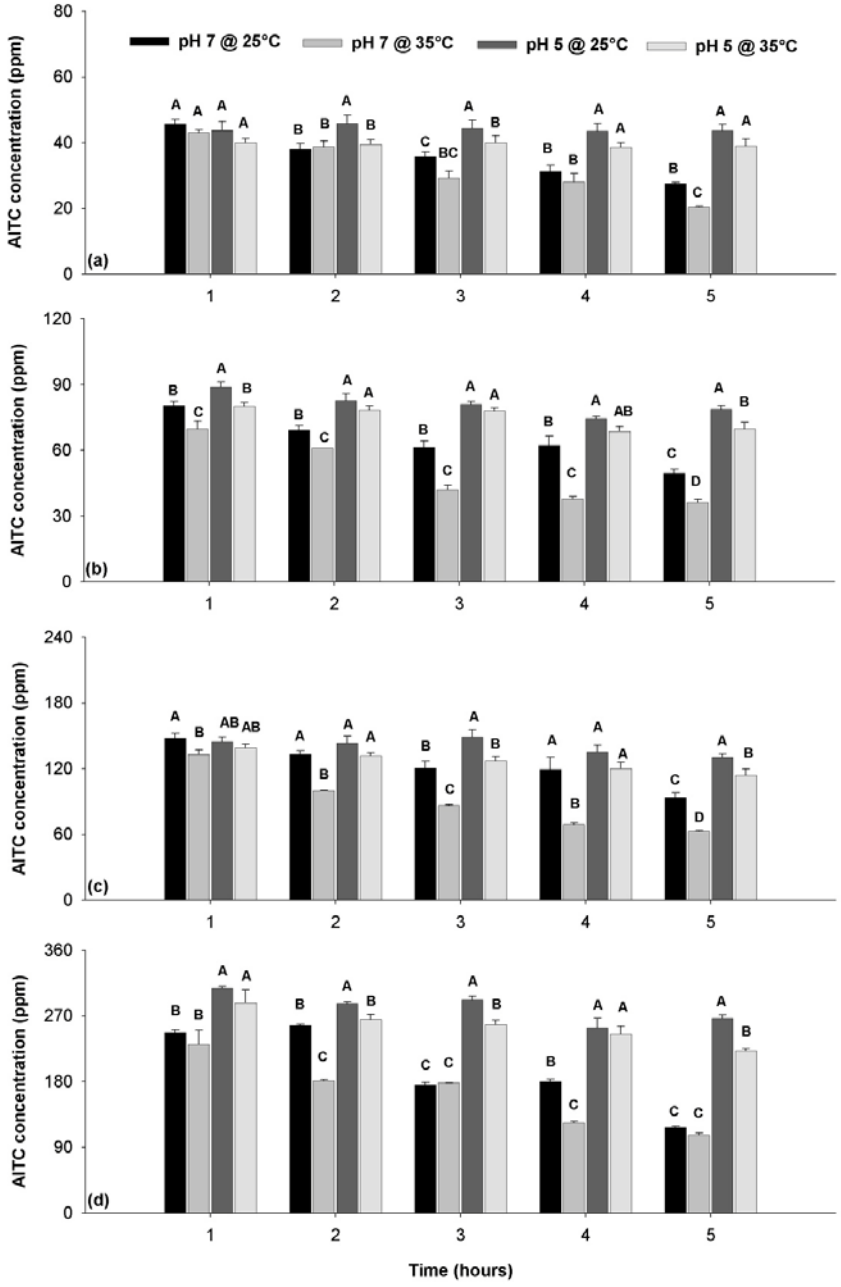

Figure 6.1: Quantification of allyl isothiocyanate (AITC) by HPLC. AITC concentrations (a) 50 ppm, (b) 100 ppm, (c) 200 ppm, and (d) 300 ppm, were tested in Mueller-Hinton broth during 5 h incubation at different pH and temperatures. Mean AITC concentrations are averages of three replicates. Different letters for means at the same concentration and same time indicate a statistically significant difference between treatments ($p < 0.05$).

The influence of pH on AITC stability found in this study is consistent with results found by others. Chen and Ho (1998) demonstrated that 5000 ppm of AITC in aqueous solution was more stable at pH 2.7 than at pH 7 and 9 when heated at 100°C for 60 min. Tsao et al. (2000) showed that the half-lives of AITC (1000 $\mu g.mL^{-1}$) at 25°C were 31, 34, 31, and 26 d at pH 5, 6, 7, and 9, respectively. These results indicated that AITC was more stable at acid to neutral rather than alkaline pH values. Moreover, Luciano and Holley (2009) showed that the inhibitory concentration of AITC against *E. coli* O157:H7 at pH 4.5 was 20-fold lower than that at pH 8.5 when incubated at 37°C. Their results suggest that AITC may be more effective as an antimicrobial in acid foods due to its increased stability. The stability pattern of AITC observed in this and their work might be explained by the fact that low pH values decrease the effects of nucleophilic attack by water molecules against AITC, which renders it more stable (Jiang et al., 2006).

In contrast to pH, few studies have addressed the effect of different temperatures on AITC stability. Pecháček et al. (1997) worked with solutions containing 1000 ppm of AITC in buffers of pH 4, 6, and 8, held at 20°C, 40°C and 80°C for 80 min. They found that the decomposition of AITC was significantly influenced by the pH of the medium, with the slowest degradation observed at pH 4. The authors also showed that solutions at 80°C were more prone to AITC decomposition because more AITC degradation products were found at this temperature. Conversely, low quantities of the same products were found at lower temperatures, which imply that AITC decomposition was reduced at 20°C and 40°C. It was noteworthy that Luciano and Holley (2009) showed the compounds generated by AITC decomposition had no effect on *E. coli* O157:H7 viability and were not able to reduce *E. coli* O157:H7 growth individually or when combined with a sub-lethal concentration of AITC. The results of the

present study suggest that the acidic pH and low temperature (16-26°C) used in dry-fermented sausage manufacture promote the stability of AITC, which in turn, enhance its ability to control *E. coli* O157:H7.

6.4.2 Adaptation of E. coli O157:H7 *to high AITC concentrations*

The MIC and MBC of AITC toward the 6 *E. coli* O157:H7 strains tested were 51 and 412 ppm, respectively. After being challenged with increased AITC concentrations, *E. coli* O157:H7 strain 02-0304 was able to grow in 206 ppm AITC, suggesting that this strain was able to overcome its inhibitory effects. However, when the AITC challenge was removed and cells were held at -20°C in 20% glycerol for a few days, upon reactivation the organism became unable to grow at AITC levels higher than its MIC. Since it was possible that AITC instability at 25°C influenced the accuracy of MIC and MBC determinations, viable bacteria present during exposure to 26 to 824 ppm AITC were monitored hourly to 5 h and then at 18 h. Results confirmed that changes in numbers occurred within the first hour of AITC exposure and numbers thereafter were not significantly ($p > 0.05$) different from those at 18 h. Thus, reductions in AITC concentration observed (Fig. 6.1) due to AITC instability did not influence the MIC and MBC values of AITC determined.

The ability to grow at high AITC concentrations seemed to have been a temporary, phenotypic response that was reversed upon removal of the AITC challenge. It is of interest that the ability of microorganisms to withstand the toxicity of essential oils has also been investigated by others. Ultee et al. (2000) showed that after culturing *B. cereus* cells in inhibitory, but not lethal concentrations of≤ 0.4 mM carvacrol, bacterial cells were less sensitive to subsequent exposure to carvacrol than cells incubated in its absence. The phenotypic response of *B. cereus*

to carvacrol was believed to be related to the reduction of membrane fluidity through changes in fatty acid and phospholipid head-group composition. In another study, stress response seemed to facilitate the ability of bacterial cells to withstand concentrations of secondary metabolites of plants. Smith et al. (2003) showed that *E. coli* was able to overcome the inhibitory effects of tannin, and spontaneous tannin tolerant mutants were isolated from a sensitive *E. coli* strain. The inhibition of *E. coli* growth was caused by the production of hydrogen peroxide from the auto-oxidation of tannin, but the resistant *E. coli* strain was able to overcome the inhibitory effect of tannin by an oxidative stress response. The isolated tannin tolerant strain showed more than a 10-fold increase in hydroperoxidase I (HPI), a hydrogen peroxide-inducible protein, while mutants lacking the HPI gene were more sensitive to the effect of tannins.

6.4.3 The role of the BaeSR two-component system in E. coli O157:H7 *response to AITC*

E. coli O157:H7 mutants lacking *baeS*, *baeR*, and *baeSR* genes were confirmed using PCR assays and sequencing analyses (Fig. 6.2). Mutants lacking the *baeS* or *baeR* genes showed MIC/MBC values for AITC similar to those of the wild-type strain (Table 6.2). Besides the similarity of MIC, both the ΔbaeS or ΔbaeR mutants also showed similar viable numbers on VRB plates at exposures to all concentrations of AITC after overnight incubation. However, viable numbers of both the ΔbaeS or ΔbaeR mutants were slightly lower than numbers of wild-type cells at concentrations of 51, 103 and 206 ppm (Fig. 6.3). In another study, Kivistik et al. (2006) showed that a mutant strain lacking the regulator gene (*colR*) of the two-component ColRS system in *P. putida* was more sensitive to elevated phenol concentrations than the wild-type strain.

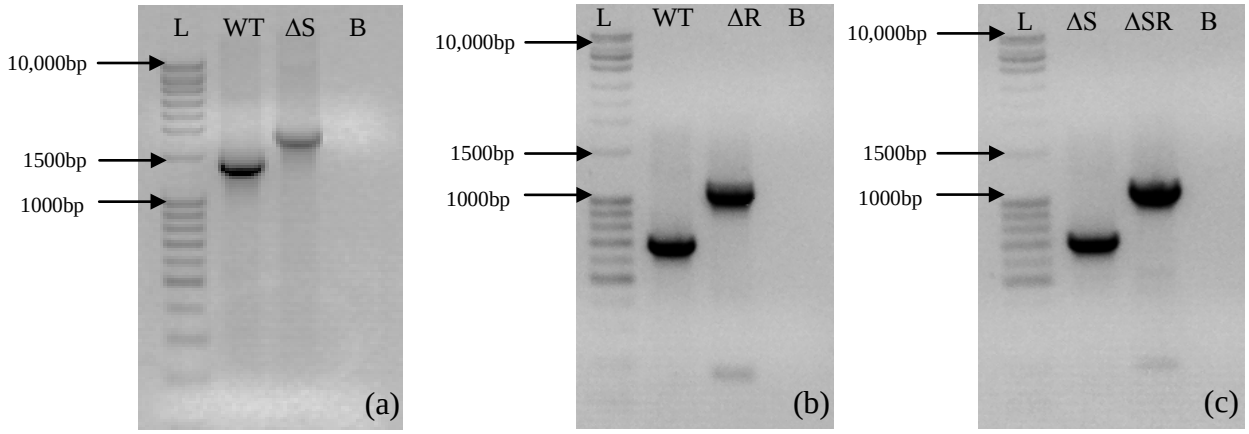

Figure 6.2: Gel-electrophoresis of PCR amplification products showing replacement of genes from the BaeSR two-component system. (a) the *baeS* gene segment (600bp) was replaced by the chloramphenicol resistance marker (1034 bp) [wild-type *baeS* (WT) = 1404bp; mutant baeS (ΔS) = 1838bp], (b) the *baeR* gene (723 bp) was replaced by the chloramphenicol resistance marker (1034 bp) [wild-type *baeR* (WT) = 723 bp; mutant baeR (ΔR) = 1034 bp], and (c) the *baeR* gene from the mutant baeS (723 bp) was replaced by the chloramphenicol resistance marker (1034 bp) [mutant baeS (ΔS) = 723 bp; double mutant baeSR (ΔSR) = 1034 bp]. B: blank. L: DNA ladder 1kb. *E. coli* O157:H7 strain 02-0304 was used for all mutations.

The similarity of cell numbers on VRB plates of both single mutants in the present study indicates there is a network of functional interaction involving cross-regulation between the BaeSR system and either a sensor or a regulator gene from another two-component system. It is likely that a sensor other than BaeS was able to activate BaeR or, conversely, that a response regulator different from BaeR was activated by BaeS. As a result of this interaction the performance of the system was slightly affected, where the single mutants did not achieve the same viable numbers as the wild-type (Fig. 6.3).

Table 6.2: Minimum inhibitory concentration (MIC) and minimum bacteridal concentration (MBC) of AITC to *E. coli* O157:H7 strains and respective mutants.

Bacteria (strain)	MIC (ppm)	MBC (ppm)
E. coli O157:H7 (1934)	51	412
E. coli O157:H7 (1931)	51	412
E. coli O157:H7 (16184)	51	412
E. coli O157:H7 (CO283)	51	412
E. coli O157:H7 (EO122)	51	412
E. coli O157:H7 (02-0304)	51	412
ΔbaeS (02-0304)	51	412
ΔbaeR (02-0304)	51	412
ΔbaeSR (02-0304)	26	206

It was noteworthy that the interaction between two-component systems was not evident from the comparison of MIC alone because both ΔbaeS and ΔbaeR and the wild-type had the same MIC. The interaction was more apparent when analyzing the viable numbers. The versatility of this functional interaction between two-component systems in *E. coli* has been reported in the literature. For example, Oshima et al. (2002) established there were functional interactions between two-component systems using a microarray profile of 36 mutants of *E. coli* K-12. High correlations between mutant gene profiles indicated cross-regulation among the BaeSR, DcuSR, YpdAB, and YehUT two-component systems. The two-component DcuSR system is involved in anaerobic fumarate respiration, whereas both YpdAB and YehUT systems are involved in the carbon control network of *E. coli* K-12. According to Oshima et al. (2002), the regulatory network formed by different two-component systems is important for *E. coli* adaption and survival in response to complex environmental changes. The functional redundancy among BaeSR and other two-component systems was also confirmed by Nishino et al. (2005).

The latter authors found a network of functional interactions in *E. coli* among BaeSR, PhoBR, and CreBC two-component systems. While the PhoBR system is associated with the assimilation of an alternative phosphate source, the CreBC system appears to be involved in carbon and energy metabolism. In that study, *baeR* amplification increased the expression levels of *phoB* and *phoR*, which are the sensor and regulator of the PhoBR system, respectively. Baranova and Nikaido (2002) supported the occurrence of cross-talk among BaeSR and other two-component systems by showing that full activity of overexpressed *BaeR* did not require the presence of the sensor kinase *BaeS*. According to the authors, the activation of *BaeR* was due to another sensor kinase present in *E. coli*. From these results, it appears that several different sets of genes may be controlled by different networks of two-component systems, expanding the adaptability of *E. coli* to different challenge conditions.

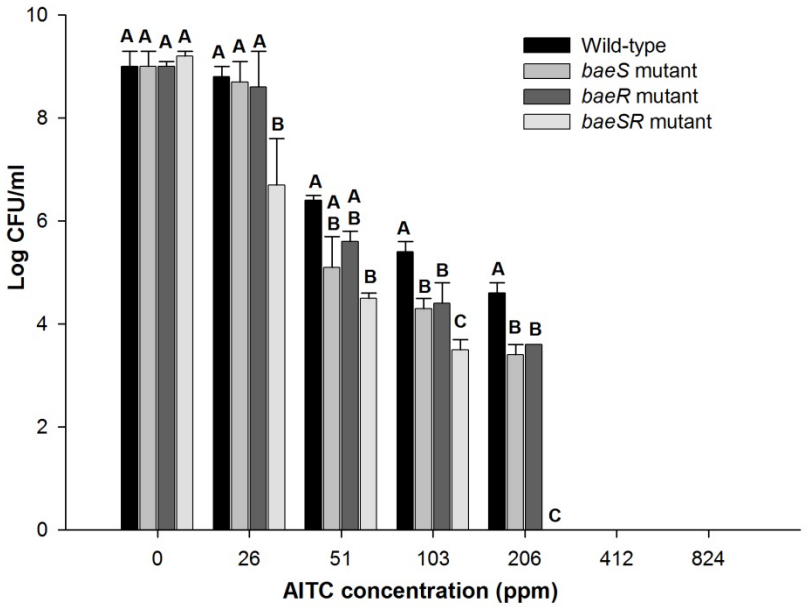

Figure 6.3: Enumeration of *E. coli* O157:H7 after overnight exposure to AITC concentrations in MHB with pH 5 and incubation at 25°C. Bacterial cells were plated on VRB and incubated at 35°C. *E. coli* O157:H7 counts are averages of three replicates. Different letters at the same AITC concentration indicate that the difference between bacterial counts of strains were statistically significant ($p < 0.05$).

In contrast to ΔbaeS, ΔbaeR, and wild-type cells, the double mutant *(Δbae*SR) lacking both the *baeS* and *baeR* genes was more sensitive to AITC. The MIC and MBC values of AITC against ΔbaeSR were 26 ppm and 206 ppm, respectively (Table 6.2). Numbers of ΔbaeSR were more than 2 Log CFU.mL^{-1} lower than wild-type cells on VRB plates after AITC exposure at 26, 51, and 103 ppm (Fig. 6.3). In addition, the ΔbaeSR strain was not able to grow at AITC concentrations > 200 ppm. These results showed that the sensitivity of the *E. coli* O157:H7 strain to AITC was increased by the simultaneous deletion of *baeS* and *baeR* genes. It seems that the complete deletion of the two-component BaeSR system limited the ability of *E. coli* O157:H7 to withstand enhanced AITC challenge. The extent to which the two-component systems control

bacterial response to environmental challenges has been described elsewhere (Leblanc et al., 2011). Zoetendal et al. (2008) found that an *E. coli* mutant strain lacking the two-component BaeSR system was more sensitive to condensed tannins than wild-type cells. Initially, wild-type *E. coli* was able to overcome the inhibitory effect of tannins by mechanisms that involved the cell envelope stress protein gene *spy* and the multidrug transporter-encoding operon *mdtABCD*. These genes, which are under the control of the two-component BaeSR regulatory system, were significantly up-regulated in the presence of tannins. This ability was decreased upon deletion of the two-component BaeSR system. Nishino et al. (2005) showed that the expression of *spy* and genes encoding the operon *mdtABCD* in *E. coli* were decreased by more than a factor of two after *baeSR* deletion. Nagakubo et al. (2002) found that overexpression of the regulator BaeR stimulated resistance to novobiocin and bile salts by upregulating the expression of the multidrug transporter mdtABC in *E. coli*. Although this two-component system responds to and regulates environmental signals such as stress adaptation and drug resistance, the results of the present study indicate that *E. coli* O157:H7 was more prone to respond to AITC toxicity as an environmental stress rather than a drug toxicity since *E. coli* O157:H7 was unable to develop permanent resistance to AITC. It is likely that the unstable adaptive resistance of *E. coli* O157:H7 to AITC was a bacterial stress response to this compound, which was lost after removal of the stress.

6.5 Conclusion

This study showed that the instability of AITC in aqueous medium was minimized at pH 5 and 25°C. These are conditions that are developed during dry-fermented sausage manufacture and would promote the stability of AITC as well as its effectiveness as an antimicrobial agent.

When sequentially exposed to increased AITC concentrations, *E. coli* O157:H7 was transiently able to adapt to concentrations above its MIC. Partial or complete deletion of the two-component BaeSR system reduced the ability of *E. coli* O157:H7 to grow in AITC, indicating that this system played a role in the response of this pathogen to AITC toxicity. Based on the known role of the two-component system as a defense mechanism, it seemed that *E. coli* O157:H7 responded to AITC challenge as an environmental stress. The observed sensitivity of *E. coli* O157:H7 to AITC and its inability to acquire stabile resistance to this compound demonstrated the potential value of AITC as an antimicrobial for use in dry-fermented sausage ripening to control this pathogen.

Chapter 7

Overall Discussion

It has been found that the use of mustard as a natural antimicrobial of plant origin controls *E. coli* O157:H7 in non-thermally treated dry-fermented sausage. As discussed in preceding chapters, the required 5 Log CFU.g^{-1} reduction of *E. coli* O157:H7 in dry-fermented sausage was easily achieved when 6% (w/w) mustard was used. However, these concentrations were not considered acceptable by consumers. The present work investigated whether reduced concentrations of yellow mustard would be capable of controlling *E. coli* O157:H7 viability in dry-fermented sausage (Chapter 3). The results showed that 4% (w/w) deodorized (autoclaved at 15°C for 15 min) mustard powder was the minimum level of mustard able to achieve the 5 Log CFU.g^{-1} reduction of *E. coli* O157:H7 in dry-fermented sausage at 28 d; however, this concentration also had low overall acceptance by consumers (Li et al., 2013). In contrast, dry-fermented sausage with 2% (w/w) mustard had good consumer acceptance; but this concentration required longer than the normal production period (i.e. 35 d) to achieve the required 5 Log CFU.g^{-1} reduction. A strategy to facilitate a reduction in mustard concentrations and still have the antimicrobial activity needed to control *E. coli* O157:H7 found was the use of mustard with a higher level of glucosinolates, which yielded greater amounts of antimicrobial ITCs. In addition, a small amount of mustard powder with plant myrosinase was combined with mustard powder containing higher levels of glucosinolates to accelerate the conversion of glucosinolates to ITC. A third approach was the use of mustard meal instead of powder. The meal is the material left after oil is pressed from the seeds and will contain a proportionately greater concentration of glucosinolates. Sausages treated with mixed mustard (deodorized + hot) would be expected to give faster reduction in *E. coli* O157:H7 viability than sausages treated

with deodorized mustard meal alone due to the presence of active myrosinase (which would enhance the hydrolysis of glucosinolates). In earlier work, contradictory results were found in different studies. Luciano et al. (2011) showed that laboratory deodorized mustard was more effective in killing *E. coli* O157:H7 than cold (commercial) or hot powders alone. Also, the combination of deodorized + hot mustard showed a more pronounced capacity for killing *E. coli* O157:H7 than the deodorized mustard alone. Conversely, Graumann and Holley (2008) found greater antimicrobial activity in the cold mustard, where a 5 Log reduction of *E. coli* O157:H7 viability was achieved at 24 d instead of the 30 d for hot mustard of the same cultivar. In the present study, sausages treated with 2% mixed mustard (1% deodorized + 1% hot) showed a reduction in *E. coli* O157:H7 viability similar to sausages treated with 2% deodorized mustard, and sausages treated with 4% mixed mustard (2% deodorized + 2% hot) required additional time to achieve a 5 Log $CFU.g^{-1}$ reduction of *E. coli* O157:H7 viability when compared to 4% deodorized mustard. This variability in the antimicrobial activity of mustard treatments was an artifact of using non-standardized thermal treatment processes. The issue was resolved by establishing the maximum depth of mustard powder at 1 cm for inactivation of plant myrosinase when heating in trays at 115°C for 15 min. In Chapter 4 several aspects influencing isothiocyanate formation due to residual myrosinase and glucosinolate content in mustard were examined. Specifically, tests examined whether the thermal treatment was sufficient to inactivate naturally occurring plant myrosinase; whether a small amount of hot mustard containing active myrosinase might accelerate the rate of reduction of *E. coli* O157:H7 viability in dry-fermented sausage; and whether mustard powder and deoiled mustard meal from yellow mustard were equivalent to the same preparations from Oriental mustard and able to achieve a ≥ 5 $Log.g^{-1}$ reduction of *E. coli* O157:H7 in sausages.

The deodorization treatment developed in previous studies typically used a 2 cm thick layer of mustard autoclaved at 115^{o}C for 15 min. However, the present study showed that this procedure was not able to completely inactivate plant myrosinase, resulting in partially deodorized (P-d) mustard. When a 1 cm thick layer of powdered mustard was autoclaved at 115^{o}C for 15 min, the myrosinase present was consistently inactivated, resulting in fully deodorized (F-d) mustard. It is possible that the variability in antimicrobial activity of mustard found in previous studies (Luciano et al. 2011 and Chapter 3) was due to the presence of residual myrosinase in mustard which was autoclaved in a 2 cm thick layer. In fact, myrosinase is a thermally stable enzyme (Van Eylen et al., 2008); although stability appears to vary widely among different *Brassica* species (Ludikhuyze et al., 1999; Ghawi et al., 2012). Inactivation of myrosinase extracted from *S. alba* L. and *Brassica napus* seeds has been reported to occur only at temperatures above 60° C (for 30 min) (Bjorkman and Lönnerdal, 1973), and 75°C (for 10 min) (Van Eylen et al., 2006). It is possible that this thermal stability contributed to the retention of some myrosinase activity in the 2 cm layer of powder because of uneven heat penetration through the thicker layer during treatment. When a small amount of hot yellow mustard containing endogenous plant myrosinase was added to fully-deodorized powders or meals from the same cultivar it had a larger effect on *E. coli* O157:H7 viability than when Oriental mustard was used (Chapter 4). The reduction of *E. coli* O157:H7 to mandatory levels was achieved in 18 d with 4% yellow mustard powder treatments containing either 0.1% or 0.2% hot mustard. In contrast, Oriental mustard powder given the same thermal treatment and proportions of hot mustard powder in the mixtures was unable to cause a 5 Log reduction by 36 d.

The greater antimicrobial effect of yellow than Oriental mustard when combined with a small proportion of hot powder is related to its greater glucosinolate content which contributed to

enhanced formation of ITC. There was about two times more glucosinolate (sinalbin) in yellow mustard than sinigrin in Oriental mustard powder samples on a molar basis. Since myrosinase activity follows first order kinetics (Ghawi et al., 2012; Van Eylen et al., 2007), a higher glucosinolate conversion rate would be expected in mustard containing greater glucosinolate content. It is also possible that the ITC formed from glucosinolate hydrolysis in yellow mustard (ρ-HBITC) has greater antimicrobial activity than AITC from Oriental mustard. To test the antimicrobial activity of deoiled mustard meal, concentrations were reduced to 2% (w/w) since this mustard fraction contains higher proportions of glucosinolates. The results showed a 5 Log reduction of *E. coli* O157:H7 within 21 d in sausages treated with 2% deodorized, deoiled yellow mustard meal containing 0.2 % hot mustard, while the treatment with 2% deodorized, deoiled Oriental mustard meal with 0.2 % hot mustard showed a reduction in *E. coli* O157:H7 numbers to the mandatory level at 35 d. Interestingly, the 2% deoiled Oriental mustard meal with 0.2% hot mustard had greater antimicrobial activity than 4% Oriental mustard powder containing 0.2% hot mustard. This can be explained by the fact that deoiled meals from either yellow or Oriental mustard contain greater glucosinolate concentrations than mustard powders of the same type. The fact that myrosinase activity follows first order kinetics leads to greater glucosinolate conversion to ITCS in mustard containing more glucosinolate. These findings suggest that the use of deoiled meal in dry-fermented sausage would allow a 50% reduction of mustard levels in industrial applications, which would allow use of concentrations acceptable to consumers which were capable of achieving the mandatory $\geq$ 5 Log CFU.g^{-1} reduction of *E. coli* O157:H7.

In experiments reported in Chapters 3 and 4, it was established that the myrosinase-like activity in *E. coli* O157:H7 contributed to glucosinolate hydrolysis in sausages where plant myrosinase was not present. To understand the genetic basis of the myrosinase activity expressed

by *E. coli* O157:H7, the role of glycoside hydrolase genes in sinigrin hydrolysis by *E. coli* O157:H7 was investigated (Chapter 5). An *in silico* analysis was the approach chosen for identifying genes associated with myrosinase activity in *E. coli* O157:H7. This was possible because the enzyme responsible for glucosinolate hydrolysis in mustard was already known and was used as a starting point. Despite the advent of genome-wide microarrays and large-scale genome sequencing, *in silico* screens can still reveal target genes not identified by microarray analysis (Wang et al., 2005). Generally, microarrays identify genes that are both up- and down-regulated at a range of different levels; however, the genes identified are limited to those regulated in the model system under investigation (Lin et al., 2013). *In silico* analysis allows the identification of potential target genes independent of their type of expression; however, it produces protein targets for which the biochemical function and/or structural "fold" assignment is unknown. In the present study, the *in silico* analysis not only showed similarities between myrosinase from Oriental mustard and the enzyme 6-phospho-*β*-glucosidase in *E. coli* O157:H7, but identified the genes encoding the 6-phospho-*β*-glucosidase in this pathogen. It also allowed the categorization of enzymes studied into the different known glycoside hydrolase families. Myrosinase from mustard and 6-phospho-*β*-glucosidase in *E. coli* O157:H7 that were encoded by genes *bglA* and *ascB* were classified within the glycoside hydrolase family 1, while a third gene, *chbF,* was classified in family 4. Similarly, Mullaney et al. (2013) confirmed that 6-phospho-*β*-glucosidase and *β*-glucosidase genes were assigned to glycoside hydrolase family 1 and that these enzymes were present in several *Lactobacillus* and *Lactococcus* strains exhibiting myrosinase activity. Thus, the practicality of *in silico* analysis was shown and the availability of information in the public databases facilitated the selection of the target genes to be investigated.

Although much is known about the role of 6-phospho-*β*-glucosidase in the hydrolysis of *β*-glucosides such as arbutin, salicin, and disaccharide cellobiose, no information has been reported for sinigrin, which is also a β-glucoside. While sinigrin is known to be a substrate for myrosinase, studies identifying sinigrin as a substrate for 6-phospho-*β*-glucosidase were not found in the literature. Gene expression profiles obtained by exposing the wild-type strain to sinigrin in this study showed that sinigrin induced the expression of the 6-phospho-*β*-glucosidase encoded by *bglA*, *ascB* and *chbF* genes in *E. coli* O157:H7 to different extents.

Among the three genes encoding 6-phospho-*β*-glucosidase in *E. coli* O157:H7, *ascB* was shown to play a role in sinigrin degradation in *E. coli* O157:H7 when hydrolysis of sinigrin and gene expression profiles were compared among single, double mutants, and wild-type strains. The hydrolysis of salicin and arbutin, which are glycosides similar to sinigrin, has been associated with the *ascB* gene (Raghunand and Mahadevan, 2004). The gene *ascB* is part of the cryptic *asc* operon (AscG, AscF, and AscB) of *Pectobacterium carotovorum* which is influenced by factors that can either activate or repress its transcription (An et al., 2005). This operon was found to be very similar to the *asc* operon of *E. coli* (An et al., 2005). Thus, it is important to consider the influence of factors that can activate or repress the metabolism of sinigrin by *ascB* in *E. coli* O157:H7.

The one-step inactivation of chromosomal genes using PCR products is a common technique used with *E. coli* for genetic modification (Datsenko and Wanner, 2000). Thus, it was the method chosen for construction of single and double mutants in the present study. However, the method showed low efficiency during the construction of double mutants, giving a high proportion of antibiotic-resistant transformants without gene disruptions. It is possible that the low efficiency observed in multiple gene disruptions impeded the construction of a triple mutant

strain. Several attempts to construct a triple mutant were made without success by using the three different double mutants (i.e. bglAascB, chbFascB, and chbFbglA). For each double mutant, different primer sequences targeting different regions of the remaining gene to be disrupted was used. Thus, the primer sequence used depended on the double mutant used in the triple mutant attempt. Also, optimized growth conditions and different antibiotic markers were tested.

The low rate of mutants observed might have been associated with the presence of "scars" produced during gene disruption. Usually when gene disruptions are made, the elimination of the antibiotic resistance gene leaves behind about an 82- to 85-nucleotide scar in place of the disrupted gene (Datsenko and Wanner, 2000). Each incoming PCR product encodes a selectable antibiotic resistance marker flanked by flippase recognition target (FRT) sites, which are the source of FRT scars. These scars may serve as recombinant hot spots at each successive step in strain construction, reducing the frequency of obtaining the desired insertion (Datsenko and Wanner, 2000). The presence of scars could become problematic if the method is used to introduce multiple mutations (Sun et al., 2008; Katashkina et al., 2009). As an alternative to the one-step inactivation of chromosomal genes using PCR products, studies have described a two-step recombination method for markerless gene deletion and insertion that can be used for repetitive genetic modification (Tischer et al., 2006; Sun et al., 2008). However, the efficiency of this method in *E. coli* O157:H7 has not been exploited. In contrast, the PCR-mediated gene replacement method has been used extensively in *E. coli* (Datsenko and Wanner, 2000; Murphy et al., 2000) and *Salmonella* Typhimurium *(*Chakravortty et al., 2002; Worlock and Smith, 2002), but less so in pathogenic species of *E. coli* such as the EHEC and EPEC because of the inconsistent results demonstrated (Murphy and Campellone, 2003). Another alternative to PCR-mediated gene replacement methods are recombination methods that create gene disruption on a

suitable plasmid before recombining it into the chromosome using restriction enzymes which cleave DNA at sequence-specific sites (Russel, 2002).

While the genetic characterization of the myrosinase activity in *E. coli* O157:H7 (Chapter 5) was significant, investigation of the potential value of AITC as a target-generated antimicrobial against *E. coli* O157:H7 (Chapter 6) was of greater significance. As an essential oil, AITC is a promising candidate as a natural antimicrobial because of its ability to kill foodborne pathogens like *E. coli* O157:H7. However, an antimicrobial agent should cause no genetic damage so that its toxicity towards higher multicellular organisms and resistance development can be minimized (Chan et al., 2013). In this study *E. coli* O157:H7 was unable to develop permanent resistance to higher AITC concentrations following repeated exposure. In addition, during this work the contribution made by the two-component BaeSR regulatory system was assessed. To better determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of AITC against *E. coli* O157:H7, optimized conditions for AITC stability in an aqueous medium were established (Chapter 6).

AITC stability was best maintained under conditions usually used during dry-fermented sausage manufacture (pH 5 and 25°C) (Pecháček et al., 1997; Tsao et al., 2000), and this promoted its effectiveness as an antimicrobial agent. Although the MIC (51 ppm) and MBC (412 ppm) of AITC toward *E. coli* O157:H7 were determined, a comparison with the values reported in the literature was difficult since standardized conditions for measuring the antimicrobial activity of AITC have not been established. Divergence between the antimicrobial concentrations of essential oils obtained by the agar dilution method and the broth dilution method have been reported (Klančnik et al., 2010), which reinforces the value of the present study where optimal environmental conditions for AITC antimicrobial activity were determined.

E. coli O157:H7 showed a temporary, phenotypic response to AITC after being challenged with increased concentrations of AITC. The ability of *E. coli* O157:H7 to adapt to concentrations of 206 ppm, which was well above its MIC, was reversed upon removal of the AITC challenge. Previous studies showed that AITC was unable to induce mutation in *E. coli* (Říhová, 1982; Chan et al., 2013). Although no mutagenic effect of AITC was demonstrated for *E. coli* O157:H7, the phenotypic response of this pathogen to AITC stimulated further investigation. After partial or complete deletion of the two-component BaeSR system, the ability of *E. coli* O157:H7 to grow in AITC was reduced, suggesting that this system played a role in the response of this pathogen to AITC toxicity. Although efflux pump genes (i.e. *mdtABCD*) and the stress protein (i.e. *spy*) are under regulation of the BaeSR two-component regulatory system (Zoetendal et al., 2008), the inability of *E. coli* O157:H7 to acquire resistance to AITC suggests that this pathogen responds to AITC challenge more as an environmental stress rather than toxic drug challenge.

Chapter 8

Conclusion

The reduction in concentrations of deodorized yellow mustard powder to 4% (w/w) enabled a ≥ 5 Log CFU.g^{-1} reduction of *E. coli* O157:H7 viability within the normal commercial production period for these products. In addition, deoiled mustard meals had greater antimicrobial activity than mustard powders due to their higher content of glucosinolates, which could be converted into antimicrobial ITCs. Suitable reductions in *E. coli* O157:H7 viability occurred when 2% deodorized mustard meal was used with 0.2% hot meal. The addition of small amounts of hot mustard containing plant myrosinase enhanced the antimicrobial activity of both mustard powders and deoiled meals. Such addition may positively influence the organoleptic characteristics of dry-fermented sausage by allowing the use of reduced mustard concentrations, which are more acceptable to consumers.

Incomplete deodorization of mustard because of the thermal resistance of plant myrosinase contributed to the antimicrobial activity of mustard powder observed during tests. This feature was taken advantage of in the development of a mixture of deodorized and spicy mustard meal at low level (2.2%) to eliminate *E. coli* O157:H7 from dry sausages. Powder and deoiled mustard meals derived from yellow mustard had increased antimicrobial activity when compared to those derived from Oriental mustard due to the greater glucosinolate and perhaps phenolic content of yellow mustard.

In silico analysis is an effective tool for identification of target genes when the function of the target protein can be associated with a known enzyme. The known function of myrosinase in mustard allowed the identification of 6-phospho-β-glucosidase in *E. coli* O157:H7 encoded by

bglA, *ascB*, and *chbF* genes as an analogous enzyme in this pathogen. Although these three genes appeared to be associated with degradation of sinigrin by *E. coli* O157:H7, the more significant ones (*bglA* and *ascB*) belong to the same glycoside hydrolase family 1 as plant myrosinase. Of these, the *ascB* gene was found to be the most important in this process due to its expression profile and its role in influencing sinigrin degradation rates.

The pH and temperature conditions used during the manufacture of dry-fermented sausage favor the stability of AITC formed following sinigrin hydrolysis. *E. coli* O157:H7 did not develop permanent resistance during AITC exposure; the resistance observed was transient in nature. The role of the two-component BaeSR system, which controls genes encoding antibiotic resistance and environmental stress, was important in the phenotypic response of *E. coli* O157:H7 to AITC since its ability to grow in higher AITC concentrations was reduced after complete deletion of the two components of the system.

Further work is recommended to explore the antimicrobial activity of deoiled mustard meal at concentrations < 2% (w/w) as well as the efficiency of reduced levels of residual myrosinase to achieve $\geq$ 5 Log $CFU.g^{-1}$ reduction in *E. coli* O157:H7 viability. In addition, sensory evaluation should be conducted to examine the consumer acceptability of dry sausages made with $\leq$ 2% deoiled mustard meal, and those containing deodorized mustard with residual myrosinase. Moreover, work is needed to determine the involvement of the other genes comprising the *asc* operon in sinigrin degradation by *E. coli* O157:H7, and whether gene expression is conditioned by any activation or repression factors. Additional work should be undertaken to isolate the glucoside hydrolase enzyme encoded by the genes examined in the present work. Once isolated, the characteristics of the enzyme(s) should be studied (e.g. kinetics, identification of active sites, specific activities, enzyme inhibition), which in turn, would allow

an understanding of the function, structure and interactions of this protein(s). These characteristics would facilitate the optimization of conditions for enzyme production and enable identifying the period of greater enzymatic activity during the manufacturing process or, even create conditions that favour this activity. A greater understanding of bacterial myrosinases in general would allow greater utilisation of this system in a broader range of foods for the control of a variety of pathogens with this activity.

Chapter 9

Future directions

The research reported in this thesis investigated many novel aspects concerning the antimicrobial effects of mustard in dry fermented sausages. However, some questions still remain to be addressed in future studies. These include optimal mustard concentrations, residual myrosinase, sinigrin as a substrate for *E. coli* O157:H7, the genetic basis of sinigrin degradation, and the resistance of *E. coli* O157:H7 to AITC. The suggestions listed below are those which should be given priority in future research effort.

- To investigate the antimicrobial activity of deoiled mustard meal at concentrations < 2% (w/w) as well as the efficiency of reduced levels of residual myrosinase to achieve ≥ 5 log CFU/g reduction in *E. coli* O157:H7 viability during dry sausage ripening;

- To conduct sensory evaluation to examine the consumer acceptability of dry sausages made with deoiled mustard meal, and those containing deodorized mustard with residual myrosinase;

- To purify the enzymes encoded by the genes *bglA*, *ascB*, and *chbF*, and to investigate the affinity of sinigrin to these enzymes;

- To investigate whether high sinigrin degradation rates observed in the present study were due to the greater affinity of sinigrin to the enzymes encoded by *bglA*, *ascB*, and *chbF*, or if they were due to the influence of each gene on the relative expression of the other two.

- To assess the involvement of the other genes comprising the *asc* operon in sinigrin degradation by *E. coli* O157:H7, and determine whether gene expression is conditioned by any activation or repression factors.

- To examine the expression of the genes in *E. coli* O157:H7 encoding 6-phospho-β-glucosidase under different growth conditions (i.e. temperature, pH, medium composition), and examine subsequent increases or decreases in sinigrin metabolism.

- To assess the role of the efflux pump gene (i.e. *mdtABCD*) and stress protein encoded by the *spy* gene in the response of *E. coli* O157:H7 to different concentrations of AITC.

- To compare the AITC resistance between *E. coli* O157:H7 strains possessing a highly active efflux pump system with a strain possessing a pumping system operating at normal rate.

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Appendix I - Tables 1 - 12 containing the average of Ct values used in RT-PCR

Table 1: Average of Ct values for *bglA* and *rpoA* used in the calculation of relative expression caused by the presence of sinigrin

Samples	Average Ct *bglA*	Average Ct *rpoA*	ΔCt = (Avg.Ct *bglA* – Avg. CT *rpoA*)	ΔΔCt= (Avg. ΔCt - Avg. $\Delta Ct_{calibrator}$)	Normalized *bglA* amount relative to calibrator $2^{-\Delta\Delta Ct}$
A. Wild-type with 0.1% sinigrin	25.79 ± 0.16	21.00± 0.14	4.79 ± 0.21	-1.20± 0.21	2.37
B. Wild-type without 0.1% sinigrin (calibrator)	24.96 ± 0.24	18.93 ± 0.41	6.03 ± 0.30	0.00 ± 0.30	1.00

Table 2: Average of Ct values for *ascB* and *rpoA* used in the calculation of relative expression caused by the presence of sinigrin

Samples	Average Ct *ascB*	Average Ct *rpoA*	ΔCt = (Avg.Ct *ascB* – Avg. CT *rpoA*)	ΔΔCt= (Avg. ΔCt - Avg. $\Delta Ct_{calibrator}$)	Normalized *ascB* amount relative to calibrator $2^{-\Delta\Delta Ct}$
A. Wild-type with 0.1% sinigrin	27.11 ± 0.27	21.00 ± 0.14	6.11 ± 0.21	-1.63± 0.21	3.08
B. Wild-type without 0.1% sinigrin (calibrator)	26.67 ± 0.56	18.93 ± 0.41	7.74 ± 0.95	0.00 ± 0.95	1.00

Table 3: Average of Ct values for *chbF* and *rpoA* used in the calculation of relative expression caused by the presence of sinigrin

Samples	Average Ct *chbF*	Average Ct *rpoA*	ΔCt = (Avg.Ct *chbF* – Avg. CT *rpoA*)	ΔΔCt = (Avg. ΔCt - Avg. $\Delta Ct_{calibrator}$)	Normalized *chbF* amount relative to calibrator $2^{-\Delta\Delta Ct}$
A. Wild-type with 0.1% sinigrin	25.77 ± 0.18	21.00 ± 0.14	4.77 ± 0.17	-2.67 ± 0.17	6.40
B. Wild-type without 0.1% sinigrin (calibrator)	26.38 ± 0.49	18.93 ± 0.41	7.45 ± 0.86	0.00 ± 0.86	1.00

Table 4: Average of Ct values for *ascB* and *rpoA* used in the calculation of relative expression caused by *bglA* deletion

Samples	Average Ct *ascB*	Average Ct *rpoA*	ΔCt = (Avg.Ct *ascB* – Avg. CT *rpoA*)	ΔΔCt = (Avg. ΔCt - Avg. $\Delta Ct_{calibrator}$)	Normalized *ascB* amount relative to calibrator $2^{-\Delta\Delta Ct}$
A. ΔbglA with 0.1% sinigrin	25.75 ± 0.25	20.54 ± 0.11	5.21 ± 0.31	-1.11 ± 0.31	2.10
B. Wild-type with 0.1% sinigrin (calibrator)	27.11 ± 0.27	20.79 ± 0.14	6.33 ± 0.28	0.00 ± 0.28	1.00

Table 5: Average of Ct values for *chbF* and *rpoA* used in the calculation of relative expression caused by *bglA* deletion

Samples	Average Ct *chbF*	Average Ct *rpoA*	ΔCt = (Avg.Ct *chbF* – Avg. CT *rpoA*)	ΔΔCt = (Avg. ΔCt - Avg. $\Delta Ct_{calibrator}$)	Normalized *chbF* amount relative to calibrator $2^{-\Delta\Delta Ct}$
A. ΔbglA with 0.1% sinigrin	25.48 ± 0.18	20.54 ± 0.11	4.94 ± 0.18	-0.04 ± 0.18	1.03
B. Wild-type with 0.1% sinigrin (calibrator)	25.77 ± 0.18	20.79 ± 0.14	4.98 ± 0.17	0.00 ± 0.17	1.00

Table 6: Average of Ct values for *bglA* and *rpoA* used in the calculation of relative expression caused by *ascB* deletion

Samples	Average Ct *bglA*	Average Ct *rpoA*	ΔCt = (Avg.Ct *bglA* – Avg. CT *rpoA*)	ΔΔCt = (Avg. ΔCt - Avg. $\Delta Ct_{calibrator}$)	Normalized *bglA* amount relative to calibrator $2^{-\Delta\Delta Ct}$
A. ΔascB with 0.1% sinigrin	26.62 ± 0.26	21.26 ± 0.21	5.36 ± 0.24	0.36 ± 0.24	0.77*
B. Wild-type with 0.1% sinigrin (calibrator)	25.79 ± 0.16	20.79 ± 0.14	5.00 ± 0.21	0.00 ± 0.21	1.00

*Value of $2^{-\Delta\Delta Ct} < 1$ implies that there was a reduction in the expression due to the treatment. The negative inverse of $2^{-\Delta\Delta Ct}$ will give the fold change reduction in expression.

Table 7: Average of Ct values for *chbF* and *rpoA* used in the calculation of relative expression caused by *ascB* deletion

Samples	Average Ct *chbF*	Average Ct *rpoA*	ΔCt = (Avg.Ct *chbF* – Avg. CT *rpoA*)	ΔΔCt = (Avg. ΔCt - Avg. $\Delta Ct_{calibrator}$)	Normalized *chbF* amount relative to calibrator $2^{-\Delta\Delta Ct}$
A. ΔascB with 0.1% sinigrin	25.30 ± 0.30	21.26 ± 0.21	4.03 ± 0.49	-0.94 ± 0.49	1.92
B. Wild-type with 0.1% sinigrin (calibrator)	25.77 ± 0.18	20.79 ± 0.14	4.98 ± 0.17	0.00 ± 0.17	1.00

Table 8: Average of Ct values for *bglA* and *rpoA* used in the calculation of relative expression caused by *chbF* deletion

Samples	Average Ct *bglA*	Average Ct *rpoA*	ΔCt = (Avg.Ct *bglA* – Avg. CT *rpoA*)	ΔΔCt = (Avg. ΔCt - Avg. $\Delta Ct_{calibrator}$)	Normalized *bglA* amount relative to calibrator $2^{-\Delta\Delta Ct}$
A. ΔchbF with 0.1% sinigrin	26.14 ± 0.32	21.70 ± 0.34	4.45 ± 0.26	-0.55 ± 0.26	1.47
B. Wild-type with 0.1% sinigrin (calibrator)	25.79 ± 0.16	20.79 ± 0.14	5.00 ± 0.21	0.00 ± 0.21	1.00

Table 9: Average of Ct values for *ascB* and *rpoA* used in the calculation of relative expression caused by *chbF* deletion

Samples	Average Ct *ascB*	Average Ct *rpoA*	ΔCt = (Avg.Ct *ascB* – Avg. CT *rpoA*)	ΔΔCt = (Avg. ΔCt - Avg. $\Delta Ct_{calibrator}$)	Normalized *ascB* amount relative to calibrator $2^{-\Delta\Delta Ct}$
A. ΔchbF with 0.1% sinigrin	30.97 ± 1.30	21.70 ± 0.34	9.28 ± 1.38	2.85 ± 1.38	0.13*
B. Wild-type with 0.1% sinigrin (calibrator)	27.22 ± 0.28	20.79 ± 0.14	6.43 ± 0.23	0.00 ± 0.23	1.00

*Value of $2^{-\Delta\Delta Ct} < 1$ implies that there was a reduction in the expression due to the treatment. The negative inverse of $2^{-\Delta\Delta Ct}$ will give the fold change reduction in expression.

Table 10: Average of Ct values for *chbF* and *rpoA* used in the calculation of relative expression caused by *bglAascB* deletion

Samples	Average Ct *chbF*	Average Ct *rpoA*	ΔCt = (Avg.Ct *chbF* – Avg. CT *rpoA*)	ΔΔCt = (Avg. ΔCt - Avg. $\Delta Ct_{calibrator}$)	Normalized *chbF* amount relative to calibrator $2^{-\Delta\Delta Ct}$
A. ΔbglAascB with 0.1% sinigrin	26.40 ± 0.41	21.42 ± 0.34	4.98 ± 0.25	-0.001 ± 0.25	1.00
B. Wild-type with 0.1% sinigrin (calibrator)	25.77 ± 0.18	20.79 ± 0.14	4.99 ± 0.25	0.00 ± 0.25	1.00

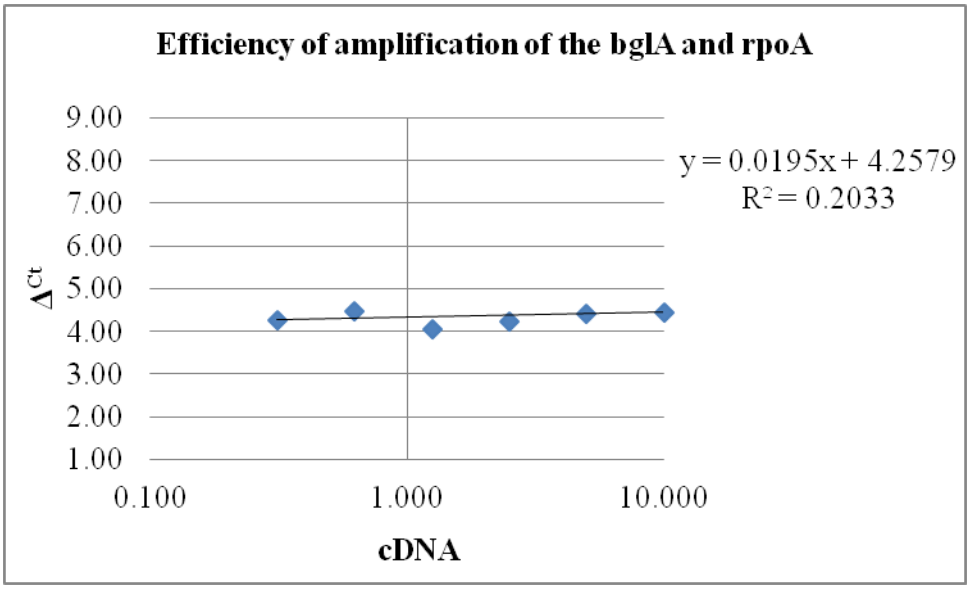
Table 11: Average of Ct values for *bglA* and *rpoA* used in the calculation of relative expression caused by *chbFascB* deletion

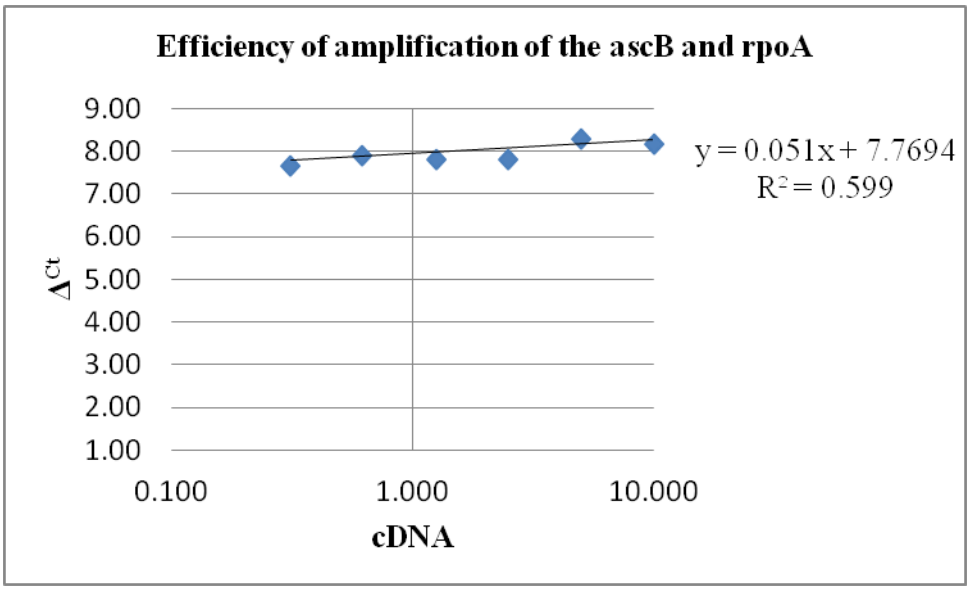
Samples	Average Ct *bglA*	Average Ct *rpoA*	ΔCt = (Avg.Ct *bglA* – Avg. CT *rpoA*)	ΔΔCt= (Avg. ΔCt - Avg. $\Delta Ct_{calibrator}$)	Normalized *bglA* amount relative to calibrator $2^{-\Delta\Delta Ct}$
A. ΔchbFascB with 0.1% sinigrin	27.37 ± 0.20	22.73 ± 0.40	4.63 ± 0.38	-0.36 ± 0.38	1.29
B. Wild-type with 0.1% sinigrin (calibrator)	25.79 ± 0.16	20.79 ± 0.14	5.00 ± 0.21	0.00 ± 0.21	1.00

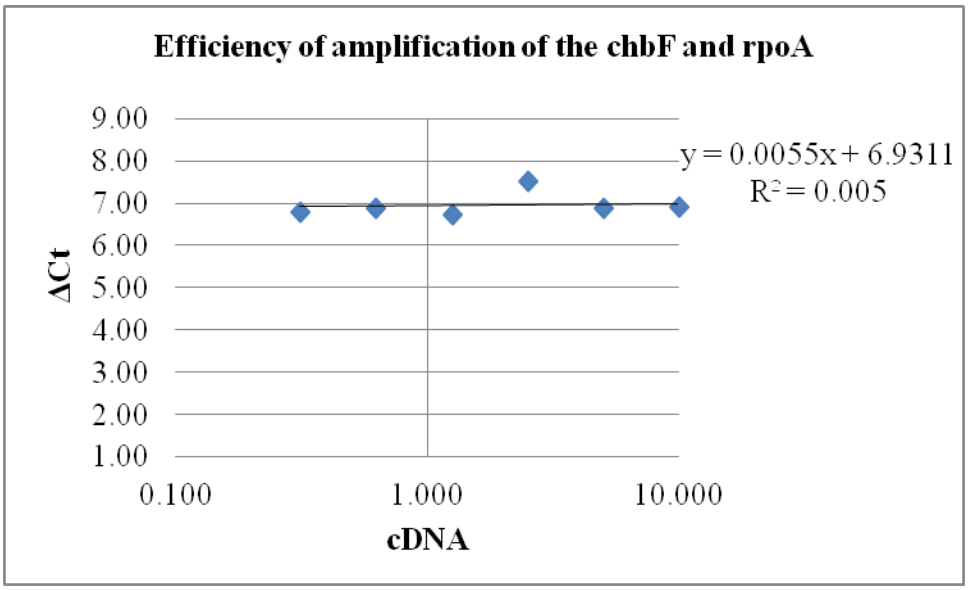
Table 12: Average of Ct values for *ascB* and *rpoA* used in the calculation of relative expression caused by *chbFbglA* deletion

Samples	Average Ct *ascB*	Average Ct *rpoA*	ΔCt = (Avg.Ct *ascB* – Avg. CT *rpoA*)	ΔΔCt = (Avg. ΔCt - Avg. $\Delta Ct_{calibrator}$)	Normalized *ascB* amount relative to calibrator $2^{-\Delta\Delta Ct}$
A. ΔchbFbglA with 0.1% sinigrin	29.25 ± 0.27	24.96 ± 0.27	4.30 ± 0.58	-2.03 ± 0.58	4.09
B. Wild-type with 0.1% sinigrin (calibrator)	27.11 ± 0.41	20.79 ± 0.14	6.33 ± 0.28	0.00 ± 0.28	1.00

Appendix II - Validation of the 2-ΔΔCt method: The efficiency of amplification of the target genes (i.e. *bglA, ascB, chbF*) and the internal control (*rpoA*) was examined using real-time PCR. Serial dilutions of cDNA were amplified using gene-specific primers. The $\Delta Ct = (Ct_{target\ gene} - Ct_{internal\ control})$ was calculated for each cDNA dilution. The data were fit using least-squares linear regression analysis (n = 3).






Efficiency of amplification of the chbF and rpoA
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
ΔCt
0.100
1.000
10.000
cDNA
y = 0.0055x + 6.9311
R² = 0.005

41