Use of Completely and Partially Deodorized Yellow and Oriental Mustards to Control *Escherichia coli* O157:H7 in Dry Fermented Sausage

BY

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ABSTRACT

Yellow and oriental mustards deodorized by a laboratory autoclave method (115^{o}C for 15 min in a 2 cm thick sample layer) have been shown to reduce the number of *E. coli* O157:H7 greater than the mandatory 5 log CFU/g reduction during dry fermented sausage manufacture. However, *E. coli* O157:H7 was inconsistently controlled in previous work using commercially deodorized mustards (Luciano et al. 2011). In addition, during current work variable results were also found among sausage trials where the same mustard was separately deodorized before repeated trials. The antimicrobial action of mustard results from the conversion of naturally present glucosinolates into inhibitory isothiocyanates by plant myrosinase in untreated hot mustard and by bacterial myrosinase-like activity when present in thermally-treated (deodorized) mustard. Variable results with deodorized mustards suggested that plant myrosinase might not have been consistently inactivated during laboratory thermal treatment using the autoclave. Therefore, an RP-HPLC method was used to detect and quantify residual myrosinase activity in deodorized mustard. Results obtained showed that when a 2 cm thick layer of mustard was used during autoclave treatment, plant myrosinase activity periodically remained in the deodorized mustard. It was found that when the thickness of the powdered mustard was reduced from 2 to 1 cm thick, consistent inactivation of plant myrosinase occurred. However, the completely deodorized yellow mustard failed to reduce bacterial viability as effectively as yellow mustard containing residual myrosinase. In sausages treated with 6 % (w/w) fully deodorized mustard, the normal 30 d period

required for a 5 log CFU/g reduction of *E. coli* O157:H7 was often extended by 14 d. When yellow mustard was partially deodorized, or when completely deodorized yellow mustard had 0.1-0.2 % hot mustard added and used together at 4 % (w/w), the required reduction of *E. coli* O157:H7 occurred within 24 d. It is likely that residual plant myrosinase contributed to the antimicrobial activity of deodorized yellow mustard. The same tests with oriental mustard in sausages revealed that residual plant myrosinase was not the factor limiting antimicrobial activity. Results indicated that the lower glucosinolate and lower total phenolic content (TPC) of oriental than yellow mustard may explain in part why yellow mustard had greater antimicrobial activity than oriental mustard.

The results of this study suggested that 4 % (w/w) yellow mustard treatments containing myrosinase activity from 0.1 or 0.2 % hot mustard were as antimicrobial as 6 % yellow mustard in sausages. However, the inability to demonstrate the same effect with oriental mustard indicates that there are other factors which have yet to be determined that influence the antimicrobial activity of oriental mustard.

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CHAPTER 1

1. INTRODUCTION

Dry fermented meats have long been considered ready-to-eat and shelf-stable products even though traditional manufacture does not involve cooking. The preservation of this product is characterized by: 1) development of low pH and microflora dominated by the starter culture added; 2) development of low water activity (a_w) resulting from long-term sausage drying and 3) addition of salt and other curing agents such as nitrite/nitrate. Foodborne illness outbreaks involving *E. coli* O157:H7 have been reported in over 30 countries and many have been associated with eating undercooked or uncooked beef-containing meat products (Besser et al. 1999, Johnsen et al. 2001, Doane et al. 2007). Additionally, *E. coli* O157:H7 is capable of surviving in acidic, low a_w environments (Arnold and Kaspar 1995, Conner and Kotrola 1995), making dry fermented sausage susceptible to *E. coli* O157:H7 related illness despite multiple antimicrobial hurdles being present. As with many other pathogens causing foodborne illness, its ability to be present in products without causing sensory change make it hard to detect and contain (Alexander et al. 1995). A mandatory 5 log CFU/g reduction of *E. coli* O157:H7 was added to the guidelines for dry fermented sausage manufacture in both the US and Canada after 23 individuals in the states of Washington and California were infected with this pathogen as a result of consumption of dry-cured salami (Reed 1995, Armstrong et al. 1996, Health Canada 2000). However, it was found that commercial dry

or semi–dry sausage processing methods cannot achieve this reduction. Thus, a novel approach or alternative method is needed for compliance.

Canada ranks highly as one of the largest mustard seed producers and exporters, accounting for 49 % of mustard seed exports worldwide, with a market value of approximately $128 million in 2009 (AAFC 2009). Three mustard types from two different species are widely cultivated in Canada, and these include oriental, brown (*Brassica juncea*) and yellow (*Sinapis alba*) mustards. Mustards are a significant source of glucosinolates (GLs) among *Brassica* vegetables and their concentration is highest in the seed (Sang et al. 1984, Zrybko et al. 1997, Clarke 2010). The production of isothiocyanate caused by endogenous myrosinase hydrolysis of GLs, which are abundant in mustard seeds, yields its typical pungent characteristic (Bones and Rossiter 1996, Clarke 2010). The use and applications for mustard seeds in the food industry are substantial but also limited by their pungency. Oriental and brown mustards are primarily used as condiments or as spices because of their very hot flavor resulting from the formation of allyl isothiocyanate (AITC) after sinigrin hydrolysis (AAFC 2007, Golz and Aakre 2010). In yellow mustard, myrosinase hydrolyzes sinalbin to form p-hydroxybenzyl isothiocyanate (ρHBIT) creating a mild flavor, allowing this type of mustard to be used as a binder in salad dressings (e.g. mayonnaise), or as a fat emulsifier and water absorptive agent in processed meat products (Lipner 1972, Shim and Wanasundara 2008).

The value of mustard seed used for processed meat manufacture was enhanced when the bio–protective properties of the essential oil isothiocyanates (Delaquis and Sholberg 1997, Mithen 2001, Saavedra et al. 2010) as well as their antimicrobial action in meat batter against *E. coli* O157:H7 (Graumann and Holley 2008, Luciano et al. 2011, Lara-Lledo et al. 2012, Nilson and Holley 2012, Cordeiro et al. 2013) were reported. Glucosinolates themselves are generally agreed not to be bioactive, and therefore are considered an ideal precursor for the biologically active, but also unstable, volatile isothiocyanate (Holst and Williamson 2004). However, negative effects of glucosinolates have also been reported, although most of their focus has been on animal feed because *Brassica* plants are a good source of protein. Some studies have shown glucosinolates caused renal dysfunction or thyroid toxicity in animals when the glucosinolate concentration was relatively high in animal diets (Heaney and Fenwick 1995; Holst and Williamson 2004).

A patent for thermal treatment of mustard seed to reduce its pungency by the denaturation of plant myrosinase was introduced by Brunn (1964). It outlined that heat between 110^{o}C to 180^{o}C for 1 to 10 min was sufficient to inactivate endogenous myrosinase. Thus, the amount of essential oil isothiocyanate produced was reduced from 0.6 % to less than 0.05 % in yellow mustard seed (w/w). Deerfield and Dougherty (1971) first reported the use of saturated steam for different periods to partially denature myrosinase so that reduced, but a more controlled amount of isothiocyanates could be produced. Additionally, the latter authors also suggested that the duration of heating might

need to be varied according to the specific conditions such as the moisture content in the mustard seed and the capacity of the particular apparatus used.

Previous studies in this laboratory used autoclave treatment (115^{o}C for 15 min, with mustard powder in a 2 cm thick layer) to denature the endogenous myrosinase in yellow mustard which was used in dry fermented meats against *E. coli* O157:H7. Interestingly, this type of deodorized yellow mustard at concentrations of 4-6 % (w/w) in sausage batter showed high antimicrobial activity against the bacteria and caused ≥ 5 log CFU/g reduction within 18 d to 28 d of ripening, depending on mustard concentration (Luciano et al. 2011, Cordeiro et al. 2013). Lara-Lledo et al. (2012) reported that *Listeria monocytogenes* inoculated at 4 log CFU/g was eliminated from bologna sausage by an antimicrobial film containing deodorized oriental mustard extract. There are several studies which have reported human colonic microflora as well as lactic acid bacteria have bacterial myrosinase-like activity, which acted similar to endogenous myrosinase to degrade GLs (Nugon-Baudon et al. 1990, Aires et al. 2009). *In vitro* tests conducted by Luciano and Holley (2010) showed that 5 strains of non-pathogenic *E. coli* O157:H7 and starter cultures used in sausage manufacture also possessed the ability to degrade purified sinalbin and sinigrin hydrates. This result, at least partially, explains the reason for high antimicrobial activity in deodorized mustard which controls *E. coli* O157:H7 in dry fermented meats. Variability in the period of time during sausage ripening required for a ≥ 5 log CFU/g reduction of *E. coli* O157:H7 was found in several studies in this laboratory from the earliest (Graumann and Holley 2008) to the later work (Cordeiro et al. 2013). It

became apparent that commercially deodorized yellow mustard had lower antimicrobial activity than laboratory deodorized mustard against *E. coli* O157:H7 (Luciano et al. 2011). It was also noted when pure sinigrin was used as a substrate during *in vitro* trials with several pathogens (*E. coli* O157:H7, *Salmonella* and *Listeria monocytogenes*), even though significant sinigrin hydrolysis occurred, bacterial inhibition either did not occur or was less than expected (Herzallah et al. 2011, Lara-Lledo et al. 2012, Olaimat and Holley 2013a). Factors known to influence the antimicrobial action of allyl isothiocyanate (AITC) produced by sinigrin hydrolysis include those that affect its formation (temperature and pH) as well as its stability (pH). Olaimat et al. (2013) found greater sinigrin hydrolysis at pH 7 than at higher or lower pH values, while Olaimat and Holley (2013b) found greater hydrolysis at 21^{o}C than at 10^{o}C or 4^{o}C. In addition, AITC was more stable at low pH and temperature which improved its antimicrobial action (Olaimat and Holley 2013a). Further, it seemed that a greater proportion of inhibitory AITC was formed by plant myrosinase from glucosinolates at pH 7 than at lower pH values (Bones and Rossiter 1996). If the same occurs with bacterial myrosinase, lowered antimicrobial effects might occur in acidic foods such as dry sausages following the initial fermentation.

Even when the above features of the myrosinase-glucosinolate system were taken into account, the variability in antimicrobial performance of mustard powder in dry sausage was still not fully explained. One hypothesis was that different lots of mustard previously used contained different proportions of agents that were antimicrobial (isothiocyanate and phenolic compounds). Another hypothesis was that there had

occasionally been some residual myrosinase activity in the heat-treated mustard powder after exposure to 115^{o}C for 15 min.

The principal objective of the present work was to investigate whether the laboratory method used to inactivate myrosinase present in commercial yellow and oriental hot mustards was adequate to achieve its complete inactivation, and whether or not its incomplete inactivation was a likely explanation for the differences in the antimicrobial performance of different lots of mustard during dry fermented sausage ripening. It was also of interest to attempt to determine how the anti-*E. coli* O157:H7 performance of deodorized mustard might be improved in order to reduce the amount of mustard needed to control *E. coli* O157:H7 and thus improve sausage organoleptic quality.

In order to complete these goals, 9 types of mustard samples from two varieties (yellow and oriental) were tested in 7 dry fermented sausage trials. It was felt that this number of types of mustard samples should be adequate to draw firm conclusions. The first 5 sausage batches were manufactured using the same procedures as in previous work (Luciano et al. 2011, Cordeiro et al. 2013), whereas the last two batches were done with modifications which included changes in the amount of glucose added to the meat batter, changes in the manner of mustard deodorization, and the combination of fully deodorized mustard with small proportions of mustard in which myrosinase was confirmed present.

A method for confirmation (RP-HPLC myrosinase assay) of the absence or presence of myrosinase activity was developed by improvement of the original myrosinase verification method reported by Luciano et al. (2011). The modified RP-HPLC

myrosinase assay was able to quantify myrosinase activity and better detect slight enzyme activity from myrosinase after mustard was autoclave-treated and used to verify whether or not glucosinolate hydrolysis in sausage was caused by residual endogenous plant myrosinase after deodorization.

CHAPTER 2

2. LITERATURE REVIEW

2.1. The characteristics and significance of *E. coli* O157:H7 illness

2.1.1. The habitats and transmission of *E. coli* O157:H7

E. coli O157:H7 has been isolated from varied sources and is a continuing, problematic foodborne pathogen. *E. coli* O157:H7 illnesses have been traced back to undercooked or uncooked meats of bovine origin (Tilden et al. 1997, Tuttle et al. 1999). However, a long list of foods also have been confirmed as sources including milk, apple juice, yogurt, cheese, water, alfalfa sprouts and salad products such as leafy greens (Williams et al. 2000, Brandl and Amundson 2008). The transmission of *E. coli* O157:H7 illness is primarily foodborne but occasionally person to person transfer occurs (Su and Brandt 1995). The pathways for contamination often involve irrigation water, transfer from manure-contaminated soil to plants, and most frequently, from feces-contaminated animal carcass to meat products (Bach et al. 2002, Ethan et al. 2002).

2.1.2. Infectious pattern and pathology of *E. coli* O157:H7 illness

Generally, illnesses caused by *E. coli* O157:H7 involve the production of shiga-like toxins which are similar to the toxin produced by *Shigella dysenteriae*. An infectious dose could be as low as 50 organisms (Tuttle et al. 1999, FDA 2012). After it enters the host through the gastrointestinal tract, the microorganism or toxin attacks the epithelial cells of the gastrointestinal tract causing hemorrhagic colitis, a condition that results in nausea, vomiting, bloody diarrhea and severe abdominal pain. Haemolytic uraemic syndrome

(HUS), a major cause of acute renal failure in children, is another typical life threatening disease caused by *E. coli* O157:H7 infection, resulting in about 3-5 % mortality. Children under 10, the elderly and immunocompromised patients are at greatest risk (Health Canada 1999, Carlos et al. 2003). It was estimated that *E. coli* O157:H7 infection contributes about 70-80 % of all the food or water-related *Escherichia coli* illnesses which result in the classic hemolytic uremic syndrome (HUS) in North America (Douglas et al. 2009).

2.1.3. Epidemiology of *E. coli* O157:H7 and its corresponding economic influence

E. coli O157:H7 is estimated to be the most common pathogenic *Escherichia coli* serovar and was first described as a foodborne pathogen after it caused 47 persons in Michigan and Oregon who ate contaminated hamburger in 1982 to develop bloody diarrhea (Riley et al. 1983, Welinder-Olsson et al. 2004). Since then, sporadic cases and outbreaks of *E. coli* O157:H7 related foodborne diseases have emerged and reports and studies continually uncover features of this microorganism. Meanwhile, surveillance and prospective studies to identify and characterize illnesses associated with *E. coli* O157:H7 also have been undertaken in North America and Europe (Duncan et al. 1986, CDC 1990)

In Canada, as a result of surveillance, the number of laboratory confirmed *E. coli* O157:H7 illnesses climbed to a peak of 2407 in 1989, and ranged from 1014 to 1700 cases/year during the 1990s (Woodward et al. 2002), which is equivalent to about 4.1 cases per 100,000 Canadians. Since 1989, *E. coli* O157:H7 infection has been a nationally notifiable illness which must be reported by health professionals to Health Canada.

The decline in the number of *E. coli* O157:H7 cases after 1990 suggests that this pathogen has been effectively reduced by industry intervention. In comparison to the United States, in a roughly parallel period from 1982 to 2002, *E. coli* O157:H7 caused 73,000 illnesses annually and 350 outbreaks were reported over 49 states (Rangel et al. 2005).

The Canadian National Enteric Surveillance Program (NESP) issued a 2010 summary of laboratory surveillance data which showed that the incidence rate of *E. coli* O157:H7 had decreased from 4 cases per 100,000 in 2001 to 1.18 cases per 100,000 in 2010, and in that year the number of confirmed cases dropped to 382 (NESP 2012). However, foodborne *E. coli* O157:H7 outbreaks remain common and, since many outbreaks and sporadic cases go unreported, it still has a significant economic impact in Canada. A study conducted by the George Morris Centre in Ontario in 2009 showed that the estimated annually cost of *E. coli* O157:H7 infection in Canada was approximately $21 million (Kevin and Claudia 2009). The United States Centers for Disease Control and Prevention reported that there were approximately 265,000 infections by *E. coli* O157 annually, after accounting for under-diagnosis and under-reporting (CDC 2012).

2.2. Dry fermented sausage

2.2.1. Product overview

The characteristics of dry fermented meat sausages vary in different countries/regions or cultures because of the use of different materials, often in different ratios. However, the basic recipes and methods of manufacture are generally the same:

fresh meat and fat are first subjected to grinding and ingredients plus curing agents are added, then the batter is stuffed into a casing. Sausages are allowed to ferment for up to a week and are dried for 3 weeks or more (Sabine et al. 2004). The development of starter cultures and their use since the mid 1900's significantly improved the control of manufacture and provided better sensory attributes (Caplice and Fitzgerald 1999, Sabine et al. 2004).

2.2.2. Ingredients and processing steps used for dry fermented sausage manufacturing

Addition of ingredients (e.g. salt and glucose) at the beginning of manufacturing provides flavor for the product as well as nutrients for the beneficial microorganisms to produce lactic acid. More importantly, salt inhibits the growth of undesirable bacteria at the beginning, before establishment of antimicrobial hurdles such as low moisture (water activity, a_w) and low pH. Subsequent processing steps like fermentation, smoking and drying ensure product shelf-stability and safety, meanwhile developing the final product flavor and appearance (Berdagué et al. 1993, Joshua and Brian 2012).

2.2.2.1. Curing (salt, nitrate and nitrite)

Curing has been used as an enduring preservation method for meat products since earliest recorded history. Sodium chloride is an essential curing agent and is the first hurdle used during manufacturing. It helps to lower the a_w, thus inhibiting the growth of many microbes. It is noteworthy that high concentration of sodium chloride ($\geq$ 4 %, w/w) influences the flavor and may also encourage the growth of staphylococci, which if

initially present in large number, can be faster than lactic acid bacteria (LAB) since the former is also salt tolerant (Martin 2012). In addition, salt is able to solubilize salt-soluble protein, improving the cohesion of the meat batter during subsequent ripening (Sabine et al. 2004).

Nitrate and nitrite are also commonly used as curing agents in processed meat. Nitrate is usually considered as the reservoir of nitrite if long ripening is required. Nitrate is reduced to nitrite by the action of nitrite reductase produced by micrococci or non-pathogenic staphylococci (Sabine et al. 2004). Curing salt containing nitrate or nitrite serves to inhibit the growth of spoilage microorganisms by the production of nitrous acid, which is capable of inhibiting bacterial enzymes leading to spore outgrowth, specifically in *Clostridium* (Lücke 1994).

After nitrite is reduced to nitric oxide, it reacts with meat pigments (myoglobin) and is converted to nitrosomyoglobin, or nitrosohemochrome if cooked, maintaining the desirable red or pink color, respectively. Nitrite also aids in development of a better flavor in cured meats. It also helps to tie up iron from myoglobin which prevents oxidation and rancidity (Ann and Nejib 2007).

The use of nitrate or nitrite has been strictly regulated since there is the potential for carcinogenic nitrosamine formation when used at a high level or when products containing nitrite are subjected to high temperature cooking. In both Canada and United States, for cured meat products except bacon, the maximum permitted use level of nitrate/nitrite is 200 ppm (Katan 2009, CFIA 2010).

2.2.2.2. Fermentation and the use of starter cultures

Fermentation is a fundamental step for dry fermented sausage manufacturing since it is the main preservative treatment used to inhibit the growth of most of the Gram-negative spoilage and pathogenic microorganisms. Fermentation is also the principal period during which much of the synthesis of final flavor occurs (Jytte and Lene 2005). Historically, fermentation has largely relied on the indigenous microflora of the meat. However, this caused inconsistency between batches and failed to ensure product safety because the indigenous microflora varied (Fidel 2005). The use of starter cultures overcomes this issue. Bacterial starters used for meat fermentation are generally made up of a balanced mixture of LAB and coccal Gram-positive bacteria. The benefit of using a combined started culture is that one microorganism produces lactic acid while the other improves desirable flavor and appearance. The main role of LAB is the acidification of meat matrices, resulting in a rapid drop of pH thus promoting product safety. Other advantages of LAB include the fact that at pH 5.4–5.5, they cause coagulation of meat proteins and promote the reaction of nitrogen monoxide with myoglobin to develop red color. In addition, carbohydrates are rapidly utilized by LAB and are unavailable for undesirable microorganisms, thus overwhelming unwanted microorganisms that may be present in the meat during the first few days of ripening (Fidel 2005, Ann and Nejib 2007). Coccal Gram-positive bacteria like *Staphylococcus* and *Micrococcaceae* contribute to the maintenance of desirable color by producing nitrate reductase and peroxidase. They also contribute to product flavor by producing aroma compounds through proteolysis and

lipolysis (Hammes and Hertel 1998, Jytte and Lene 2005) . In general, starter cultures can decrease the fermentation time compared to natural fermentation by 15-20 % and increase the product yield by 5-7 %. This results in a reduction of the time required for ripening and usually improves product sensory properties (Herbert and Lopa 2010). The addition of starter cultures also provides better control of fermentation than possible with the indigenous microflora (Jytte and Lene 2005, Régine and Sabine 2006). Herbert and Lopa (2010) reported that to obtain a pH of 4.8 - 5.0 requires approximately 25 g of lactic acid / kg of meat. Additionally, sugar such as glucose, which can be readily used by all LAB, improves the production of lactic acid and it is known that 0.1 unit pH reduction requires 0.62 g glucose/kg of meat. In comparison, other sugars like sucrose, maltose, and lactose will also contribute to bacterial fermentation, although the rate of acid production is slower and their usefulness depends upon the starter culture employed (Lücke 1994). Consequently, processing conditions as well as the microorganism used as the starter culture can influence the rate of acid production and the ultimate pH of products. According to the temperature of fermentation used, time (duration) also needs to be controlled to ensure the desired pH is achieved within the limits of the degree•hour regulation (Health Canada 2000).

2.2.2.3. Smoking and drying

Smoking can be conducted intermittently during the fermentation and subsequent ripening of sausages. It gives products a distinctive flavor and odor, improving the color, and preventing the development of rancidity as well as early spoilage by retarding the

growth of molds and yeast (Jensen 1943). Smoking can be done using either liquid preparations or by burning wood or wood chips (Joshua and Brian 2012)

Drying is used to remove approximately 20-50 % of the total moisture from the product by evaporation or sublimation to reach a moisture-to-protein ratio (MP) lower than 2.3 : 1 (USDA 2005). The principle of drying is to reduce the a_w so that microbial growth and relevant biochemical reactions are inhibited (Santchurn et al. 2012). An inappropriate drying procedure, for instance drying too fast which can result from the use of high temperature or too low a relative humidity in the drying chamber, especially for large diameter sausage, can cause an imbalance in moisture content between the inner and outer areas of the sausage. An outer dry ring prevents further evaporation from the inner core, yielding sausage with uneven texture. This defect is called “case hardening” and can be visually observed in a cross-section of the sausage as a darker color in the outside surface due to low moisture content (Fidel et al. 2004).

2.2.3. Pathogen survival in dry fermented sausage

Dry fermented meats are considered ready-to-eat (RTE), are normally shelf-stable for long periods and are directly consumable without further preparation (Farber and Harwig 1996). Thus the confirmation of their safety is important. The potential risks of pathogens in dry fermented sausage that cause foodborne illness are generally associated with the survival of *Staphylococcus aureus*, *Salmonella* and *E. coli* O157:H7 in these products. *Listeria monocytogenes* is problematic in cooked cured meat products, and

although sometimes present at low numbers in dry fermented sausages, these products are not known to have caused listeriosis in humans.

2.2.3.1. *Salmonella*

Dry fermented sausage usually contains lean pork which has been described as a vehicle for *salmonellosis*. *Salmonella* strains are commonly found in pork carcasses and cuts (Giovannacci et al. 2001). They have also been found in animal feed and sometimes in cattle (Divya et al. 2010). *Salmonella* contaminated dry sausages are a periodic cause of foodborne illness in humans (Pontello et al. 1998).

2.2.3.2. *Staphylococcus aureus*

Staphylococcus aureus intoxication is commonly a result of post-processing contamination since it is ubiquitous in the processing environment (Oliver et al. 2005). Staphylococcal food poisoning (SFP) is the most common type of related illness (Atanassova et al. 2001). The enterotoxins are produced and constitute a risk in food when this pathogen grows to a level of about 10^6 – 10^8 CFU/g. Production of toxins is limited at an a_w value of 0.86 and below (Wong and Bergdoll 2002). In the United States, the growth of *S. aureus* and the presence of enterotoxins in fermented sausages, particularly in Genoa and Italian-type dry salami, have caused various outbreaks of food poisoning (Bergdoll and Wong 2011).

2.2.3.3. *Listeria monocytogenes*

In most cases, the issue of *L. monocytogenes* related foodborne illness with dry cured meat is that cross-contamination may occur during processing or handling. Even

when the initial contamination is small in number, this pathogen is psychrotrophic and able to grow at refrigeration temperatures. The organism can become dominant if there is a starter culture failure, however, this would be unusual. With cooked cured meats, at high pH (6.1) and with shelf-lives under vacuum of at least 40 d, the pathogen can multiply and be problematic (Jacquet et al. 1995).

With respect to the control of *Salmonella*, *Listeria monocytogenes*, and *Staphylococcus aureus*, the use of good manufacture practice (GMP) helps to minimize the chances of cross-contamination during handling and processing. Because the first hours and days (high a_w and pH) are critical, the rapid development of LAB is very important, and normally results in a rapid reduction of pH to 5.3 or below within the required times according to the temperature of ripening (degree•hour regulation). The acidic environment, low a_w as well as use of hygienic raw materials and a sanitary processing environment exert synergy to prevent contamination by foodborne pathogens during manufacture.

2.2.3.4. *Escherichia coli* O157:H7

Escherichia coli O157:H7 is known to be acid tolerant and survives well in fermented and acidic foods. Most of the *E. coli* O157:H7 related foodborne illness is associated with the consumption of undercooked or uncooked meats (Bach et al. 2002, Rangel et al. 2005). Studies have shown that *E. coli* O157:H7 can survive typical dry fermentation processing conditions (Faith et al. 1998, Williams et al. 2000, Divya et al. 2010). However, this issue was not taken seriously until an *E. coli* O157:H7 illness

outbreak occurred involving 23 individuals in Washington and California in 1994, as a result of the consumption of dry cured salami (Alexander et al. 1995). Tilden et al. (1997) investigated this new route of transmission for *E. coli* O157:H7 in salami and suggested the that current manufacturing methods used for dry and semi-dry sausages were inadequate for the elimination of this pathogen. This is because *E. coli* O157:H7 is often present in raw ground beef and because it is tolerant to acid and low moisture. More importantly, dry cured sausage is an uncooked, ready-to-eat product and when contaminated by *E. coli* O157:H7 there is no flavor change. Regulations were adopted in both Canada and the US requiring dry fermented sausage manufacturing methods to be capable of reducing *E. coli* O157:H7 to more than 5 log CFU/g (Reed 1995, CFIA 2010). The lethality of *E. coli* O157:H7 required was established after a 1 log safety margin was added to an assumed worst case of 4 log CFU/g as recommended by the Task Force on Technical Issues of the National Advisory Committee for Microbiological Criteria for Foods (NACMCF) and accepted by the Food Safety and Inspection Service (USDA 2001). However, several reports indicated that dry fermented sausage manufacturing methods were only capable of causing from 2 to 3 log CFU/g reductions of *E. coli* O157:H7 within a commercially acceptable period of ripening (Graumann and Holley 2008, Luciano et al. 2011).

2.3. Use of mustard glucosinolate as an antimicrobial agent in dry fermented meats

2.3.1. Overview

Mustard is a vegetable belonging to the family *Brassicaceae* and there are several varieties. Because of the geographic location and climate, Canada is one of the largest mustard producers and exporters worldwide (AAFC 2008, AAFC 2009). Brown, oriental (*Brassica juncea*) and yellow (*Sinapis alba*) mustards are two major varieties that are cultivated in Canada. Mustards are traditionally used as oilseeds or for the condiment industry. Mustard leaves are called mustard greens, and are also used as a prepared vegetable (Charles 2013). Mustard seeds are usually ground from whole or dehulled seeds before use, yielding mustard powder and flour, respectively.

Mustards are also well known as a significant source of glucosinolates and the seeds usually have the highest concentration (Bennett et al. 2003, Bennett et al. 2004). Mustard glucosinolates are water-soluble, anionic, amino acid-derived secondary metabolites that vary in composition, depending on their genus, or more specifically, their chemical side chains or R groups (Wittstock et al. 2003). The diversity of R groups leads to variation in polarity and biological activity. In plant tissues, glucosinolate degradation is considered as a part of the plant self-defense system. The mechanism involves endogenous myrosinase hydrolysis of glucosinolates following physical injury since they are located separately in the plant tissue. This action forms various products like isothiocyanates, thiocyanates, nitriles and minor compounds depending on the degradation conditions such as pH, temperature, the presence of metal ions and the nature of the R group. When

glucosinolate degradation occurs it yields a typical sharp pungent flavor. This could happen in prepared mustard products (e.g. flour and powder) when moist, or during mustard seed milling if a suitable amount of moisture is present (Fenwick et al. 1982, Mithen 2001).

Since the bioavailability (Mithen et al. 2000, Holst and Williamson 2004) and bio-protective effect (Graumann and Holley 2008, Aires et al. 2009, Luciano et al. 2011, Nilson and Holley 2012) of the derived glucosinolate products (the isothiocyanates), have been widely reported, mustard seeds are considered to have extra value because of the high concentration of glucosinolates they contain (Bennett et al. 2004). However, one of the disadvantages is the simultaneous production of the sharp odor by isothiocyanates which might be undesirable for some food products.

2.3.2. Relationship between mustard seed applications and pungency

Beside oil extraction, the traditional applications of mustard seed in the food industry highly depend upon its pungent intensity. Oriental mustard is primarily used as a hot, spicy condiment since it is the hotter of the two kinds, which results from the formation of AITC (AAFC 2007, Golz and Aakre 2010). In yellow mustard the glucosinolate sinalbin is hydrolyzed and forms ρHBIT creating the milder flavor. It is used as an emulsifier, or is available for use as a binder in many salad dressings and processed meat products (Lipner 1972, Shim and Wanasundara 2008).

To process mustard seeds for use as a spicy condiment, moderate heat is applied to reduce the moisture content in order to prevent the enzymatic hydrolysis of the

glucosinolate during milling. The moderate heating (commonly lower than 60^{o}C) does not affect the activity of the endogenous myrosinase. As a result, condiment mustard seeds are able to develop a sharp flavor after being mixed with water (Sakai and Ebisawa 1985, Holst and Williamson 2004).

Several early studies reported that the thermal stability of myrosinase varied substantially with the plant source as well as the sample storage form (e.g. crude extract, juice, dry seed). For instance, myrosinase in fresh broccoli and cabbage had the lowest thermal stability. The inactivation started at temperatures above 30^{o}C and 90 % inactivation was achieved at 60^{o}C for 3 min (Ludikhuyze et al. 1999). In contrast, Tsao et al. (2002a) reported myrosinase in oriental mustard bran was not completely inactivated after heating at 100^{o}C for 1 h. This variation was most likely caused by the differences in moisture content present. It seemed that myrosinase started to deactivate once heating reached higher than 60^{o}C while the moisture content was higher than 10 % (Van Eylen et al. 2008, Veto-Kiszter et al. 2009). Further, McGregor et al. (1983) reported higher temperature and duration was needed to achieve similar myrosinase inactivation if the moisture content was lower than 8 % in mustard seeds. Nevertheless, there are no comprehensive validated data available describing the complete inactivation of myrosinase which specify temperature, its duration, sample pH and moisture content for use with specific equipment.

A patent for dry heat treatment of mustard seed for sausage manufacture developed by Brunn (1964) showed that high temperature heating (110^{o}C to 180 ^{o}C) for 1 to 10 min

was sufficient to inactivate the myrosinase present. Using this process, the amount of volatile oil resulting from glucosinolate hydrolysis was reduced from 0.6 % to less than 0.05 % in yellow mustard seed (w/w), and the mustard product obtained following this procedure lacked the sharp flavor. A later patent introduced by Deerfield and Dougherty (1971) described the use of various durations of saturated steam which inactivated myrosinase to various degrees, yielding prepared mustard products with controlled levels of essential oil, and different levels of pungency.

2.3.3. Antimicrobial activity of deodorized mustards and glucosinolate derivatives in dry fermented meats against *E. coli* O157:H7

For the last decade, the glucosinolates hydrolysis byproducts, isothiocyanates, have been studied as potent antimicrobials that kill *E. coli* O157:H7 and other pathogens (Delaquis and Sholberg 1997, Shofran et al. 1998). Nadarajah et al. (2005) reported AITC derived from sinigrin was successfully used for the elimination of 2.7 log CFU/g *E. coli* O157:H7 from ground beef, roast beef and hamburger patties. Because isothiocyanates are volatile oils which are somewhat unstable, the glucosinolate precursor is generally considered a better alternative than direct use of the bioactive isothiocyanates. However, isothiocyanate formation relies on the enzymatic conversion from glucosinolate (Holst and Williamson 2004). Later applications that used the hydrolytic derivatives, isothiocyanates, or mustard powder containing them in dry fermented meat products also showed a significant reduction of *E. coli* O157:H7 during the ripening process (Nadarajah et al. 2005, Chacon et al. 2006, Graumann and Holley 2008). A drawback that needed to

be overcome was the simultaneous production of sharp flavor with isothiocyanates by the enzymatic conversion of glucosinolates, causing negative effects on the organoleptic qualities of dry fermented meats. The introduction of the use of deodorized mustard, produced by treatment of hot mustard in an autoclave at 115^{o}C for 15 min, resulted in the denaturation of the endogenous plant myrosinase, and was able to eliminate the undesirable pungency associated with hot mustard. Surprisingly, this type of deodorized mustard was also observed to have significant bactericidal activity against *E. coli* O157:H7 in dry fermented meats (Luciano et al. 2011, Nilson and Holley 2012, Cordeiro et al. 2013).

It is widely reported that a number of microorganisms, including LAB, have the ability to hydrolyze glucosinolates by means of their myrosinase-like activity (Nugon-Baudon et al. 1990, Getahun and Chung 1999, Krul et al. 2002). Luciano and Holley (2010) and Herzallah et al. (2011) reported pathogenic, non-pathogenic bacteria and the starter cultures that were used for meat fermentation also possessed this myrosinase-like activity *in vitro*. It was believed that these organisms in an environment which has limited carbohydrates, or under stressful conditions, will degrade glucosinolates to gain glucose as an energy source using myrosinase-like enzyme(s). However the unstable aglucone, after glucose is released from glucosinolate, rearranges to form the isothiocyanate which is lethal to bacteria. This at least partially explains the reason why deodorized yellow mustard, which did not have apparent myrosinase activity, showed substantial antimicrobial activity against *E. coli* O157:H7 in dry fermented

sausage. It is thought that when *E. coli* O157:H7 is stressed by the sausage processing conditions it is less able than LAB to access nutrients, and turns to the glucosinolate as a source of glucose and the antimicrobial isothiocyanate is spontaneously produced. Since this is an intracellular reaction (Luciano and Holley 2010), the isothiocyanate formed has minimum effects on the sausage organoleptic qualities.

CHAPTER 3

3. QUANTIFICATION OF GLUCOSINOLATE AND MYROSINASE ACTIVITY IN MUSTARD SEEDS BY RP-HPLC

3.1. Abstract

The use of RP-HPLC to quantify glucosinolates from sample mixtures is widely accepted. The method can be used either for intact glucosinolates where an ion pair agent is involved (e.g. tetrabutylammonium hydrogen sulphate), or for desulfurated glucosinolate where a long process of desulfation is required. The mechanism of glucosinolate–isothiocyanate transformation has received significant attention because of its potential antibacterial activity. Mustard is characterized by its abundant glucosinolate content. Because of its widespread application in foods where it is used as an emulsifier or binder, mustard is considered a unique and novel antimicrobial candidate. However, the accompanying pungency produced by the formation of volatile isothiocyanates has restricted its utilization.

In this study, commercial yellow and oriental mustard seed products were analyzed for their (intact) glucosinolates by ion pair RP-HPLC. Yellow mustards showed higher glucosinolate concentration than oriental mustard and sinalbin was exclusively found. However, while two of 4 oriental mustards contained sinigrin as expected, they also unexpectedly contained sinalbin. Additionally, this method was further modified to estimate myrosinase activity by measuring the substrate (glucosinolate) decline caused by commercial myrosinase standards and mustard extracts containing myrosinase. Pure

sinigrin standard and two other crude glucosinolate extracts were used as substrates and also tested for feasibility. Comparable linearity ($R^2 \geq 0.99$) of substrate decline caused by a series of myrosinase concentrations from 0.1 to 1 unit was observed with all three substrates tested. This result indicated that the RP-HPLC method can also be used to quantify myrosinase activity. Moreover, it is suggested that these crude substrates might be an alternative to replace the expensive sinigrin standard in this assay because of the high sensitivity and specificity of the RP-HPLC method to detect glucosinolates in these mixtures.

3.2. Introduction

Myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147) is an indigenous enzyme that exists exclusively in *Brassica* vegetables which hydrolyzes glucosinolates following cell disruption since these two compounds are located separately within cells (Bridges et al. 2002). Upon enzymatic conversion, glucosinolates are transformed mainly into isothiocyanates, which result in the typical pungent flavor, plus other compounds which vary in nature depending on the pH and the specific substrate (Fenwick et al. 1982, Bones and Rossiter 1996, Mithen 2001). Several studies of the glucosinolate–isothiocyanate transformation have shown desirable bio-protective effects with food products (Luciano and Holley 2010, Luciano et al. 2011, Lara-Lledo et al. 2012, Nilson and Holley 2012) as well as benefits for human health (Mithen et al. 2000, Holst and Williamson 2004, Wang et al. 2004). Because of the volatile and pungent properties of isothiocyanates, glucosinolates are a convenient reservoir of the bioactive isothiocyanates (Clarke 2010).

However, a source of myrosinase must be present in order for them to be of value as antimicrobial precursors. This may come from the target bacteria themselves, but its generation appears to be slower than desirable (Herzallah et al. 2011), and so a small amount of plant myrosinase in the mustard may be of value if it does not lead to undesirable organoleptic effects.

Thus, a rapid and simple method for the determination of glucosinolate(s) as well as the measurement of myrosinase activity becomes significant, since these two factors affect the production of isothiocyanates. The myrosinase assay should also be practical for the quantification of low levels of myrosinase activity, and be able to measure varying velocities of enzymatic hydrolysis of glucosinolate after different extents of thermal treatment.

Two spectrophotometric methods for myrosinase determination have been previously introduced, including: 1) the Direct Spectrophotometric Assay (DSA) which involves the direct assay of substrate decrease (Palmieri et al. 1982); and 2) the Spectrophotometric Enzyme-Coupled Assay (SCEA) which involves measurement of the formation of NADPH from NADP after its reaction with glucose produced from glucosinolate degradation (Wilkinson et al. 1984). Sinigrin is the most frequently used substrate for myrosinase determination since it is commercially available at reasonable cost, although more than 120 different types of glucosinolates have been reported (Fahey et al. 2001, Clarke 2010).

RP-HPLC coupled with an ion-pair buffer has been comprehensively studied as a direct and fast method for separation and quantification of intact glucosinolates from plant extract mixtures which does not involve time consuming desulfation (Jen et al. 2001, Tsao et al. 2002b, Herzallah and Holley 2012). Herzallah et al. (2012) successfully separated and quantified sinigrin and sinalbin from mustard extracts with a relatively short retention time, and a low detection limit (0.1-0.5 μg/ml) was also reported. As a result, this method seems theoretically feasible to use to determine myrosinase activity by measuring the substrate decline in conjunction with a myrosinase activity calibration curve. With the high sensitivity and selectivity of the RP-HPLC method to detect glucosinolates in sample mixtures, background interference that was the main concern associated with the spectrophotometric myrosinase assay should be largely eliminated.

Sinigrin standards are expensive and myrosinase determination requires concentrated substrate to ensure sufficient enzymatic activity, since the enzyme reaction follows first order kinetics (Van Eylen et al. 2007). As a result, the present work also explored the use of crude glucosinolate extracts from plant tissue (e.g. mustard) as an alternative substrate to replace the standard sinigrin.

The aim of this work was to measure the glucosinolate concentration present in different mustard samples, and examine whether the modified RP-HPLC method was suitable for the quantification of myrosinase activity. A third objective was to examine the possibility of using crude glucosinolate extracts as alternative substrates to replace the sinigrin standard solution. To verify these results, myrosinase activity was independently

evaluated using enzyme concentrations ranging from 0.1 to 1 unit with three different substrates. These substrates were sinigrin solution prepared from an anhydrous sinigrin standard as well as crude sinalbin and sinigrin extracts from yellow (containing only sinalbin) and oriental (containing only sinigrin) mustards. With each substrate, the linearity of substrate decline in a given period caused by varying amounts of myrosinase was studied. Subsequently, a myrosinase calibration curve was established with each substrate by plotting the concentration of myrosinase added for each test as the X axis versus the substrate decline per min (μmol/min) as the Y axis. Substrate decline per min at each concentration of myrosinase tested was calculated by the difference of substrate concentration divided by the time between each of two sampling intervals. In order to accurately quantify myrosinase activity it was necessary to develop a method to terminate the enzyme reaction after samples were taken which would not affect the substrate concentration. Boiling reaction mixtures for 1 min in a water bath was evaluated and found suitable for this purpose.

3.3. Materials and methods

3.3.1. Chemicals and materials

Sinigrin hydrate and thioglucoside glucohydrolase (EC 3.2.1.147) were from Sigma-Aldrich (St. Louis, MO, USA). Sinalbin hydrate was from AppliChem, Inc. (St. Louis, MO, USA). HPLC grade acetonitrile and analytical grade hexane were purchased from Fisher Scientific (Whitby, ON, Canada). Tetrabutylammonium hydrogen sulphate

(TBA) was from J.T. Baker (Phillipsburg, NJ, USA). Mustard samples tested are summarized in Table 3-1.

3.3.2. Preparation of sinigrin, sinalbin and myrosinase standard solutions

For glucosinolate quantification, sinigrin and sinalbin calibration curves were prepared in the range of 50–4000 ppm using 10 mM sodium phosphate buffer (pH 6.5). For myrosinase determination, different concentrations of myrosinase standard solution (0.1 to 1.0 enzyme units) were freshly prepared and refrigerated before analysis. Water used for all standard solutions was HPLC grade (Milli-Q Direct water system, Millipore SAS, Molsheim Cedex, France).

3.3.3. Glucosinolate extraction and quantification by HPLC

In addition to commercially deodorized mustard powder (CDP), hot mustards were used but first autoclaved at 115^{o}C for 15 min to inactivate the myrosinase, and 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 down to ambient temperature, 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, Toronto, Canada). All of the mustard samples were extracted in triplicate. Glucosinolate determination and separation from the mustard extract was conducted 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. 2002b, Luciano et al. 2011, Herzallah and Holley 2012).

3.3.4. RP-HPLC myrosinase assay and the preparation of crude glucosinolate substrates

To evaluate the sensitivity and linearity of substrate decline caused by different amounts of myrosinase as a function of time, myrosinase concentrations of 0.1, 0.3, 0.5, 0.8 and 1.0 unit were independently tested. Briefly, for each myrosinase concentration tested, 7 sampling intervals were used, and at each interval separate tubes each containing a reaction mixture of 1 ml substrate and 50 µl myrosinase solution were examined. Substrate solutions contained approximately 8 mM sinigrin in 10 mM sodium phosphate buffer (pH 6.5). Reaction mixtures were agitated by vortex for 30 sec. When samples were removed from incubation they were placed in a boiling water bath for 1 min to inactivate myrosinase before being transferred to HPLC vials for the analysis.

To examine the feasibility of using crude glucosinolate extract as an alternative substrate, two other independent trials were conducted using crude sinalbin and sinigrin extracts from mustards #106 and #107F, respectively, following the extraction method described above, but were filtered through No. 40 ashless Whatman paper (Fisher Scientific, Whitby, ON, Canada). Sinigrin and sinalbin concentration in the extracts were adjusted to about 8 mM using specific sample–water ratios during extraction. As a result,

glucosinolate concentration and pH were considered identical among the three substrates. All assays were conducted at ambient temperature (22^{o}C ± 1) with duplication.

Both sample interval and the total measurement period were adjusted according to the intensity of myrosinase activity in samples in order to allow detection of differences between each interval at low myrosinase activity, as well as avoid the loss of linearity at high myrosinase concentrations. Samples with 0.1, 0.3, 0.5, 0.8 and 1.0 enzyme units were incubated 6 h, 1 h, 48 min, 36 min and 30 min, respectively. Samples were clarified before RP-HPLC analysis by passage through a 0.22 µm PES syringe filter (VWR Co., Toronto, ON, Canada) fitted to a 1 ml syringe (Becton Dickson, Franklin Lakes, NJ, USA). All samples were stored at 4^{o}C before analysis.

3.3.5. Conversion of substrate decline to myrosinase activity

Presuming that acceptable linearity would be obtained for each test (from different amounts of myrosinase), the amount of decrease in substrate is needed to convert the result to a uniform and comparable index since different sample intervals and periods were used for each test. Therefore, myrosinase activity was expressed by substrate decline rate per minute (µmol/min). To address this issue, a formula was developed as follows:

$$\text{Substrate decline rate}\left(\mu mol/_{min}\right) = \frac{C_{n+1} - C_n}{\Delta t}$$

- C represents the substrate concentration in solution
- N represents the sample interval
- Δt represents the time between two sample intervals

To verify the linearity as well as feasibility of the RP-HPLC myrosinase assay, a myrosinase calibration curve was drawn for each substrate tested by plotting different amounts of myrosinase as the independent variable (X) versus the substrate decline caused by the corresponding myrosinase concentration as the dependent variable (Y). The resulting calibration curve was used for the conversion of the substrate decline rate (μmol/min) to actual myrosinase activity (units), allowing an assessment of the myrosinase activity present in the actual sample solution.

3.3.6. Validation of the boiling water bath treatment for the elimination of myrosinase activity

In order to stop the enzyme reaction at precise intervals to prevent further substrate degradation after samples were taken, a method for enzyme inactivation was needed. To achieve this, a 1 min boiling water bath treatment was examined for its ability to eliminate myrosinase without affecting the glucosinolate. Reaction mixtures (sinigrin substrate with myrosinase addition) were taken from 7 intervals and immersed in a boiling water bath for 1 min. To examine its ability to stop myrosinase action, post-boiling samples (boiling 1d) were analyzed for their substrate concentration by RP-HPLC directly and then re-analyzed the next day (boiling 2d) to see if further degradation had occurred. To verify whether further degradation occurred without the thermal treatment, unboiled samples (unboiled) were also analyzed. In order to examine the thermal stability of glucosinolate, pure sinigrin solutions without the addition of

myrosinase (negative control) were also treated 1 min in the boiling water bath. All samples except boiling 1d were stored at 4^{o}C before RP-HPLC assay.

3.3.7. Statistical analysis

The statistical significance of mean differences at $\alpha = 0.05$ were assessed by analysis of variance using JMP 10.00 (SAS Institute Inc., Cary, NC, USA) and statistical differences among treatments were compared using Tukey's test.

3.4. Results and discussion

3.4.1. The capability of RP-HPLC to detect intact sinigrin and sinalbin

Because both glucosinolate determination as well as myrosinase quantification were dependent upon the ability of the RP-HPLC method to accurately measure glucosinolates (sinigrin and sinalbin), validation of glucosinolate detection by this method was important. Sinigrin and sinalbin standards at concentrations ranging from 50 to 4000 ppm were tested. With 10 µl sample injection, sinalbin recovery at the high concentrations did not show any reduction in linearity when the AU (area under the peak) was < 30,000,000. On the other hand, sinigrin recovery showed poor linearity once the AU value become ≥ 20,000,000 as the concentration rose (Figure 3-1). As a result, the injection volume was modified to 5 µl from the original work (Luciano et al. 2011, Herzallah and Holley 2012) for both sinigrin and sinalbin analysis so that the AU value for sinigrin was lower than the upper detection limit (20,000,000). With 5 µl injection, the coefficient of determination (R^2) showed high linearity for both sinigrin (R^2=0.9997) and sinalbin (R^2=0.9999)

calibration curves (Figure 3-2), and thus, both calibration curves were used for the conversion and determination of glucosinolate concentration.

3.4.2. Glucosinolate concentration in different mustard samples

Nine commercially available mustard products (5 yellow and 4 oriental) were analyzed for the presence of glucosinolate types and their concentrations (Table 3-1). Sinalbin was found exclusively in yellow mustards and its concentration ranged from 174 to 271 μmol/g in different samples tested (Table 3-2). Bennett et al. (2004) reported *Sinapis alba* seed contained sinalbin concentrations as high as 250 μmol/g, which was consistent with the current results. In terms of oriental mustard, it was a surprise to find that two of the 4 samples (#107 and #202) contained both sinigrin and the unexpected sinalbin. It is generally agreed that oriental mustards contain predominately sinigrin (Tsao et al. 2000, Rangkadilok et al. 2002), thus it was unclear if this difference was caused by analysis of unusual mustard genotypes, or whether yellow mustard was mixed deliberately or accidentally during milling by the manufacturer. The mixing of yellow and oriental mustard for condiment preparation may be done to reduce the sharp flavor of oriental mustard since yellow mustard has a milder taste. It was found here that yellow mustards contained higher glucosinolate concentrations than oriental mustards and the deoiled mustard cakes (both yellow and oriental) contained the highest concentrations. This is reasonable since the later had been used for oil extraction which would concentrate the glucosinolates in the residual meal or “cake” (Table 3-2). It is noteworthy that sinigrin and sinalbin have a remarkable difference in molecular mass (397.46 and

734.79, respectively) (AppliChem 2012, Sigma-Aldrich 2012). Thus, use of uniform weights (e.g. ppm) of sinigrin and sinalbin will cause obvious differences in moles (e.g. mM), although the latter seems to be a more suitable unit to use for chemical and enzymatic analyses.

3.4.3. Validation of myrosinase elimination by a 1 min boiling water bath treatment

It has been comprehensively reported that glucosinolates themselves are heat stable, whereas myrosinase is heat labile (Drew et al. 2007, Tang et al. 2008). As a result, a 1 min boiling water bath was tested to determine whether it would eliminate myrosinase without affecting the substrate concentration. The results (Figure 3-3) showed the 1 min boiling water bath treatment did not affect the sinigrin concentration (negative control). With the samples treated 1 min by boiling and analyzed immediately (boiling 1d) and one day later (boiling 2d), identical sinigrin recovery was observed and the correlation between these two analyses was 0.9997 (Table 3-3). For samples without boiling (unboiled), a significantly ($p < 0.05$) lower sinigrin concentration was recovered. The correlation between the unboiled treatment and treatments with boiling water was also reduced to 0.9412 (1d) and 0.9409 (2d). These results suggested that a 1 min boiling water bath treatment was sufficient for the prevention of further glucosinolate degradation after sampling and it did not affect the substrate concentration. On the basis of these results, the 1 min boiling water bath treatment was used for myrosinase inactivation before the RP-HPLC myrosinase assay.

3.4.4. Linearity and feasibility of the RP-HPLC myrosinase assay

For all 3 substrates (sinigrin standard and the two crude extracts) tested, good linearity of substrate decline caused by the myrosinase from low to high concentration with time was obtained. A reasonable sample interval as well as total measuring period were critical to establish good linearity, because differences in enzyme concentrations used lead to differences in hydrolysis rates of the glucosinolates. For instance, 1 h and 5 min sample intervals were used for 0.1 unit and 1 unit myrosinase analysis, respectively, and this permitted observing a linear and measureable difference in glucosinolate concentrations between each of the two intervals at both myrosinase concentrations (Figure 3-4, Figure 3-5, Figure 3-6, Figure 3-7 and Figure 3-8).

This result suggested that it was feasible to use RP-HPLC for the determination of myrosinase activity by measuring glucosinolate degradation in the substrate caused by myrosinase. Additionally, it showed that besides using a pure sinigrin solution, crude sinalbin and sinigrin extracts could be used in this assay due to their comparable linearity in substrate decline with the sinigrin standard. Crude extracts are theoretically not ideal substrates for enzymatic activity analyses, and are not feasible for use with a spectrophotometer-based method because of interference caused by the mixture. However, due to the high specificity of RP-HPLC, independent peaks of sinalbin and sinigrin can be easily separated from other compounds in the sample mixture (Rangkadilok et al. 2002, Bennett et al. 2004, Herzallah and Holley 2012). More importantly, the specificity of

myrosinase action in degrading glucosinolate yielded unique changes in peak area (indicator change), making the measurement possible and practical.

It is notable that myrosinase at the same concentration showed varying ability to hydrolyze the substrates from different sources (sinigrin standard and crude extracts), while pH, temperature and substrate concentration were identical. This suggests that it may also be possible to use other glucosinolates to evaluate myrosinase activity from other *Brassica* plants which contain glucosinolates other than sinigrin.

3.4.5. Myrosinase total calibration curves in different substrates

After separate examination of the linearity of substrate decline caused by myrosinase with time, it was important to examine whether the substrate decline proceeded in a linear manner with increasing concentrations of myrosinase. Because in the series of myrosinase trials different sample intervals were used to better characterize substrate decline, an overall substrate decline rate (μmol/min) was calculated using the equation mentioned before (section 3.3.5) to generate a uniform and comparable indicator. A total calibration curve was then constructed using myrosinase units as the independent variable (X) versus substrate decline rate as the dependent variable (Y). Thus one calibration curve was established for each of the 3 substrates and used for myrosinase quantification in subsequent work (Figure 3-9). The scatter plot figure showed that substrate decline rate caused by myrosinase action at increasing concentration also had good linearity, suggesting the RP-HPLC myrosinase assay was feasible and reliable for the measurement of a wide range of myrosinase activity. The decline rate of sinigrin in the standard

solution (R^2=0.9951) did not show substantially better linearity than sinalbin (R^2=0.9942) and sinigrin (R^2=0.9946) in crude extracts. This also suggested that crude sinalbin and sinigrin extracts could be used to replace the sinigrin standard in this assay. Sinigrin standard is expensive and sinalbin standard is prohibitively expensive. Use of the RP-HPLC myrosinase assay with crude glucosinolate extracts seemed to be a useful alternative, providing an economic option, and more importantly, providing an opportunity to use other extracted plant materials as glucosinolate substrates for the measurement of myrosinase activity.

3.4.6. Ascorbic acid and myrosinase activation

Ascorbic acid has been proposed as a necessary enzyme activator during use of one of the two spectrophotometric methods (SCEA) for myrosinase determination (Wilkinson et al. 1984, Sakorn et al. 2002), otherwise its activity was not measureable. In contrast, according to Palmieri et al. (1982), the DSA spectrophotometric method does not require using ascorbic acid to activate the hydrolysis of sinigrin. This is probably because different indicators are used in these two assays. The SCEA method measures the NADPH formed after glucose is released from sinigrin hydrolysis and reacts with NADP+, and thus a relatively small amount of NADPH must be detected in the very concentrated sinigrin solution at the beginning of the assay. As a result, ascorbic acid enhancement of the reaction seems to be required to accelerate the synthesis of NADPH, or in other words, to accelerate sinigrin hydrolysis, releasing more glucose. On the other hand, the DSA method measures the decrease of sinigrin directly in the sample solution;

however, the method is limited to the detection of sinigrin levels < 0.5 mM, otherwise spectrophotometric detection is not linear.

In the present work, measureable degradation was obtained without the use of ascorbic acid even at the lowest myrosinase concentration tested (0.1 unit). It is believed this was possible because at lower concentration the incubation periods and sampling intervals were extended which allowed measurement of small changes in glucosinolate concentration.

Another reason to avoid spectrophotometric myrosinase assays was that at suitable sinigrin concentrations in the substrate there was large disagreement in the results of the two accepted methods. For the DSA measurement, relatively low concentration of sinigrin ranging from 0.2 mM to 0.5 mM were preferred because of the limited capacity of the spectrophotometer to measure sinigrin in quartz cells (Palmieri et al. 1987). On the other hand, the use of a wide range of sinigrin concentrations was reported in the SCEA method; the original method used up to 5 mM sinigrin (Wilkinson et al. 1984). However, several later reports suggested sinigrin concentrations from 30 mg/ml to 300 mg/ml were optimal (Charron et al. 2005, Van Eylen et al. 2006, Travers-Martin et al. 2008, Ghawi et al. 2012). This huge variation suggested there might be a problem with substrate saturation occurring during the test. In the present work, the establishment of the calibration curves for myrosinase is likely to at least partially eliminate this uncertainty since excellent linearity was shown with substrate concentration and its detection.

3.5. Conclusion

The RP-HPLC method was able to separate and quantify intact glucosinolates without time-consuming pre-desulfation. Data showed that myrosinase activity could be measured by estimating the change in glucosinolate concentration in the substrate. Results also showed that the RP-HPLC myrosinase assay was feasible over a wide range of myrosinase activity. It is suggested that this method might help to improve the detection of myrosinase when present at low levels of activity, particularly after moderate heat treatment. The present work also demonstrated that reasonable measurement periods as well as sampling intervals were critical for method sensitivity, and that these should be modified based on the myrosinase activity anticipated. A myrosinase calibration curve constructed by plotting myrosinase concentration (X axis) versus substrate decline per min (Y axis) eliminated uncertainty associated with the action of ascorbic acid and potential for interference by substrate saturation. Use of crude glucosinolate extracts as alternative substrates for sinigrin and sinalbin standard was validated and has significant cost advantages for preliminary studies. It is necessary to further characterize this RP-HPLC myrosinase assay using classic enzymatic kinetics-based procedures with a view to the possible application of the method for the study of myrosinase hydrolysis of a variety of glucosinolate substrates.

Table 3-1: Summary of mustard samples examined.

Type	Condition	Supplier	Abbr.[3]
Yellow mustard	Commercially deodorized powder	Viterra, Minneapolis, MN, USA	CDP
	Deoiled cake	Sakai Spice, Lethbridge, AB, Canada	DYM
	Powder[1]	Viterra, Minneapolis, MN, USA	YM
	Flour[2]	G.S.Dunn, Hamilton, ON, Canada	#106
	Powder[1]	G.S.Dunn, Hamilton, ON, Canada	#201
Oriental mustard	Deoiled cake	Sakai Spice, Lethbridge, AB, Canada	DOM
	Flour[2]	G.S.Dunn, Hamilton, ON, Canada	#107
	Flour[2]	G.S.Dunn, Hamilton, ON, Canada	#107F
	Powder[1]	G.S.Dunn, Hamilton, ON, Canada	#202

1 Ground from the whole seed.

2 Ground from the dehulled seed.

3 Abbreviation shown for each mustard sample is used throughout the thesis.

Table 3-2: Glucosinolate concentration (sinalbin and sinigrin) in mustards.

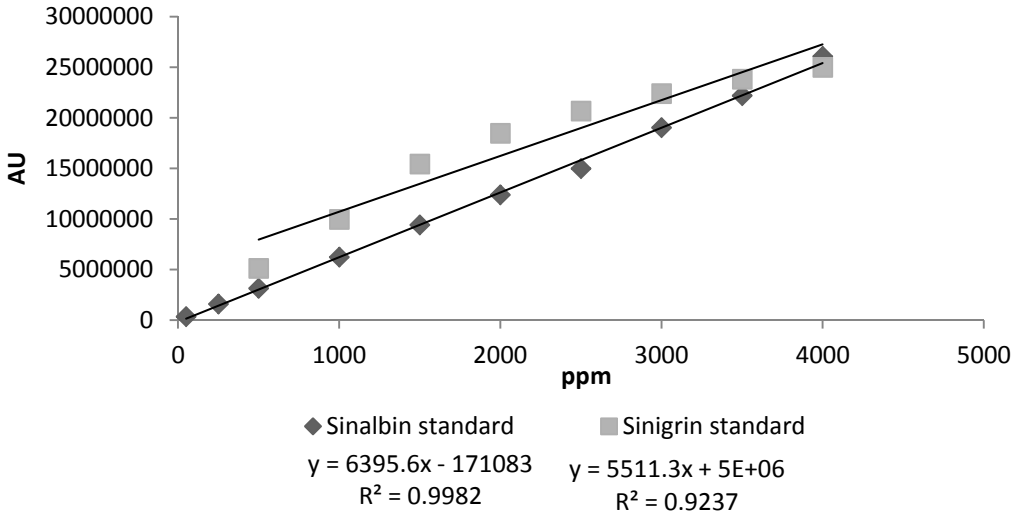
Mustard type	Mustard sample	Glucosinolate concentration (μmol/g)	
		Sinalbin	Sinigrin
Yellow	CDP	175.18±2.54	-
	YM	174.31±15.16	-
	DYM	271.40±7.14	-
	#106	245.92±12.11	-
	#201	188.56±3.27	-
Oriental	DOM	-	147.52±6.86
	#107	41.94±2.19	97.82±4.33
	#107F	-	81.77±8.18
	#202	32.35±4.29	85.44±8.60

Values in each cell represent mean ± SD (n=3).
- Not detectable.

Table 3-3: Correlation analysis (R^2) of the 1 min boiling water bath treatment.

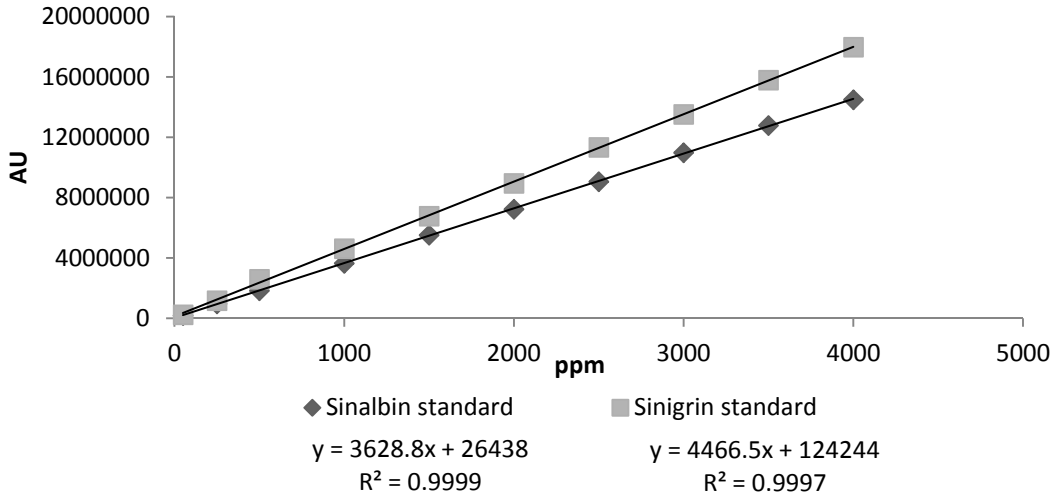
	Boiling 1d	Boiling 2d	Unboiled
Boiling 1d	1		
Boiling 2d	0.9997	1	
Unboiled	0.9412	0.9409	1

Figure 3-1: Sinigrin and sinalbin standard curves quantified by RP-HPLC (10 µl injection).



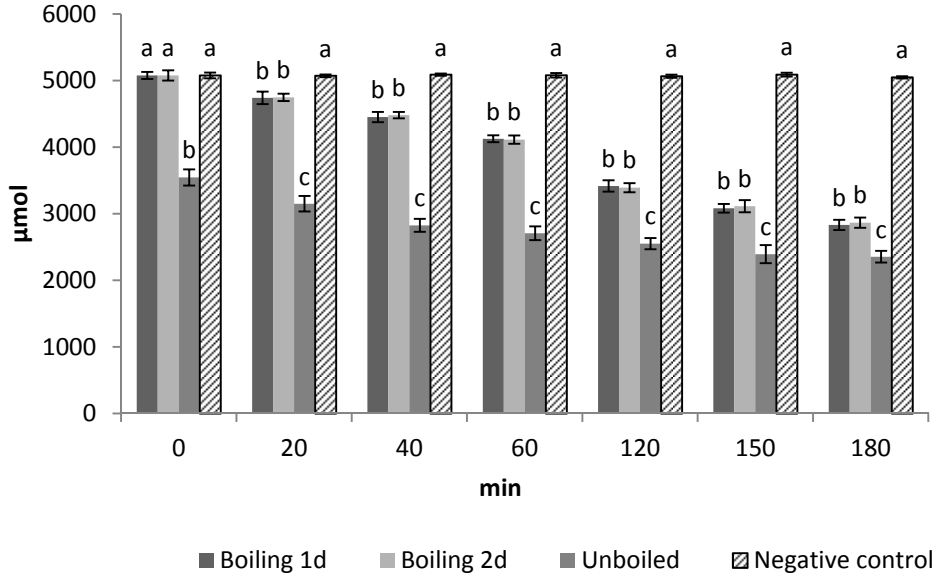
Measurement was conducted in triplicate (n=3).

Figure 3-2: Sinigrin and sinalbin standard curves quantified by RP-HPLC (5 µl injection).



Measurement was conducted in triplicate (n=3).

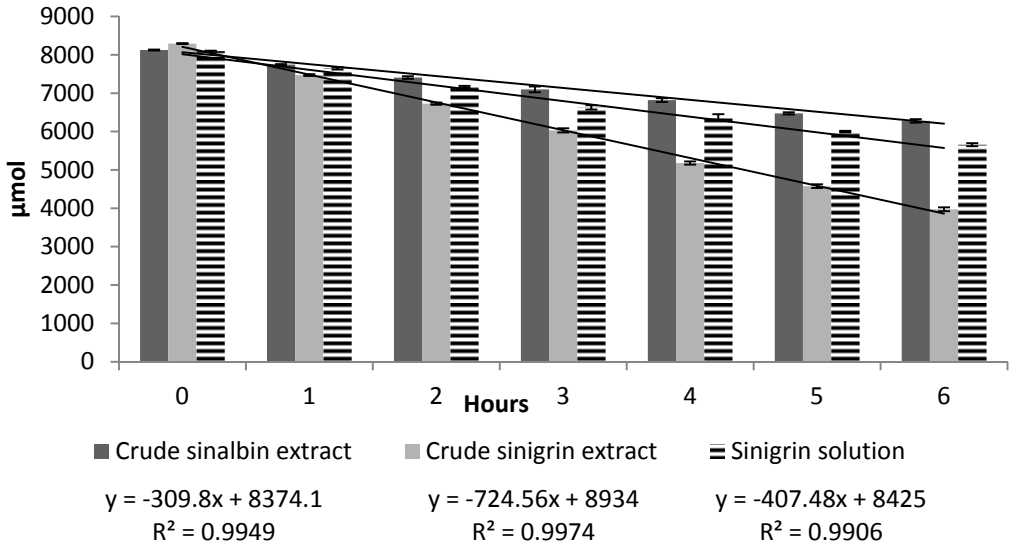
Figure 3-3: Validation of the 1 min boiling water bath treatment to eliminate myrosinase activity.



Different letters on vertical bars in the same group indicate a significant difference ($P < 0.05$) from others within the group.
Values were calculated from two trials with duplication (n=4).

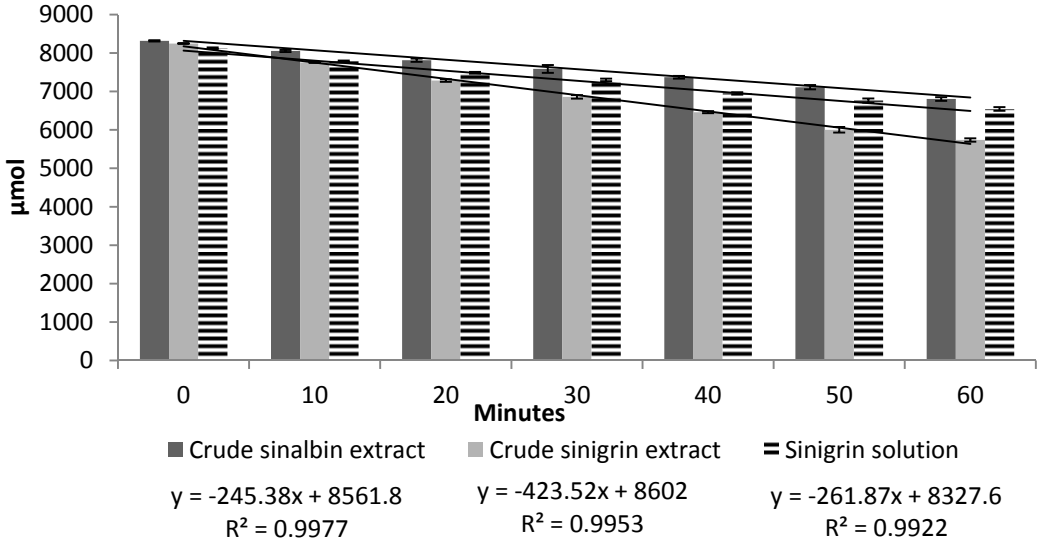
1. Boiling 1d indicates the sample solution was treated 1 min in a boiling water bath and analyzed by HPLC immediately (one time).
2. Boiling 2d indicates the Boiling 1d sample was stored at 4^{o}C and re-analyzed the next day.
3. Unboiled indicates the sample was not thermally-treated.
4. Negative control indicates sinigrin substrate (no myrosinase) was treated 1 min in the boiling water bath.

Figure 3-4: Substrate declines caused by 0.1 unit of myrosinase activity with time.



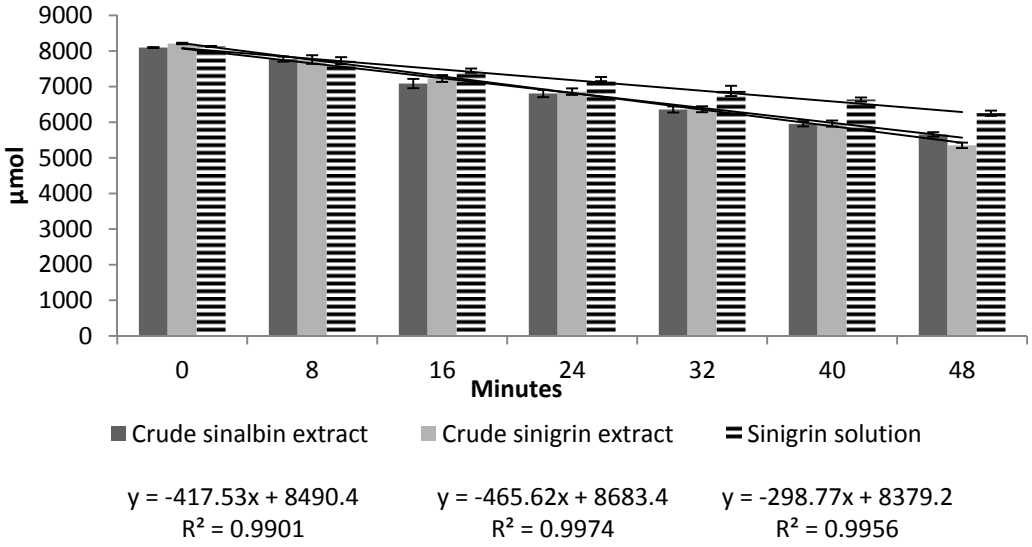
Values were calculated from two trials with duplication (n=4).

Figure 3-5: Substrate declines caused by 0.3 unit of myrosinase activity with time.



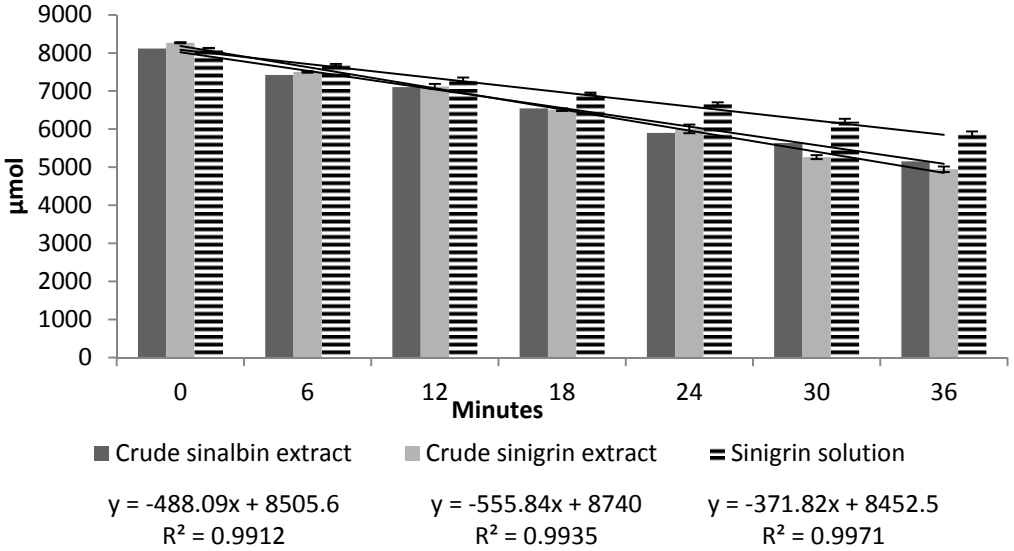
Values were calculated from two trials with duplication (n=4).

Figure 3-6: Substrate declines caused by 0.5 unit of myrosinase activity with time.



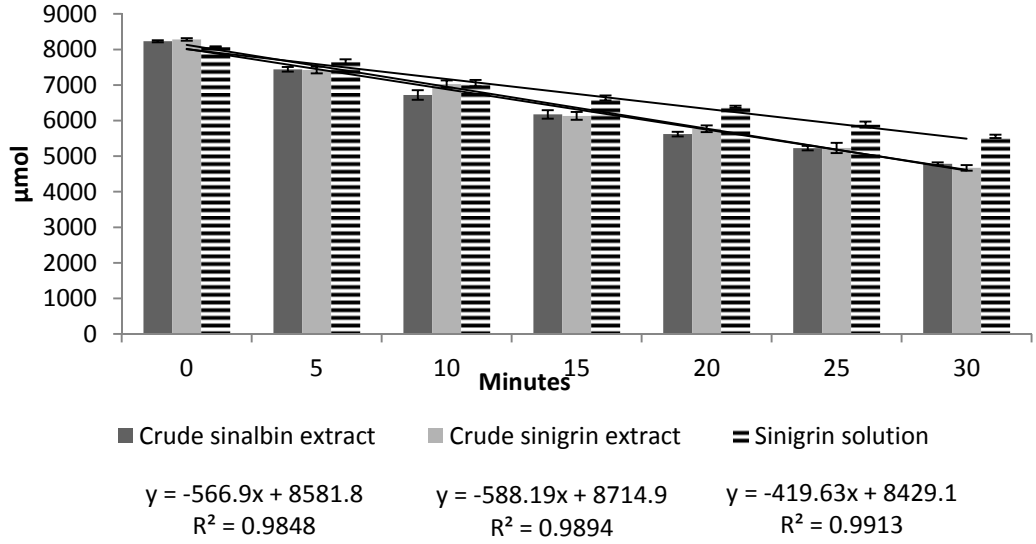
Values were calculated from two trials with duplication (n=4).

Figure 3-7: Substrate declines caused by 0.8 unit of myrosinase activity with time.



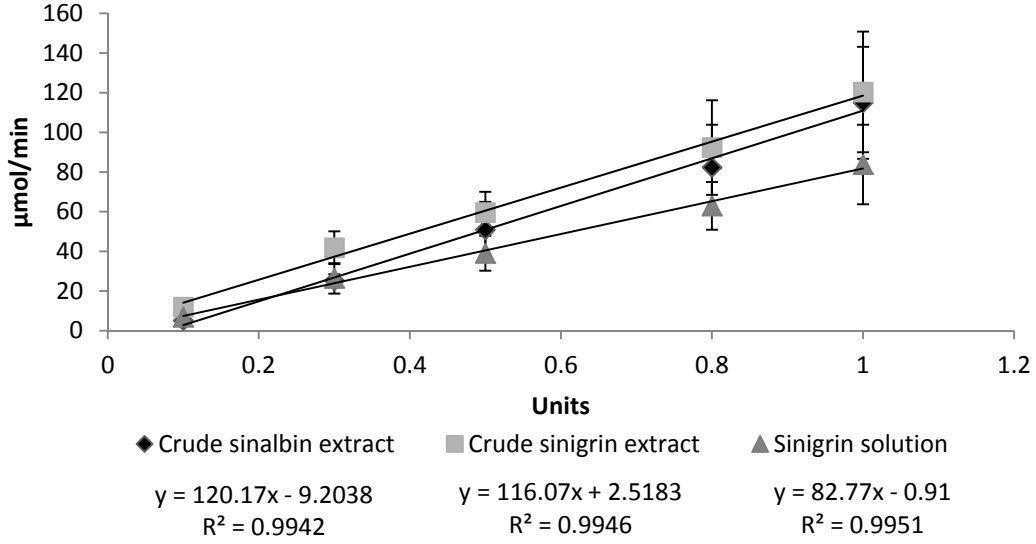
Values were calculated from two trials with duplication (n=4).

Figure 3-8: Substrate declines caused by 1 unit of myrosinase activity with time.



Values were calculated from two trials with duplication (n=4).

Figure 3-9: Substrate decline rate versus increasing myrosinase concentration (myrosinase calibration curve) tested with the three different substrates.



Values were calculated from two trials with duplication (n=4).

CHAPTER 4

4. USE OF DEODORIZED MUSTARD TO INHIBIT *E. COLI* O157:H7 IN DRY FERMENTED SAUSAGE – A PRELIMINARY STUDY

4.1. Abstract:

In previously published work, deodorized yellow mustard in which the endogenous myrosinase was inactivated by autoclave treatment (115^{o}C for 15 min using a 2 cm thick layer) showed significant antimicrobial activity against *E. coli* O157:H7 in dry fermented meats (Luciano et al. 2011, Nilson and Holley 2012, Cordeiro et al. 2013). Because no apparent myrosinase was observed, *E. coli* O157:H7 and the starter cultures which had myrosinase-like activity were believed responsible for glucosinolate degradation in deodorized mustard. Glucosinolate hydrolysis, as a result of the action of bacterial myrosinase-like activity has also been widely reported in the human colonic microflora (Nugon-Baudon et al. 1990, Krul et al. 2002). In the present study, 9 types of prepared mustard products (powder and flour) from two varieties (yellow and oriental) were deodorized, the absence of endogenous myrosinase was verified, and then examined for their antimicrobial activity in 5 independent dry fermented sausage trials. Surprisingly, the time required for *E. coli* O157:H7 elimination varied by more than a week in different independent sausage trials even when the same mustard sample was used and separately deodorized before each trial. It was unlikely that sample variation was the reason for this difference. It was suspected that endogenous myrosinase in mustard may not have been completely inactivated in some batches of autoclave treated (115^{o}C for 15 min) material

when the thickness of the mustard layer was ≥ 2 cm, and this could have contributed to its high antimicrobial activity against *E. coli* O157:H7 in dry fermented sausage. Further investigation indicated the thickness of the mustard powder had a substantial influence on the ability of the thermal treatment to inactivate the endogenous myrosinase in the mustard.

4.2. Introduction

Dry fermented sausage is traditionally uncooked and protected by low pH and water activity resulting from fermentation and drying. The vehicles of foodborne *E. coli* O157:H7 transmission are largely undercooked or raw foods of bovine origin. The ability of *E. coli* O157:H7 to survive in acidic as well as low a_w environments makes this pathogen problematic in dry fermented sausage (Doyle and Schoeni 1984, Alexander et al. 1995, Bach et al. 2002). Glucosinolates exclusively exist in the *Brassicaceae* family and their hydrolysis products, the isothiocyanates, have remarkable antimicrobial activity (Bones and Rossiter 1996, Bennett et al. 2004, Holst and Williamson 2004). As a result, prepared mustard seed products (e.g. flour or powder) which have traditionally been used as emulsifiers in cooked processed meats have extra value, because they contain high levels of glucosinolates (Bennett et al. 2004, Clarke 2010). Several previous studies reported that microencapsulated allyl isothiocyanate (AITC) or untreated hot yellow mustard powder significantly inhibited *E. coli* O157:H7 in ground beef, hamburger patties and dry fermented meats (Nadarajah et al. 2005, Chacon et al. 2006, Graumann and Holley 2008). Because of economic and manufacturing constraints necessary when

working with isothiocyanates, the direct application of mustard is more practical. However, one disadvantage with its direct addition to moist foods is the subsequent pungent flavor development from isothiocyanate production, which is undesirable. Brunn (1964) and Deerfield and Dougherty (1971) developed thermal treatments to inactivate mustard myrosinase, and thus the thermally-treated mustard had a negligible or minimal taste since glucosinolate hydrolysis was prevented. To prevent development of the undesirable pungency, an autoclave-based deodorization method was used for mustard and the inactivation of endogenous myrosinase was verified by quantitative recovery of glucosinolates from the sample mixture after mixing it with water. Further work with deodorized yellow mustard at 4 to 6 % (w/w) was found to have similar or better bactericidal activity in dry fermented meats (Luciano et al. 2011, Nilson and Holley 2012, Cordeiro et al. 2013). Additionally, Luciano and Holley (2010) reported that *E. coli* O157:H7 and starter cultures which are commonly used in meat fermentations possessed bacterial myrosinase-like activity which degraded sinigrin *in vitro*. From these results, it was believed the *E. coli* O157:H7 and starter cultures were responsible for the significant antimicrobial activity of deodorized yellow mustards.

The present work was designed to compare the inhibitory potential against *E. coli* O157:H7 of different forms of ground mustard (deoiled cake, powder and flour) from yellow and oriental varieties (Table 3-1). Another goal was to explore whether reduced levels of mustard (< 6 % w/w) could eliminate *E. coli* O157:H7 during dry fermented sausage manufacture. Further, a more sensitive method for measuring myrosinase activity

using RP-HPLC was used to confirm whether there was complete or partial inactivation of myrosinase activity in deodorized mustards following autoclave treatment (115^{o}C for 15 min).

4.3. Materials and methods

4.3.1. Preparation of bacterial cultures and dry fermented sausage manufacture

Bacterial culture methods and dry fermented sausage manufacture used followed previous studies (Luciano et al. 2011, Cordeiro et al. 2013). Briefly, 5 strains of non-pathogenic *E. coli* O157:H7 (00:3581, 02:0304, 02:0627, 02:0628 and non-motile strain 02:1840) plus starter cultures including *Staphylococcus carnosus* UM109M as well as *Pediococcus pentosaceus* UM116P were selected, which were identical to those used in the previous studies. The duration of sausage ripening was extended from 30 d to 42 d, compared to the Luciano et al. (2011) work.

4.3.2. Microbial, pH and water activity analyses from dry fermented sausage during ripening

Sampling and analysis methods followed were the same as in previous studies (Luciano et al. 2011, Cordeiro et al. 2013) which were based on the protocol issued by Health Canada (2000) with modification. Sausage samples from each treatment were taken at day 0 and subsequently at 6 d intervals until the end of ripening. Enrichment and immunomagnetic separation were conducted when the number of *E. coli* O157:H7 was lower than the detection limit (≤ 1 log CFU/g). Both pH and water activity analyses used also followed those in previous studies (Luciano et al. 2011, Cordeiro et al. 2013).

4.3.3. Confirmation of myrosinase inactivation in deodorized mustards following the method of Luciano et al. (2011)

In addition to commercially deodorized mustard powder, 8 types of hot mustards (Table 3-1) were freshly deodorized individually in 2 cm thick layers using the autoclave at 115^{o}C for 15 min before each sausage trial. After deodorization, mustard was chopped into a fine powder using a commercial blender (33BL37, Waring, New Hartford, CT, USA). The absence of mustard myrosinase was verified as in the previous work (Luciano et al. 2011). Briefly, 5 g mustard were mixed with 250 ml distilled water for 3 h. After analysis by RP-HPLC, if the glucosinolate concentrations were stable (sinigrin in oriental mustard or sinalbin in yellow mustard), myrosinase in the deodorized mustard was considered inactivated and used for sausage manufacture.

4.3.4. Confirmation of myrosinase inactivation and quantification of residual myrosinase in deodorized mustards by RP-HPLC myrosinase assay

To better study and verify the reliability of the autoclave deodorization method for the inactivation of mustard myrosinase, myrosinase activity was confirmed absent, or quantified by the RP-HPLC myrosinase assay as described in Chapter 3. Myrosinase extraction was adopted from Yen and Wei (1993) as modified by Ghawi et al. (2012). For each of the deodorized mustards, a 10 g sample was stored at -20^{o}C overnight and then extracted with 50 ml ice cold 0.1 M sodium phosphate buffer (pH 6.5) containing 0.01 M mercaptoethanol for 30 min. The sample mixture was centrifuged (12000 ×g, 20 min, 0^{o}C) to separate the insoluble components and the supernatant was retained. Myrosinase was

then extracted by fractional precipitation using ammonium sulfate. Myrosinase was pelleted by centrifugation (12000 ×g, 20 min, 0^{o}C) after ammonium sulfate had been added to achieve 30–80 % saturation (Ghawi et al. 2012) in the sample solution. The pellet was collected and re-suspended in 10-fold diluted sodium phosphate buffer as noted above. The whole procedure was conducted on ice as much as possible to prevent enzyme loss. Extracted myrosinase solution (50 µl) was then individually added to 7 tubes, each containing 1 ml substrate (glucosinolate, 8 mM), yielding the reaction sample mixtures. The substrate concentration was measured at 7 regular sampling intervals, and the glucosinolate concentration was compared between each of two contiguous intervals. If the glucosinolate concentration was unchanged, myrosinase was confirmed absent from the deodorized mustard. When measureable changes of substrate concentration in the reaction mixtures were obtained, protein concentration in the extracted myrosinase solution was determined by the Bradford method (Bradford 1976) according to the manufacturer's protocol. Myrosinase activity was converted from volume to units using a calibration curve as described in Chapter 3 and then expressed as units/mg protein.

4.3.5. Statistical analysis

Results were analyzed using JMP 9.0, Statistical Analysis System software (SAS Institute, Cary, NC, USA). Among treatments significant differences ($p < 0.05$) were detected using the Tukey test.

4.4. Results and discussion

4.4.1. Viability of *E. coli* O157:H7 in different mustard-treated dry fermented sausages during ripening

In trial 1 and 2, deoiled yellow and oriental mustards in 4 concentrations (3 %, 3.5 %, 4 % and 6 % w/w) were tested. Unexpectedly, none of these treatments achieved a 5 log CFU/g reduction of *E. coli* O157:H7 at the end of 30 d ripening. The best outcome was from 6 % (w/w) deoiled yellow mustard which yielded about a 4 log CFU/g reduction. In general, deoiled yellow mustard treatments showed higher bactericidal activity than deoiled oriental mustard, and the mustard bactericidal activity paralleled use of lower concentrations of mustards in the sausage; that is, 6 % > 4 % > 3.5 % > 3 % (Figure 4-1 and Figure 4-2). However, these results did not agree with previously published results which showed that 18 to 28 d of sausage ripening were adequate to cause a ≥ 5 log reduction in pathogen viability when 4 % to 6 % (w/w) yellow mustard were added to the meat batter (Graumann and Holley 2008, Luciano et al. 2011, Cordeiro et al. 2013).

To understand this unexpected result, trials 3 to 5 were done in the same manner, but with different types of mustard samples at 6 % (w/w) in sausage, and sausage ripening was extended to 42 d. Trials 3 and 4 included 4 identical mustard samples, consisting of commercially deodorized yellow powder (CDP), yellow mustard powder (YM), deoiled yellow and oriental mustards (DYM & DOM). In both trial 3 and trial 4, all mustard treatments reduced the number of *E. coli* O157:H7 lower than the detection limit (1 log CFU/g) after extension of the ripening to 42 d (Figure 4-3 and Figure 4-4). Even though

the 5 log reduction goal was achieved, it was interesting that there was variability in the rates of *E. coli* O157:H7 elimination. Treatment DYM, in both trial 3 and 4, reduced *E. coli* O157:H7 numbers > 5 log CFU/g within 18 d of ripening. In contrast, the same type of mustard did not reduce the pathogen by 5 log CFU/g within 30 d in trial 1. Treatments YM and DOM showed better antimicrobial activity in trial 4, causing a 5 log CFU/g reduction at 24 d of ripening.

Trial 5 was conducted using 4 other types of mustards (two yellow and two oriental) that were obtained from the same supplier (G.S. Dunn, Hamilton, ON, Canada). After the same deodorization procedure, two yellow mustards (#106 and #201) were found to reduce pathogen viability below the detection limit (< 1 log CFU/g) at 24 d, and two oriental mustards took 36 d to achieve an identical outcome (Figure 4-5).

It seemed that variation in the rate of *E. coli* O157:H7 elimination from sausages not only existed among different mustard treatments, but also existed when the same mustard was tested in different sausage trials. As a result, sample variation was most likely not an acceptable explanation for this variation. When a 5 log CFU/g reduction of *E. coli* O157:H7 was achieved at 42 d of ripening, the control group with no mustard also showed about a 4 log CFU/g reduction. In contrast, in those treatments where a 5 log CFU/g reduction occurred within 24 d of ripening, the corresponding reduction of *E. coli* O157:H7 in the controls was ≤ 2 log CFU/g. In the former instance it seemed that extending drying to 42 d was more effective in reducing the pathogen than the addition of mustard. Nonetheless, high antimicrobial activity of deodorized mustard was observed

which resulted in a 5 log CFU/g *E. coli* O157:H7 reduction within < 24 d. As a result, it became important to understand the reason for variability in the antimicrobial action of mustard. It is noteworthy that the commercially deodorized yellow mustard powder (CDP), which was tested in both trial 3 and 4, showed poor but identical ability to eliminate *E. coli* O157:H7 (Figure 4-3 and Figure 4-4). Thus, it seemed reasonable to question whether the laboratory mustard deodorization method was adequate to completely inactivate the endogenous myrosinase present in the hot mustards.

4.4.2. Changes of pH and a_w during sausage ripening

The initial pH values of dry fermented sausages from trial 1 to 5 varied from 5.7 to 6.1 and rapidly decreased during fermentation to less than 5.3 within 48 h. This was consistent with the regulatory required degree•hour pH reduction which specifies that the pH must fall to or lower than 5.3 within 63.8 h at a fermentation temperature of 26^{o}C (CFIA 2010). The final pH of mustard-treated sausages ranged from 4.4 to 4.5 and was 4.6 to 4.7 in the controls without mustard addition. The pH values observed were within the range of commercial dry fermented sausage in the market place; however, mustard addition slightly reduced pH values, possibly because mustard was an additional source of fermentable sugars. Water activity varied from 0.97 to 0.78 during the ripening process. Compared to mustard-treated sausage, controls had the lowest a_w in each trial, suggesting mustard reduced moisture loss from the product (Appendix I).

Between mustard-treated sausages with high and low antimicrobial activity against *E .coli* O157:H7 among trials, there were no apparent differences in pH and a_w values,

suggesting pH and a_w were not responsible for differences in the rate of *E .coli* O157:H7 elimination.

4.4.3. Comparison of the two myrosinase assays and measurement of residual myrosinase activity

According to the description by Luciano et al. (2011), myrosinase was verified inactivated in deodorized mustard if glucosinolate levels were unchanged in the mixture by RP-HPLC, after deodorized mustard sample was mixed with distilled water and held for 3 h. To better verify the absence or presence of myrosinase activity after mustard deodorization, and to quantify the residual myrosinase activity once it was present in deodorized mustard; myrosinase was first extracted from deodorized mustard samples and its ability to cause substrate (glucosinolate) decline was measured using the RP-HPLC myrosinase assay as noted in Chapter 3. Deodorized mustard samples with both high and low antimicrobial activity found in previous sausage trials (3 – 5) were selected for the assay. In order to depict substrate decline, a scatter plot chart was drawn (Figure 4-6). Data showed yellow mustard in trial 3 (YM_T3), deoiled yellow mustard in trials 3 and 4 (DYM_T3 and DYM_T4), and #106 in trial 5 (#106_T5) were able to cause substantial substrate decline as measured by RP-HPLC in 72 h. Coincidentally, these samples also had high antimicrobial activity in sausage trials and were able to cause a 5 log CFU/g reduction of *E. coli* O157:H7 within 24 d of ripening. In contrast, the remaining two samples, which included commercially deodorized yellow powder in trial 4 (CDP_T4) and yellow mustard in trial 3 (YM_T3), showed consistent levels of

substrate throughout the measurement. Moreover, the antimicrobial activity of these two mustards was weak; requiring 42 d of ripening to reduce the pathogen to > 5 log CFU/g. This result showed that the laboratory method for mustard deodorization (autoclave at 115^{o}C for 15 min, 2 cm thick layer) was not adequate to consistently eliminate the endogenous mustard myrosinase and that the earlier tests used did not detect residual myrosinase in deodorized mustard. It is worth noting that the residual myrosinase initially caused a linear substrate decline which tended to slow as the measurement period was extended beyond 48 h. (Figure 4-6). Several studies have reported myrosinase activity follows first order kinetics, meaning the enzyme activity is dependent upon substrate concentration (Palmieri et al. 1982, Ludikhuyze et al. 1999, Van Eylen et al. 2007). Moisture content, which normally has an important influence on thermal efficiency, seemed unrelated to the inactivation of myrosinase during deodorization. All mustard samples had low moisture content (< 8 %, w/w) and significant residual myrosinase was observed present in mustards with moisture levels at both the high and low extremes of those observed (Table 4-2).

To better explore the residual myrosinase after mustard deodorization, enzyme activity and protein concentration from both hot and deodorized mustards were quantified and compared as described previously. The linear substrate decline rates were converted to myrosinase activity (units) using myrosinase calibration curves (Figure 3-9). Protein in the myrosinase extract was quantified and myrosinase activity was expressed as units/mg protein. The result showed myrosinase activity from hot mustard ranged from 5 to 10

units/mg protein. In deodorized mustards that showed positive residual myrosinase, the enzyme activity was reduced 10 to 20-fold and ranged from 0.2 to 1 units/mg protein (Table 4-1). The highest and lowest residual myrosinase activities were present in yellow mustard (YM) in trial 4 and #201 in trial 5, respectively. However, when the antimicrobial activity of these two treatments was compared, yellow mustard in trial 4 (Figure 4-4) and #201 in trial 5 (Figure 4-5) showed almost the same high levels. This finding partially indicated that even low residual myrosinase activity seemed to improve the antimicrobial activity of deodorized mustard.

Enzyme reactivation is rare after thermal inactivation and peroxidase, so far, is the only enzyme from *Brassica* plants where activity was restored after thermal treatment (Lu and Whitaker 1974; Thongsook and Barrett 2005). There is no data available that shows whether myrosinase is able to be reactivated after thermal treatment. Nonetheless, this did not seem to be the reason for differences in the effectiveness of *E. coli* O157:H7 elimination from dry sausages. Myrosinase assays were conducted after 5 sausage trials and residual myrosinase was only found in those mustard samples which previously showed high antimicrobial activity in fermented sausages.

4.5. Conclusion

The present work confirmed that use of deodorized mustards in dry fermented meat manufacture is valuable for reducing the viability of *E .coli* O157:H7 in those products. Results suggested the mustard deodorization method used (115^{o}C for 15 min, in a 2 cm

thick layer) did not consistently eliminate residual myrosinase. Subsequently, this residual myrosinase significantly improved the antimicrobial activity of deodorized mustards.

Deerfield and Dougherty (1971) suggested commercial mustard products can be further processed by saturated steam with different durations yielding prepared mustards with controlled formation of isothiocyanate, generated by different levels of residual myrosinase. The laboratory mustard deodorization method used seemed to produce deodorized mustard products with such character. Further studies are needed to better understand how important the role of residual myrosinase is on the antimicrobial activity of mustard in dry fermented meats. It is necessary to modify the deodorization method so that deodorized mustards for testing do not contain residual myrosinase, and such fully deodorized mustard should be compared with mustards containing residual myrosinase for their antimicrobial activity in sausage against *E .coli* O157:H7.

Table 4-1: Myrosinase activity quantified by RP-HPLC myrosinase assay from hot and deodorized mustards used in sausage trials 3 – 5.

Mustard sample	Myrosinase activity (units/mg protein)			
	Hot mustard	Deodorized mustard used in sausage		
		Trial 3	Trial 4	Trial 5
CDP	ND	-	-	ND
DYM	9.51±0.06	0.48±0.08	0.57±0.11	ND
YM	10.24±0.31	-	1.02±0.14	ND
DOM	5.72±0.82	-	0.28±0.05	ND
#106	6.86±0.98	ND	ND	0.41±0.11
#201	4.26±1.15	ND	ND	0.23±0.07

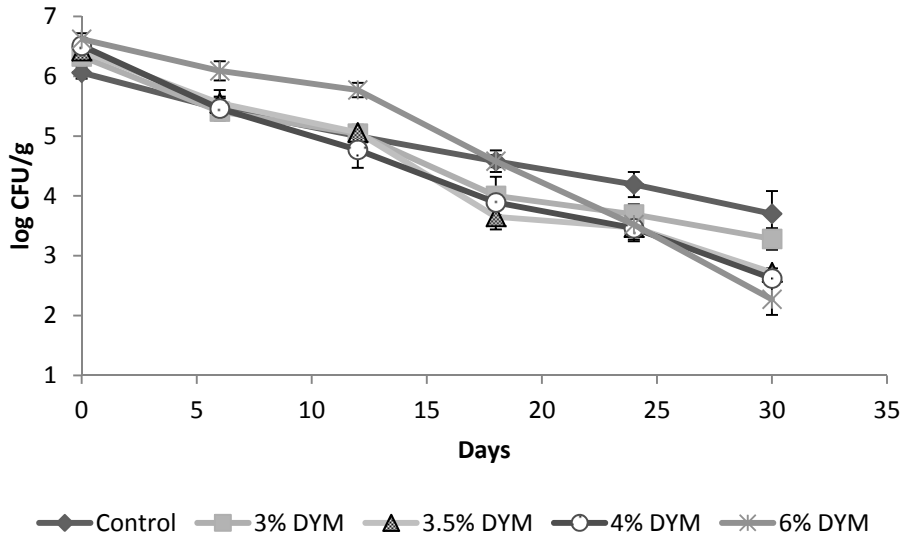
- Not detectable.
Values are means of triplicate analyses (mean ± SD, n=3).
Myrosinase extracted from yellow and oriental mustards were tested against relevant crude sinalbin and sinigrin substrate, respectively.

Table 4-2: Moisture content (%, w/w) in different mustard samples.

Deheated YM	YM	Deoiled YM	Deoiled OM	#106	#201	#107	#202
3.43 ± 0.07^{d}	4.94 ± 0.29^{c}	6.37 ± 0.49^{ab}	6.13 ± 0.17^{b}	5.26 ± 0.17^{c}	3.72 ± 0.48^{d}	4.06 ± 0.09^{d}	7.09 ± 0.15^{a}

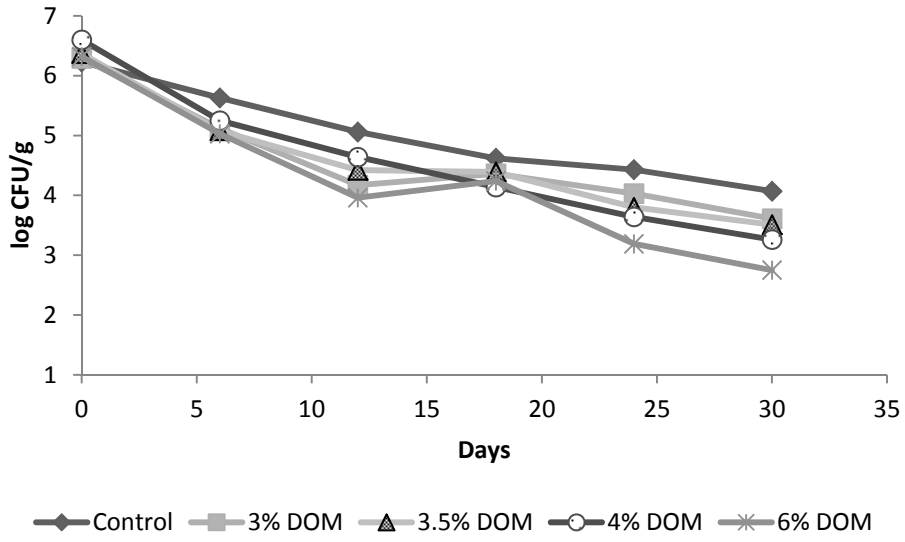
Values in each cell represent mean ± SD (n=3).
Different letters indicate a significant difference ($p < 0.05$) within the same row.

Figure 4-1 : *E. coli* O157:H7 reduction in dry fermented sausage caused by different concentrations of deoiled yellow mustard (deodorized)-trial 1.



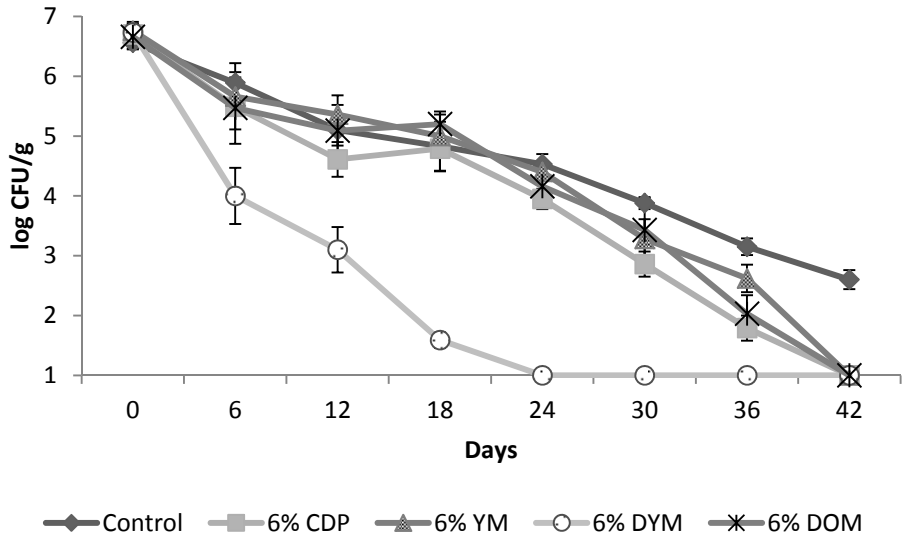
Values are means of two trials replicated 3 times (mean ± SD, n=6).

Figure 4-2: *E. coli* O157:H7 reduction in dry fermented sausage caused by different concentrations of deoiled oriental mustard (deodorized)-trial 2.



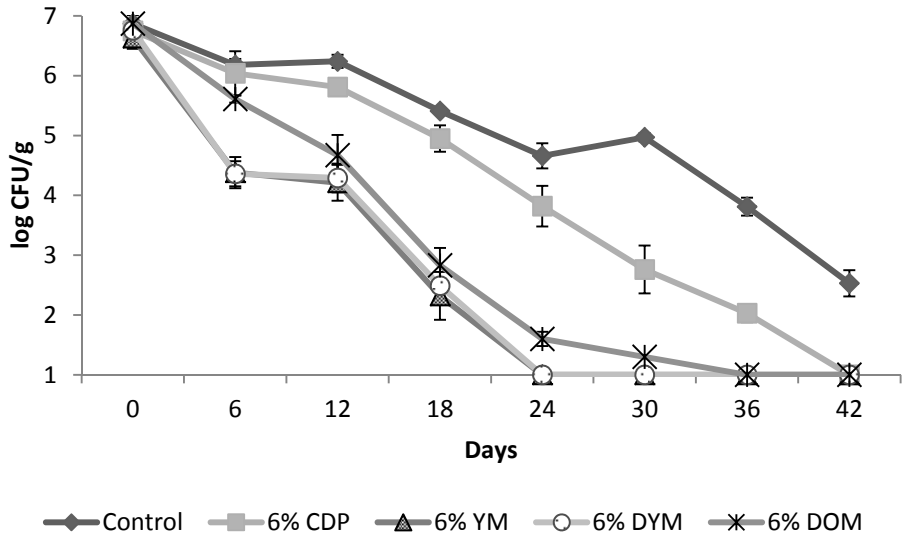
Values are means of two trials replicated 3 times (mean ± SD, n=6).

Figure 4-3: *E. coli* O157:H7 reduction in dry fermented sausage caused by 4 types of deodorized mustards at 6 % (w/w)-trial 3.



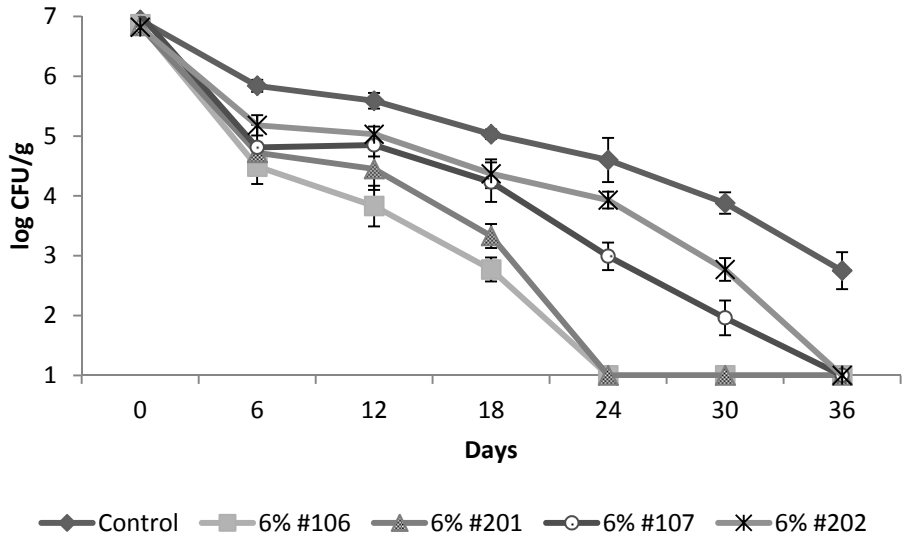
Values are means of two trials replicated 3 times (mean ± SD, n=6).
When *E. coli* O157:H7 numbers were lower than the detection limit, recovery of the pathogen by enrichment and immunomagnetic separation was positive.

Figure 4-4: *E. coli* O157:H7 reduction in dry fermented sausage caused by 4 types of deodorized mustards at 6 % (w/w)-trial 4.



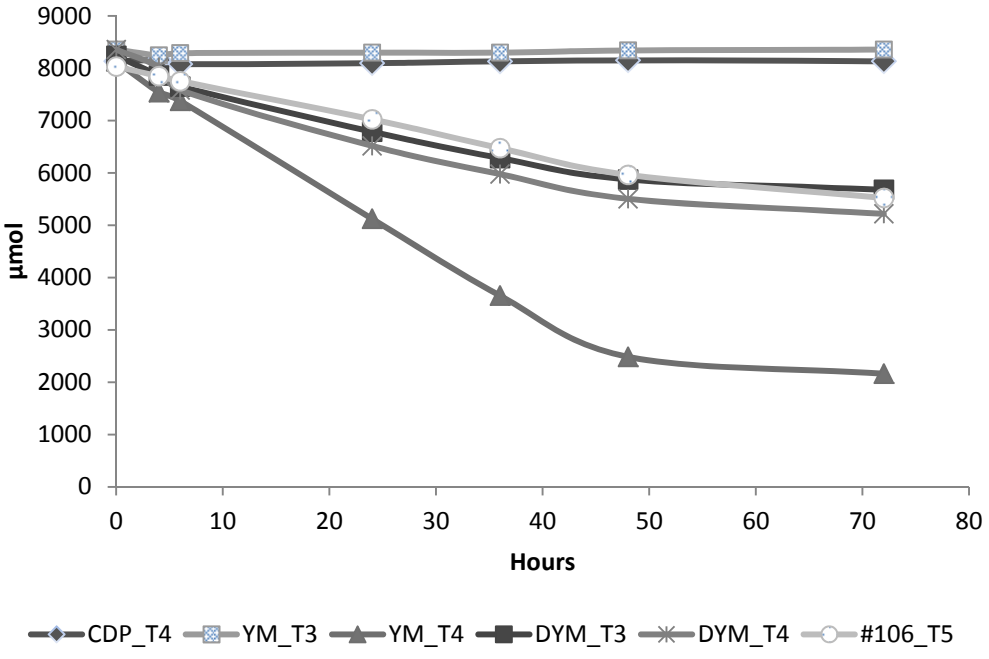
Values are means of two trials replicated 3 times (mean ± SD, n=6).
When *E. coli* O157:H7 numbers were lower than the detection limit, recovery of the pathogen by enrichment and immunomagnetic separation was positive.

Figure 4-5: *E. coli* O157: H7 reduction in dry fermented sausage caused by 4 types of deodorized mustards at 6 % (w/w)-trial 5.



Values are means of two trials replicated 3 times (mean ± SD, n=6).
When *E. coli* O157:H7 numbers were lower than the detection limit, recovery of the pathogen by enrichment and immunomagnetic separation was positive.

Figure 4-6: Substrate decline (glucosinolate degradation) caused by residual myrosinase extracted from deodorized mustards used for dry fermented sausage trials at ambient temperature (22^{o}C).



CHAPTER 5

5. THE CONTRIBUTION OF RESIDUAL MYROSINASE IN DEODORIZED MUSTARDS TO THE INHIBITION OF *E. COLI* O157:H7 IN DRY FERMENTED SAUSAGE

5.1. Abstract

The present work explored the contribution made by residual myrosinase to the overall antimicrobial activity of deodorized mustard against *E. coli* O15:H7 in dry fermented sausage. Fully deodorized mustards and mustard mixtures with known increments of myrosinase activity were tested in two sausage trials. Trial 6 used 4 types of 6 % (w/w) yellow mustards in sausage manufacture which showed high antimicrobial activity in previous work, but here mustards were fully deodorized using the same method, but with reduced sample thickness (≈ 1 cm)[1]. In trial 7, with both yellow and oriental mustards, mixtures with small but known increments of myrosinase activity were made by the addition of 2.5 % and 5 % (w/w) hot mustards to fully deodorized mustards. The mixtures were then used at 4 % (w/w) mustard to manufacture sausages, containing either 0.1 % or 0.2 % (w/w) hot mustard in meat batter[2]. Additionally, partially

[1] **Fully deodorized (F-d) mustard:** autoclaved at 115°C for 15 min while the sample thickness was 1 cm and the complete inactivation of plant myrosinase was verified by the RP-HPLC myrosinase assay. The appearance of the sample after autoclave treatment was dark brown.

[2] **2.5 % mustard mixture:** 2.5 % (w/w) hot mustard + 97.5 % fully deodorized mustard (at 4 % mustard, 0.1 % hot mustard was used in sausages).

5 % mustard mixture: 5 % (w/w) hot mustard + 95 % fully deodorized mustard (at 4 % mustard, 0.2 % hot mustard was used in sausages).

deodorized yellow and oriental mustards[3] were prepared by the same method but with increased thickness (≈ 2 cm). It was found that 6 % fully deodorized mustards in trial 6 failed to inhibit *E. coli* O15:H7 as effectively as previously shown, and only one of the 4 mustard treatments caused a 5 log CFU/g reduction of *E. coli* O15:H7 within 42 d of ripening. In contrast, at 4 % (w/w) in trial 7, the two yellow mustard mixtures and the partially deodorized yellow mustard reduced viable bacteria to below the detection limit by 18 and 24 d of ripening, respectively. Results suggested that yellow mustard containing plant myrosinase activity contributed significantly to the elimination of *E. coli* O15:H7 from dry fermented sausage. Interestingly, at the same levels oriental mustard treatments with similar hot and deodorized proportions did not suitably inhibit the pathogen by the end of ripening. Yellow mustards contained significant higher total phenolic compounds than oriental mustards, which may have contributed to the overall *E. coli* O15:H7 reduction. Additionally, sinigrin in oriental mustard was present at lower concentrations than sinalbin in yellow mustard, which meant there was a difference in glucosinolate concentration in sausages made with the same amounts of yellow or oriental mustards. Because myrosinase action obeys first order kinetics, the lower substrate concentrations probably also affected glucosinolate hydrolysis and subsequent isothiocyanate formation. It is also not clear if ρ-hydroxybenzyl isothiocyanate (ρHBIT) from sinalbin hydrolysis is consistently more antimicrobial than allyl isothiocyanate

[3] **Partially deodorized (P-d) mustard:** autoclaved at 115°C for 15 min while the sample thickness was ≈ 2 cm, and the presence of residual plant myrosinase was verified by the RP-HPLC myrosinase assay. The appearance of the sample after autoclave treatment was yellowish.

(AITC) from sinigrin. Consequently, further study to verify those uncertainties is necessary.

5.2. Introduction

The ability of *Escherichia coli* O157:H7 to survive in traditionally uncooked dry fermented sausage has been reported and has led to both illness outbreaks and sporadic cases (CDC 1990, Tilden et al. 1997). Regulations have been implemented in both Canada and the United States that require fermented dry sausage manufacturing methods to be capable of causing ≥ 5 log CFU/g reduction of *E. coli* O157:H7 (Reed 1995, Health Canada 2000).

Several studies have reported the use of 4 % and 6 % (w/w) deodorized yellow mustard as an ingredient in dry fermented sausage to be capable of causing > 5 log reduction of *E. coli* O157:H7. The antimicrobial mechanism responsible was the production of bio-functional isothiocyanates after glucosinolate hydrolysis in the mustards. However, the source of myrosinase activity that caused early and substantial glucosinolate hydrolysis to yield isothiocyanate seemed unclear, because the rates of *E. coli* O157:H7 elimination from sausage were inconsistent. Graumann and Holley (2008) initially reported that 6 % commercially deodorized[4] yellow mustard caused a 5 log reduction of *E. coli* O157:H7 in dry fermented sausage at 18 d of ripening. Luciano et al. (2011) used 6 % deodorized (autoclaved at 115^{o}C for 15 min; with no apparent plant myrosinase) and 6 % of half hot/half deodorized yellow mustard which eliminated the

[4] **Commercially deodorized mustard:** thermally treated (deodorized) by the supplier, method unknown

pathogen by 18 d of sausage ripening. Because the endogenous myrosinase was perceived as inactivated in deodorized mustard, it was believed glucosinolate hydrolysis was caused by *E. coli* O157:H7 and the starter cultures themselves which possessed bacterial myrosinase-like activity. Nevertheless, Luciano et al. (2011) also reported that in treatments where commercially deodorized yellow mustard was used, 38 d was required to reduce *E. coli* O157:H7 viability by 5 log CFU/g, and the delayed lethality of this treatment raised uncertainty. The authors explained this unexpected result by suggesting that autoclave treatment increased the release of phenolic compounds which enhanced the overall antimicrobial activity of the deodorized mustard. Cordeiro et al. (2013) tried to reduce the concentration of mustard in sausage necessary to satisfy the mandatory 5 log CFU/g reduction of *E. coli* O157:H7 and reported that 4 % yellow mustard deodorized by autoclave treatment as used previously, took 28 d to achieve a 5 log reduction goal, but a 2 % hot / 2 % deodorized treatment failed to reduce the pathogen > 5 log CFU/g, causing only a 4.4 log CFU/g reduction by 42 d. This was unexpected because Luciano et al. (2011) found this mixed (hot / deodorized) mustard had greater antimicrobial effects than deodorized yellow mustard.

In the preliminary experiments during the current study (Chapter 4), it was suspected that endogenous myrosinase may not have been completely inactivated during autoclave treatment (115^{o}C, 15 min) when the thickness of the mustard layer was approximately 2 cm. As a result, partially deodorized mustard containing some myrosinase activity may have been occasionally produced and contributed to its high antimicrobial activity against

E. coli O157:H7 within 24 d of ripening in dry fermented sausage. In contrast, it was possible that when mustards were fully deodorized, it resulted in weak *E. coli* O157:H7 inhibition and might explain the requirement for 36 d to 42 d of ripening to yield a 5 log CFU/g reduction.

The present work was undertaken to determine the antimicrobial contribution made by residual and small amounts of myrosinase activity in deodorized mustard and mustard mixtures, respectively. To achieve the goal two sausage trials were done. In trial 6, 4 types of yellow mustards chosen from previous work which showed significant antimicrobial activity, were verified as being fully deodorized and used in sausage batters. In trial 7, the mustard concentration in sausage was reduced to 4 % (w/w) because in the preliminary tests deodorized mustards with residual myrosinase activity showed significant antimicrobial activity against *E. coli* O157:H7. Treatments in this trial included fully deodorized yellow and oriental mustards mixed with identical types of hot mustards, yielding mixtures with small but known amounts of myrosinase activity. For comparison partially deodorized mustards produced by autoclave treatment in a ≈ 2 cm thick layer were also tested, to determine whether the thickness of the mustard layer during autoclave treatment was responsible for inconsistent reduction in *E. coli* O157:H7 viability noted in previous studies. The RP-HPLC myrosinase assay (Chapter 3) was used to verify the complete inactivation or the presence of plant myrosinase in each treatment. The phenolic content of mustard samples before and after deodorization was also measured since it was suspected that thermal treatment might increase their concentration in mustards (Luciano

et al. 2011) and subsequently contribute to the overall inhibitory effect on *E. coli* O157:H7.

5.3. Materials and methods

5.3.1. Chemicals

Sinigrin hydrate, myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147), protein standard (albumin from bovine serum, EC 231-791-2) and Bradford reagent (protein dye) for protein determination were from Sigma-Aldrich (St. Louis, MO, USA). Sinalbin hydrate was from AppliChem Inc. (St. Louis, MO, USA). HPLC grade acetonitrile and ACS grade ammonium sulphate, reagent grade mercaptoethanol, Folin & Ciocalteu's phenol reagent and spectrophotometric grade methanol were from Fisher Scientific (Whitby, ON, Canada). Tetrabutylammonium hydrogen sulphate (TBA) was from J.T. Baker (Phillipsburg, NJ, USA). Ingredients and curing agents for dry fermented sausage manufacture were from Canada Compound Western Corp. (Winnipeg, MB, Canada).

5.3.2. Mustard samples and the preparation of mustard treatments

In trial 6, the 4 yellow mustard samples used included yellow mustard powder (YM) from Viterra (Minneapolis, MN, USA), deoiled yellow mustard cake (DYM) supplied by Sakai spice (Lethbridge, AB, Canada); mustard flour (#106) and powder (#201) supplied by G.S. Dunn (Hamilton, ON, Canada). In trial 7, yellow (#106) and oriental (#107F) mustard flours from G.S. Dunn were selected for the study.

In trial 6, mustard samples were deodorized using a reduced sample layer of 1 cm thickness during autoclave treatment at 115^{o}C for 15 min which yielded fully deodorized

mustards. In trial 7, these fully deodorized mustards were combined with either 2.5 % or 5 % (w/w) untreated hot mustards yielding mixtures with known amounts of myrosinase activity. Partially deodorized mustards were prepared in the same manner but a 2 cm thick layer of sample was used. The different mustard mixtures were added to sausages at a final level of 4 % (w/w). Thus for 2.5 % and 5 % mustard mixtures, 0.1 % and 0.2 % of hot mustards were present in the meat batters (w/w), respectively. Partially deodorized yellow and oriental mustards at 4 % (w/w) were also used as two independent treatments. For mustard treatments, the absence or presence of myrosinase activity was confirmed by the RP-HPLC myrosinase assay before their use in sausage manufacture.

5.3.3. Preparation of *E. coli* O157:H7 and starter cultures *S. carnosus* UM109M and *P. pentosaceus* UM116P

Bacterial strains used and their preparation were described by Luciano et al. (2011) with adaptation. Briefly, 5 strains of *E. coli* O157:H7 (00: 3581, 02: 0304, 02: 0627, 02: 0628 and non-motile 02:1840) and *Staphylococcus carnosus* UM109M were cultured overnight twice in 10 ml Brain Heart Infusion (BHI) broth at 37^{o}C, then transferred and incubated in 500 ml BHI before use. *Pediococcus pentosaceus* UM116P was incubated at 37^{o}C but in deMan Rogosa Sharpe (MRS) broth. Bacterial cultures were centrifuged (Avanti® J-26 XP, Beckman Coulter Inc, Mississauga, ON, Canada) twice at 4225 xg for 20 min at 4^{o}C and washed with 0.1 % peptone water before being added to the meat batter.

5.3.4. Dry fermented sausage manufacture

Methods for formulation and manufacture of dry fermented sausages were adopted from Luciano et al. (2011) with adjustments. Glucose content was reduced from 0.6 % to 0.4 % (w/w). The relative humidity of the smoke house (AFR-Fishmaster, Rauch und Warmetchnik GmbH, Reichenau, Germany) during the drying stage was increased from 75 % to 85 % because adjustments made recently in the fan housing increased airflow over the salami causing them to dry faster than previously observed.

5.3.5. Microbial, pH and a_w analyses during sausage ripening

Duplicate samples of each treatment (two sausages from each treatment) were taken at day 0 and subsequently at 6 d intervals 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).

5.3.6. Standard operating procedure (SOP) for plate count and the calculation of means

Standard operating procedures for plate counts and determination of the means as recommended by the American Society for Testing and Materials (ASTM 1998) were followed. During sausage ripening, the viable number of *E. coli* O157:H7 cells decreased and unavoidably become lower than the countable range. To minimize underestimation and maximize accuracy, plates with colony numbers outside the countable range (20-200/plate) not obtained from the lowest dilution (1:10) and greatest deposited volume

(500 µl) were discarded and excluded from mean calculations. However, when the lowest dilution and greatest deposited volume were used, if the result was obtained from outside the countable range, it was reported as an estimated count. The bacterial detection limit/meat sample was 1.3 log CFU/g for the greatest sample concentration in a dilution and deposited volume when the spiral plating system (Advanced Instruments, Inc., Norwood, MA, USA) was used. The detection limit used in the present work was based on the sum of colonies on duplicate plates (500 µl deposited, 1 ml in total) from the most concentrated sample dilution. Since a 1 ml sample was used, the detection limit was reduced to ≤ 1 log CFU/g.

5.3.7. Confirmation of myrosinase activity from mustard samples by the RP-HPLC myrosinase assay

The methods for myrosinase extraction from mustard samples and the RP-HPLC myrosinase assay used were as previously descried (Chapters 3 and 4). Plant myrosinase in sample mixtures was inactivated by immersion in a boiling water bath for 1 min before analysis. A negative control without the addition of myrosinase extract was included to examine the stability of the substrate after boiling water bath treatment. The presence of myrosinase activity was reflected by continuous substrate decline at several intervals, whereas the absence of myrosinase was confirmed by no change in substrate concentration. Samples were clarified before RP-HPLC by passage through a 0.22 µm PES syringe filter (VWR Co., Toronto, ON, Canada) fitted to a 1 ml syringe (Becton Dickson, Franklin Lakes, NJ, USA).

5.3.8. Determination of total phenolic content (TPC) in mustards

Yellow and oriental mustard samples were examined before and after autoclave treatment (115^{o}C for 15 min, in a 2 cm thick layer) to explore the variation of total phenolic content (TPC) in mustard varieties, as well as mustard samples from the same variety, and examine the effect of autoclave treatment on the changes in total phenolic content. Mustard samples of 5 g were mixed with 30 ml 99 % methanol in a screw-capped tube. The mixture was agitated using a rotary shaker (G-33, New Brunswick Scientific Co., Inc, New Brunswick, NJ, USA) operated at 300 rpm for 3 h at ambient temperature. The mixture was held for 1 h and 1 ml of the supernatant was transferred to 9 ml methanol. The TPC of samples was evaluated using the Folin & Ciocalteu phenol assay adapted from Gutfinger (1981). One ml sample mixture, gallic acid standard solution or blank water solution was transferred to 5 ml freshly prepared Folin & Ciocalteu reagent diluted 1:10 with distilled water. Four ml 0.5 M sodium carbonate solution at 50^{o}C was added and 5 min later the volume was made to 10 ml. Treated samples were held in the dark for 1 h and passed through a 0.2 µm PES syringe filter before transfer to a 1 cm cuvette for absorbance measurement at 765 nm (Ultrospec 1100 pro spectrophotometer, Biochrom Ltd, Holliston, MA, USA). Duplicate results from each sample were determined by reference to the standard curve established with the gallic acid standard (mg gallic acid/g mustard, where $y = 0.0088x - 0.2459, R^2 = 0.9938$).

5.3.9. Statistical analysis

The statistical significance of mean differences at $\alpha = 0.05$ were assessed by analysis of variance using JMP 10.00 (SAS Institute Inc., Cary, NC, USA), and statistical differences among treatments were compared using Tukey's test.

5.4. Results and discussion

5.4.1. Verification of the absence or presence of active myrosinase in mustard treatments used in trials 6 and 7

Before the sausage trials, mustard samples were freshly prepared and their myrosinase status verified. The absence or presence of myrosinase activity in mustard treatments were confirmed by changes of glucosinolate concentration in the substrate during 120 h measurement (after myrosinase had been extracted from each mustard sample and added to the substrate). The method used for confirmation of myrosinase activity was the RP-HPLC myrosinase assay as noted in Chapter 3. No change of substrate concentration in the negative control (without myrosinase extract addition) was used to indicate substrate stability after the 1 min boiling water bath treatment. With 4 fully deodorized yellow mustard treatments used in trial 6, there was no residual myrosinase activity after deodorization, which was reflected by a consistent concentration of substrate during incubation (Figure 5-1). In contrast, with mixed (hot/mixed) mustard treatments used for sausages in trial 7, enzyme activity was present as indicated by a decline in substrate levels over 120 h. The intensity of myrosinase activity among mixed mustard samples of both types (yellow and oriental) paralled the decrease in proportion of

hot mustard in the mixture, that is, 5 % contained more myrosinase than 2.5 %. The partially deodorized yellow and oriental mustards also showed a measureable substrate decline (Figure 5-2). In comparison to fully deodorized mustards (Figure 5-1), sample thickness was most likely the key factor affecting the inactivation of plant endogenous myrosinase when deodorization was done by autoclave treatment at 115^{o}C for 15 min. Fully deodorized mustard with completely inactivated myrosinase was obtained consistently when a 1 cm thick layer of powder or flour was used; whereas partially deodorized mustard with measureable myrosinase activity was produced when the thickness of the mustard layer was about 2 cm. It was also worth mentioning that mustards autoclaved in 2 cm layer had a light yellowish appearance, whereas those autoclaved in a thinner (1 cm) layer were dark brown in color. It is unlikely that differences in moisture content were responsible for variation in myrosinase activity because all mustard samples had relatively low moisture (< 8 %, w/w), however, low moisture may have contributed to myrosinase thermal stability.

Further, it was interesting that with the same amount of hot mustard in the mixed samples, yellow mustard mixtures showed greater myrosinase activity and ability to hydrolyze substrate than oriental mixtures. This may have been related to differences in enzyme efficiency because of different amounts of sinalbin in yellow and sinigrin in oriental mustards. Substrate declines caused by myrosinase in all samples were linear at the beginning and tended to become more gradual, thus glucosinolate was still recovered at the end of the trial. This result was consistent with reports that myrosinase activity

seemed to follow first order kinetics (Van Eylen et al. 2007, Ghawi et al. 2012). Thus, enzyme activity was reduced over time as the substrate concentration was reduced. As a result, it was possible that earlier measurements used to detect residual myrosinase activity (Luciano et al. 2011, Nilson and Holley 2012) were not sensitive enough to take this feature into consideration.

5.4.2. Antimicrobial activity of different mustard treatments against *E. coli* O157:H7 during sausage ripening

After confirmation by the RP-HPLC myrosinase assay, it was clear that endogenous myrosinase was absent from the 4 types of fully deodorized yellow mustards used in sausage trial 6. Interestingly, when the method of Luciano et al. (2011) was used to deodorize these 4 mustards, each showed considerable antimicrobial activity and reduced the number of *E. coli* O157:H7 ≥ 5 log CFU/g within 24 d in the preliminary work (Chapter 4). However, after being deodorized in a thin layer (1 cm) and similarly used in sausage, the same mustards did not show satisfactory antimicrobial activity. At 42 d ripening, treatments DYM and #106 caused a 5 log CFU/g reduction of *E. coli* O157:H7. Nonetheless, only in #106 was there no viable (< 1 log CFU/g) *E. coli* O157:H7 recovered on CT-SMAC agar, but the sample was positive after enrichment and immunomagnetic separation (Table 5-1). Treatments YM and #201 were not significantly different or less antimicrobial than the control, respectively.

From a re-examination of samples used in earlier trials (3-6) it appears that enzyme re-activation during storage of deodorized mustards was less likely an explanation for

residual myrosinase in the samples than the partial inactivation of myrosinase during the initial deodorization treatment.

In trial 7 when 0.1 % and 0.2 % hot yellow or oriental mustard were used, no significant differences in the rates of *E. coli* O157:H7 elimination were noted. Since the pathogen reduction target was achieved with yellow mustard treatments, a lower proportion of hot yellow mustard in the mixture might be successfully used for *E. coli* O157:H7 control.

Results from trial 7 also showed yellow mustard #106 caused a 5 log reduction of *E. coli* O157:H7 within 18 d in treatments where 0.1 % hot (2.5 % mixture) and 0.2 % hot (5 % mixture) yellow mustard were added to the sausages. Similar results were obtained with partially deodorized yellow mustard sample #106. After 24 d *E. coli* O157:H7 was not detectable in any of these three yellow mustard treatments (< 1 log CFU/g). Again, *E. coli* O157:H7 was confirmed present following enrichment and immunomagnetic separation.

In contrast, with the same autoclave treatment and proportions of hot mustard in mixtures, the antimicrobial activity of oriental mustard (#107F) treatments was weak and failed to cause a 5 log reduction even at a 36 d of ripening (Table 5-2). In the preliminary study, while residual myrosinase was present in deodorized mustards when used at 6 % (w/w) in sausage formulations, yellow mustards seemed to have better antimicrobial activity then oriental mustards. However, it was unexpected that when used at 4 % (w/w),

oriental mustard mixtures as well as the partially deodorized treatment would fail to effectively control the bacteria.

Several different features between yellow and oriental mustards may have caused this outcome. Yellow mustards showed higher myrosinase activity than oriental samples (Figure 5-2). Theoretically, yellow mustard treatments were able to produce more ρHBIT and thus were better able to inhibit the pathogen. It is also notable that the sinalbin content in yellow mustards was substantially higher than the sinigrin content in oriental mustards (Table 3-2). This difference may have been another factor which affected the glucosinolate conversion rate. Since myrosinase activity follows first order kinetics and the same amount of mustards were added to sausages, the glucosinolate concentration in yellow mustard treated sausages would have been higher than in sausages containing oriental mustard. This would have enabled faster substrate hydrolysis in the presence of an adequate enzyme concentration. AITC derived from sinigrin hydrolysis, has been widely reported to be an effective antimicrobial compound (Fujita et al. 1999, Suppakul et al. 2003, Nadarajah et al. 2005). Consequently, the use of its precursor, oriental mustard containing sinigrin, should have been antimicrobial once suitable levels of myrosinase activity and substrate concentration were present to produce enough AITC. It is possible that 4 % (w/w) oriental mustard did not provide sufficient substrate to enable the AITC produced to reach threshold levels adequate to eliminate *E. coli* O157:H7 from the sausages.

It is generally acknowledged that isothiocyanates are volatile (Bartlet et al. 1993, Meija et al. 2002) and unstable in aqueous media and food matrices (Bailey et al. 1961, Lewis and Papavizas 1971). Since isothiocyanates can affect the sensory properties of food, the antimicrobial potency must be high enough that sensory effects are not evident. To address this issue, AITC was microencapsulated (Chacon et al. 2006), or incorporated in a film (Li et al. 2012), enclosed in a sachet (Seo et al. 2012), and then introduced into food packages to eliminate foodborne pathogens. These approaches reduced the undesirable organoleptic effects of the isothiocyanates by controlling their release into food packages. More importantly, the control of AITC release can promote its antimicrobial activity by reducing its volatility (Plackett et al. 2007, Vega-Lugo and Lim 2009). As a result, it is possible that there may be similar benefits in terms of antimicrobial efficacy in sausage by the gradual generation of isothiocyanates from glucosinolates in mustard powders or flours, in which low levels of plant myrosinase are also present.

5.4.3. Changes of pH, a_w and changes in enumeration numbers of starter cultures

pH

In both trials 6 and 7, 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 (Table 5-3 and Table 5-4). This slight increase in pH did not occur in preliminary experiments when the amount of glucose used was 0.6 % (w/w). This adjustment was for the purpose of reducing available carbohydrate in the sausage formulation to stimulate bacterial

myrosinase-like activity and prevent sausage pH from reaching values lower than 5.0. Effects of glucose reduction on pH were evident in control sausages but the additional carbohydrate in mustard treatments facilitated reductions below pH 5.0 in those samples. Thus effects of glucose reduction on bacterial myrosinase-like activity could not be measured.

Water activity (a_w)

Water activity (a_w) was about 0.95 at the beginning of ripening and was reduced to ≤ 0.8 by the end of the trials (Table 5-5 and Table 5-6). For marketing purpose an a_w of about 0.8 is desirable and was achieved here within 30 d. Reduced air flow and better humidity control in the air conditioned drying room would have prevented further moisture loss, but was difficult to achieve when the number of sausages in the chamber became small toward the end of the tests. Sausage containing mustard better retained moisture and had significantly higher a_w values than controls, regardless of the variety or type of mustard used.

Starter cultures

P. pentosaceus produces lactic acid and lowers the pH, but also generates hydrogen peroxide which can cause the oxidation of fat as well as the formation of an undesirable green colour in meat. *S. carnosus* produces peroxidase which maintains a desirable red colour and nitrate reductase which reduces nitrate to nitrite, promoting microbial stability as well as maintaining the color.

In trial 6, the number of *P. pentosaceus* in controls was identical at day 0 and day 42 of ripening. However, sausages treated with fully deodorized mustards showed about a 1 log increase of *P. pentosaceus*, suggesting mustard may have contributed some readily utilizable nutrients that enhanced its growth. In trial 7, the effect of mustard addition on the numbers of *P. pentosaceus* was negligible (Table 5-7 and Table 5-8).

With *S. carnosus*, about a 0.5 log reduction was observed in the controls which was due to the acid sensitivity of this organism. The numbers of *S. carnosus* were not affected by the addition of fully deodorized mustards, except in treatment #106 which showed an additional 0.5 log reduction. It is interesting that treatment #106 in trial 6 also had the most substantial antimicrobial activity (Table 5-7). In trial 7, mustard addition caused a 1-2 log reduction of *S. carnosus* by 36 d ripening (Table 5-8). *S. carnosus* is not tolerant of low pH values, and so the results obtained from trial 6 and 7 were not unexpected. However, the addition of mustards containing myrosinase activity in sausage trial 7 seemed to affect the viability of *S. carnosus* to a greater extent than those treated with fully deodorized mustards in trial 6.

In trials 6 and 7, it seemed that high antimicrobial activity in mustards also affected the number of starter cultures to some extent. This was most evident with *S. carnosus* but some reduction in the final numbers of *P. pentosaceus* was noted in trial 7. These results are similar to those previously reported (Chacon et al. 2006, Graumann and Holley 2008, Luciano et al. 2011).

5.4.4. Total phenolic content (TPC) in mustard samples before and after autoclave treatment

Phenolic compounds are well-known antioxidant and antimicrobial compounds. Total phenolic content (TPC) was evaluated by using Gallic acid as standard in mustards before and after the deodorization treatment to study the effects of autoclave treatment on TPC recovery. Regardless of the autoclave treatment, the TPC was not significantly different among different types of samples of the same variety. However, the TPC in different mustard varieties (yellow and oriental) were significantly different, with yellow mustards containing higher TPC than oriental mustards. Autoclave treatment did not have a significant effect on TPC in mustards (Figure 5-3). Luciano et al. (2011) explained the difference in antimicrobial activity between commercially deodorized and autoclave deodorized mustards by suggesting that autoclave treatment facilitated the release of phenolic compounds and thus mustard samples treated with the autoclave had stronger overall bactericidal properties. However, results from the present work did not find that autoclave treatment caused a significant difference in TPC.

5.5. Conclusion

The use of autoclave treatment (115^{o}C, 15 min) seemed inadequate for the dependable inactivation of plant myrosinase in mustards when the sample thickness was about 2 cm. The thicker layer appeared to prevent thorough penetration of steam into the sample and enabled retention of some myrosinase activity. This residual enzyme contributed to the high antimicrobial activity in sausage against *E. coli* O157:H7 through

its hydrolysis of glucosinolates. This effect was sporadic and caused unpredictable antimicrobial performance by mustards used to control *E. coli* O157:H7 in dry sausages. Autoclave equipment performance also contributed to the variable antimicrobial results because there were differences in treatment thermal cycles that were affected by variations in steam supply pressure and autoclave exhaust performance. When the layer of mustard powder or flour was reduced to 1 cm the problem appeared to be resolved without any negative effects, yielding fully deodorized mustard.

When 6 % (w/w) fully deodorized yellow mustards without myrosinase activity were used in trial 6, ≥ 42 d sausage ripening were needed to reduce *E. coli* O157:H7 ≥ 5 log CFU/g. Nevertheless, this showed that mustard without plant myrosinase was still antimicrobial. This was probably because bacterial myrosinase-like activity developed slowly, taking at least 12 d to cause bacterial inhibition (Herzallah et al. 2011).

In comparison, when deodorized yellow mustard contained low myrosinase activity such as in trial 7, it generally took 18-24 d of ripening to reduce the *E. coli* O157:H7 numbers below the detection limit (< 1 log CFU/g), even when 4 % (w/w) mustard was added to the sausages. However, a significant difference in bactericidal activity was observed between yellow and oriental mustard treatments where oriental mustard was less antimicrobial. This may have been because the oriental sample examined (#107F) was a variety with the lowest glucosinolate (sinigrin) concentration of those tested (Table 3-2). Additionally, it is possible, but not clear, whether AITC produced from sinigrin, is less antimicrobial than ρHBIT produced from sinalbin hydrolysis. Enzyme kinetics may also

be a factor since the rate of enzyme action influences the amount of antimicrobial isothiocyanate formed over a period of time. Myrosinase activity seemed to follow first order kinetics which means its activity was dependent on substrate concentration. Total phenolic compounds might be another factor contributing to the overall antimicrobial activity since yellow mustards contained significantly higher TPC than oriental mustards. However, TPC was most likely not a significant contributor because in trial 6, when there was no endogenous myrosinase, three of 4 yellow mustard treatments failed to achieve a 5 log CFU/g reduction of *E. coli* O157:H7 within 36 d. It is evident that residual or low endogenous myrosinase activity in mustards contributed significantly to the early and substantial reduction of *E. coli* O157:H7 viability during dry sausage ripening.

Table 5-1: *E. coli* O157:H7 viability during sausage ripening in trial 6 with 6 % (w/w) fully deodorized yellow mustards.

Day	Control	YM	DYM	#106	#201
0	6.02±0.07^{c}	6.02±0.06^{c}	6.22±0.08^{b}	6.31±0.12^{ab}	6.39±0.05^{a}
6	5.15±0.19^{b}	4.73±0.12^{d}	5.37±0.05^{a}	4.93±0.12^{c}	5.13±0.08^{b}
12	4.73±0.06^{bc}	4.86±0.06^{a}	4.68±0.09^{c}	4.38±0.13^{d}	4.82±0.07^{ab}
18	4.54±0.20^{a}	3.85±0.25^{bc}	3.71±0.88^{bc}	3.33±0.33^{c}	4.04±0.28^{ab}
24	4.35±0.28^{a}	4.03±0.38^{ab}	3.03±0.28^{c}	3.25±0.20^{c}	3.84±0.11^{b}
30	3.10±0.22^{a}	2.84±0.06^{a}	2.34±0.15^{b}	2.81±0.17^{a}	2.94±0.30^{a}
36	2.26±0.29^{bc*}	2.40±0.29^{ab}	1.66±0.39^{cd*}	1.53±0.50^{d*}	2.78±0.44^{a}
42	1.90±0.21^{b*}	2.31±0.21^{ab*}	1.10±0.63^{c*}	<1^{d+}	2.56±0.13^{a}

Values are the mean of two trials replicated three times (mean ± SD, n=6); different letters indicate a significant difference ($p < 0.05$) within the same row.

+ Indicates recovery of *E. coli* O157:H7 after enrichment and immunomagnetic separation.

* indicates data in that cell were estimated because colony numbers were out of the countable range (20-200 colonies/plate).

Table 5-2: *E. coli* O157:H7 viability during sausage ripening in trial 7 with 4 % (w/w) mustards containing myrosinase.

Day	Control	Mustard treatments					
		0.1 %[1]/#106	0.2 %[1]/#106	P-d[1]/#106	0.1 %/#107F	0.2 %/107F	P-d/107F
0	6.59±0.07^{b}	6.92±0.06^{a}	6.82±0.09^{ab}	6.70±0.20^{b}	6.61±0.20^{b}	6.72±0.03^{ab}	6.94±0.03^{a}
6	5.79±0.14^{a}	3.47±0.21^{c}*	3.49±0.08^{dc}*	3.69±0.24^{c}	5.09±0.25^{b}	4.95±0.22^{b}	5.30±0.25^{ab}
12	5.40±0.12^{a}	2.23±0.11^{e}*	2.61±0.01^{d}*	3.17±0.14^{c}	4.94±0.02^{b}	4.90±0.10^{b}	5.18±0.09^{a}
18	4.97±0.09^{a}	1.78±0.14^{e}*	1.51±0.20^{e}*	2.47±0.05^{d}	3.85±0.14^{c}	4.29±0.58^{bc}	4.78±0.04^{ab}
24	4.58±0.13^{a}	≤1^{d+}	≤1^{d+}	≤1^{d+}	3.44±0.14^{bc}	3.16±0.07^{c}	3.62±0.08^{b}
30	3.84±0.08^{a}	≤1^{c+}	≤1^{c+}	≤1^{c+}	3.07±0.44^{ab}	2.81±0.47^{b}	2.54±0.65^{b}*
36	3.41±0.17^{a}	≤1^{c+}	≤1^{c+}	≤1^{c+}	2.50±0.32^{b}*	2.14±0.82^{b}*	2.49±0.11^{b}*

Values are means of two trials replicated 3 times (mean ± SD, n=6); different letters indicate a significant difference ($p < 0.05$) within the same row.

+ indicates recovery of *E. coli* O157:H7 after enrichment and immunomagnetic separation.

* indicates data in that cell were estimated because colony numbers were out of countable range (20-200 colonies/plate).

[1] 0.1 % and 0.2 % represent the proportion of hot mustard in sausage. These were from mixtures of mustard containing 2.5 % and 5 % hot mustard, respectively; P-d = partially deodorized mustard.

Table 5-3: pH changes in sausage during ripening in trial 6.

Day	Control	YM	DYM	#106	#201
0	5.75±0.04^{b}	5.84±0.04^{a}	5.86±0.03^{a}	5.9±0.01^{a}	5.84±0.03^{a}
6	4.85±0.03^{a}	4.63±0.01^{b}	4.65±0.03^{b}	4.6±0.01^{b}	4.62±0.01^{b}
12	4.84±0.04^{a}	4.63±0.03^{b}	4.65±0.01^{b}	4.62±0.01^{b}	4.61±0.01^{b}
18	4.89±0.02^{a}	4.65±0.01^{b}	4.64±0.02^{b}	4.63±0.02^{b}	4.64±0.01^{b}
24	4.95±0.02^{a}	4.69±0.03^{b}	4.64±0.02^{c}	4.64±0.02^{c}	4.65±0.02^{c}
30	4.94±0.01^{a}	4.63±0.01^{b}	4.60±0.01^{bc}	4.57±0.01^{c}	4.61±0.02^{b}
36	5.06±0.02^{a}	4.67±0.05^{b}	4.66±0.03 ^{b}	4.66±0.01 ^{b}	4.69±0.06 ^{b}
42	5.07±0.03^{a}	4.75±0.04^{b}	4.75±0.03^{b}	4.70±0.02^{c}	4.73±0.02^{bc}

Values are means of two trials replicated twice (mean ± SD, n=4).
Different letters indicate a significant difference ($p < 0.05$) within the same row.

Table 5-4: pH changes in sausage during ripening in trial 7.

Day	Control	Mustard treatments					
		0.1 %[1]/#106	0.2 %[1]/#106	P-d[1]/#106	0.1 %/#107F	0.2 %/#107F	P-d/#107F
0	5.74±0.01^{a}	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±0.10^{a}	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^{bcd}	4.56±0.01^{cd}	4.54±0.01^{d}	4.61±0.02^{bc}
18	4.81±0.01^{a}	4.59±0.02^{bc}	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^{bc}	4.63±0.04^{bc}	4.61±0.01^{cd}	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±0.01^{d}	4.55±0.01^{d}	4.62±0.01^{b}
36	4.90±0.02^{a}	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 are means of two trials replicated twice (mean ± SD, n=4).
Different letters indicate a significant difference ($p < 0.05$) within the same row.
[1]0.1 % and 0.2 % represent the proportion of hot mustard in sausage. These were from mixtures of mustard containing 2.5 % and 5 % hot mustard, respectively; P-d = partially deodorized mustard.

Table 5-5: a_w changes in sausage during ripening in trial 6.

Day	Control	YM	DYM	#106	#201
0	0.954±0.002^{a}	0.949±0.002^{a}	0.949±0.003^{a}	0.949±0.001^{a}	0.948±0.001^{a}
6	0.921±0.005^{a}	0.926±0.003^{a}	0.925±0.002^{a}	0.919±0.003^{a}	0.916±0.001^{a}
12	0.897±0.004^{ab}	0.886±0.002^{b}	0.896±0.004^{ab}	0.898±0.005^{a}	0.896±0.004^{ab}
18	0.870±0.001^{a}	0.882±0.011^{a}	0.881±0.005^{a}	0.878±0.001^{a}	0.875±0.004^{a}
24	0.863±0.036^{a}	0.873±0.024^{a}	0.854±0.003^{a}	0.865±0.013^{a}	0.849±0.008^{a}
30	0.827±0.001^{d}	0.821±0.001^{d}	0.847±0.005^{b}	0.866±0.001^{a}	0.836±0.001^{c}
36	0.780±0.033^{c}	0.807±0.003^{bc}	0.805±0.013^{bc}	0.839±0.020^{a}	0.826±0.008^{ab}
42	0.742±0.012^{c}	0.807±0.001^{a}	0.806±0^{a}	0.777±0.011^{b}	0.787±0.009^{b}

Values are mean of two trials replicated twice (mean ± SD, n=4).

Different letters indicate a significant difference ($p < 0.05$) within the same row.

Table 5-6: a_w changes in sausage during ripening in trial 7.

Day	Control	Mustard treatments					
		0.1 %[1]/#106	0.2 %[1]/#106	P-d[1]/#106	0.1 %/#107F	0.2 %/#107F	P-d/107#F
0	0.945±0.001[a]	0.944±0[a]	0.943±0.001[a]	0.943±0[a]	0.944±0.001[a]	0.945±0.002[a]	0.946±0[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[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[a]	0.877±0.001[a]	0.884±0.002[a]	0.876±0.006[a]	0.873±0.006[a]
24	0.854±0.004[ab]	0.866±0.008[a]	0.856±0.002[ab]	0.851±0.006[ab]	0.858±0.001[ab]	0.841±0.003[b]	0.853±0.006[ab]
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±0.01[b]
36	0.801±0.004[d]	0.821±0.006[ab]	0.818±0.003[abc]	0.823±0.004[a]	0.808±0.005[bcd]	0.806±0[cd]	0.812±0.001[abcd]

Values are mean of two trials (mean ± SD, n=2).
Different letters indicate a significant difference ($p < 0.05$) within the same row.
[1] 0.1 % and 0.2 % represent the proportion of hot mustard in sausage. These were from mixtures of mustard containing 2.5 % and 5 % hot mustard, respectively; P-d = partially deodorized mustard.

Table 5-7: Changes in numbers of starter cultures during sausage ripening in trial 6.

Treatments	*Staphylococcus carnosus* UM109M		*Pediococcus pentosaceus* UM116P	
	Day 0	Day 42	Day 0	Day 42
Control	6.09±0.09^{ab}	5.51±0.31^{a}	7.66±0.15^{a}	7.85±0.69^{b}
YM	6.21±0.04^{a}	5.58±0.07^{a}	7.63±0.08^{a}	8.64±0.02^{a}
DYM	5.95±0.07^{c}	5.43±0.08^{a}	7.57±0.14^{a}	8.67±0.57^{a}
#106	6.06±0.06^{bc}	4.81±0.13^{b}	7.55±0.04^{a}	8.52±0.06^{a}
#201	6.17±0.06^{ab}	5.52±0.07^{a}	7.72±0.04^{a}	8.64±0.09^{a}

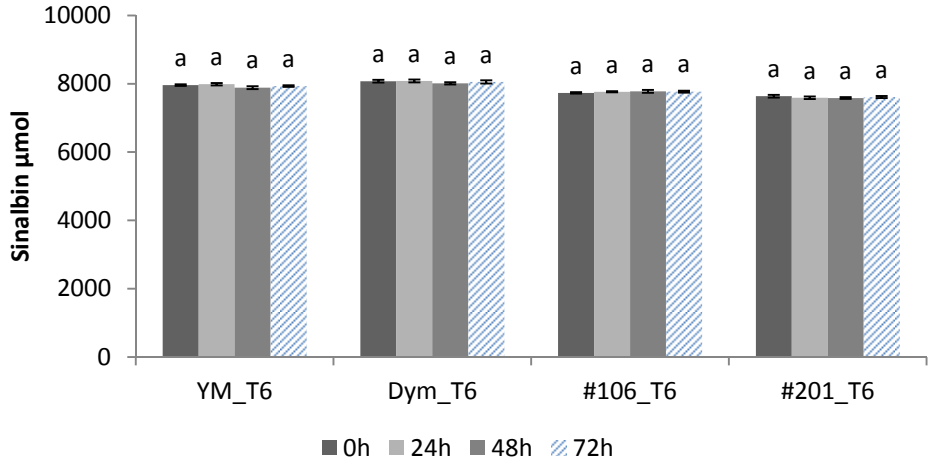
Values are mean of two trials replicated three times (mean ± SD, n=6).
Different letters indicate a significant difference ($p < 0.05$) within the same column.

Table 5-8: Changes in numbers of starter cultures during sausage ripening in trial 7.

Treatments	*S. carnosus* UM109M		*P. pentosaceus* UM116P	
	Day 0	Day 36	Day 0	Day 36
Control	6.22±0.04^{a}	5.22±0.11^{a}	7.78±0.07^{a}	8.05±0.16^{a}
0.1 %[1]/#106	5.79±0.07^{b}	3.11±0.16^{d}	7.70±0.09^{a}	7.17±0.07^{b}
0.2 %[1]/#106	5.91±0.04^{b}	3.16±0.11^{d}	7.47±0.09^{a}	7.08±0.07^{b}
P-d[1]/#106	5.88±0.06^{b}	3.67±0.08^{c}	7.67±0.06^{a}	7.50±0.40^{ab}
0.1 %/#107F	6.15±0.10^{a}	4.91±0.10^{ab}	7.73±0.02^{a}	7.66±0.19^{a}
0.2 %/#107F	6.25±0.06^{a}	4.69±0.16^{b}	7.65±0.05^{a}	7.48±0.35^{ab}
P-d/#107F	5.92±0.05^{b}	3.58±0.20^{c}	7.64±0.06^{a}	7.85±0.28^{a}

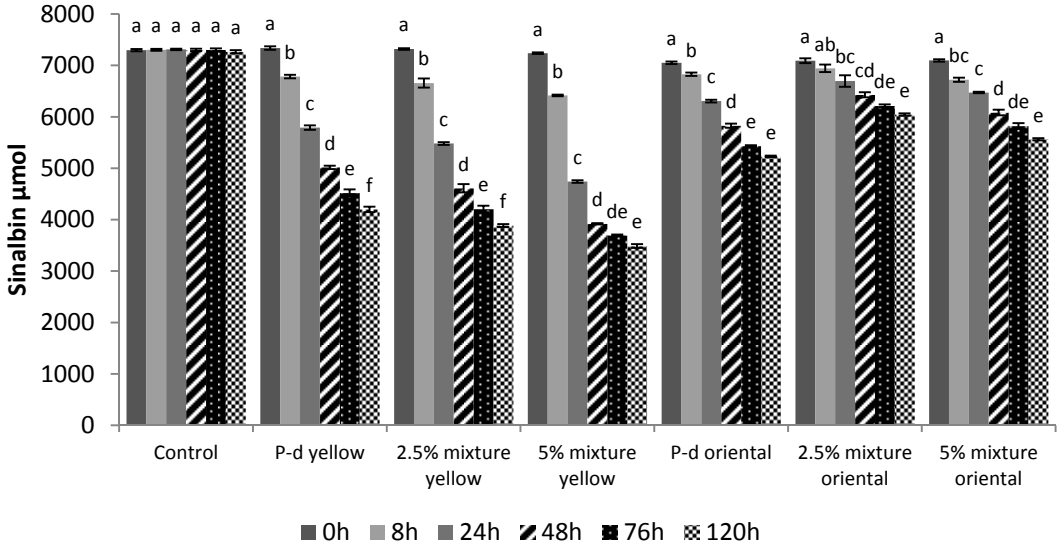
Values are means of two trials replicated three times (mean ± SD, n=6).
Different letters indicate a significant difference ($p < 0.05$) within the same column.
[1] 0.1 % and 0.2 % represent the proportion of hot mustard in sausage. These were from mixtures of mustard containing 2.5 % and 5 % hot mustard, respectively; P-d = partially deodorized mustard.

Figure 5-1: Measurement of the lack of myrosinase activity by substrate decline in fully deodorized mustard treatments from trial 6.



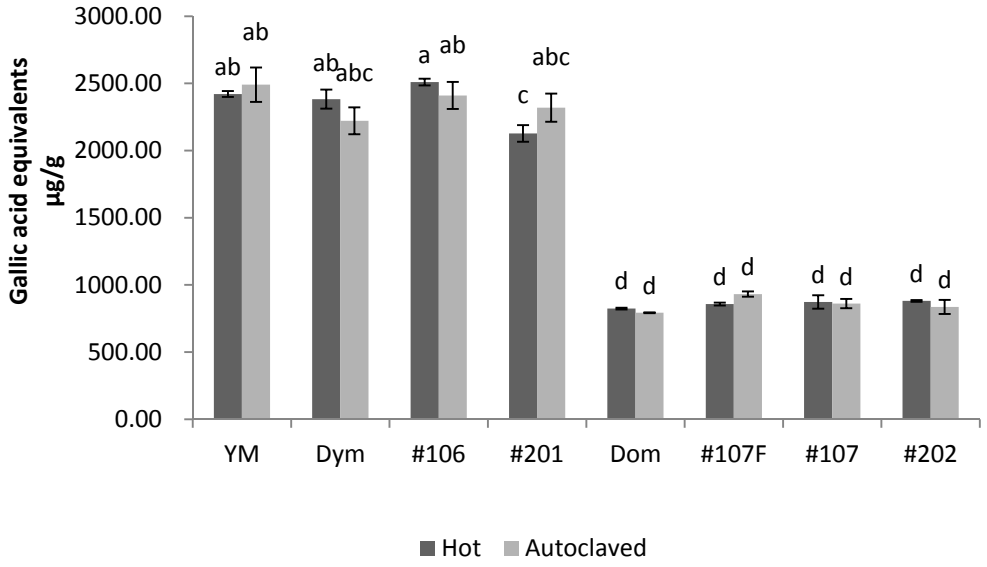
Values are means of triplicate (mean ± SD, n=3).
The same letter on the same vertical bars within the same group indicates no significant difference ($P > 0.05$).

Figure 5-2: Measurement of the presence of myrosinase activity by substrate decline in mustard treatments used for sausage trial 7.



Values are means of triplicate (mean ± SD, n=3).
Different letters on vertical bars in the same group indicate a significant difference ($P < 0.05$) from others within the group.
Yellow and oriental mustard samples were tested in sinalbin and sinigrin substrate, respectively.

Figure 5-3: Total phenolic content (TPC) of yellow and oriental mustards, before and after autoclave (115^{o}C 15 min) treatment.



Values are means of triplicate (mean ± SD, n=3).
Different letters on each bar in all groups indicate a significant difference ($p < 0.05$) from the others.

CHAPTER 6

6. GENERAL DISCUSSION

Physicochemical properties and the use of starter cultures in dry fermented sausage

Both pH and a_w values of dry fermented sausages were monitored at regular intervals during product ripening. Acidification is an important preservation technique in food by lowering the pH to prevent the growth of spoilage bacteria as well as some foodborne pathogens (e.g. *S. aureus*). In the present work when the amount of glucose added to sausage batters was 0.4 % (w/w), the pH values were on average 5.8 at day 0 and ranged from 4.9 (control) to 4.6 (mustard-treated) at the end of ripening. In all trials, sausages experienced a rapid drop of pH at the beginning and were reduced to ≤ 5.3. This step complied with the degree•hour requirement that stipulates fermented sausage pH must be less than 5.3 within a period defined by the fermentation temperature used (Health Canada 2000). The changes of pH in sausages were largely associated with the amount of glucose in the sausage formulations and its subsequent fermentation by *Pediococcus pentosaceus* to produce lactic acid. In the preliminary study and in previous work, 0.6 % (w/w) glucose in the sausage formulation was able to yield a consistent but slightly low terminal pH (4.4-4.7). When the glucose level was reduced to 0.4 % (w/w) there was no significant difference in pH, but a slight rise of pH at the end of ripening was observed in sausages, indicating the exhaustion of fermentable carbohydrate by *P. pentosaceus*. This suggested that 0.4 % glucose was probably the minimum level that should be used for dry fermented sausage, if ≥ 30 d ripening is used. All mustard-treated

sausages had a significantly lower terminal pH than controls. This suggested mustard provided additional fermentable carbohydrates for the lactic acid bacteria which contributed to further reduction of sausage pH. Moreover, residual or slight myrosinase activity in deodorized mustards, which was a significant contributor to its high antimicrobial activity, did not have an apparent effect on pH changes.

Water activity was reduced progressively throughout sausage ripening. The initial a_w in meat batters (day 0) was about 0.95. During 30 d ripening, a_w was reduced to approximately 0.82 in controls, while sausages treated with mustard had a_w values which ranged from 0.82 to 0.86. These values corresponded to those of commercial dry fermented sausages in the market place. On the other hand, with an extension of ripening to 42 d, a_w was reduced to lower than 0.8, which would be unfavorable for commercial purposes and would contribute to survival of contaminating pathogens like *E. coli* O157:H7 and *Salmonella*. The drying procedure is a critical step in sausage manufacture. Poor control of drying may lead to unbalanced moisture content in sausages, yielding undesirable appearance (e.g. product distortion and surface wrinkling) or lead to uneven drying of external and internal areas of the sausage. More importantly, poor drying control can cause an increased risk of foodborne illness since the low moisture content of this type of uncooked sausage is the key for preservation and safety (Sabine et al. 2004).

A pair of starter cultures consisting of *P. pentosaceus* and *S. carnosus* was used throughout the present study. Holley and Blaszyk (1998) reported *P. pentosaceus* and *S. carnosus* were most frequently found in 7 different kinds of commercial starter cultures

among other species of *Lactobacillus*, *Pediococcus* and *Staphylococcus*. The use of starter cultures ensures an initial rapid drop of pH and desirable red color formation, as well as maintenance of the characteristic flavor of dry fermented sausage from batch to batch. Additionally, starter cultures accelerate the development of a Gram-positive, beneficial microflora in sausage, which overwhelms the growth of undesirable or pathogenic bacteria. *P. pentosaceus* is mainly responsible for pH reduction by lactic acid production. In the current study, *P. pentosaceus* numbers were maintained at a high level, up to 8 log CFU/g throughout processing, which corresponded to results from other studies reporting that LAB grow rapidly at the beginning of sausage fermentation and reach values of 7-8 log CFU/g within the first 3 d (Drosinos et al. 2005, Cocolin et al. 2009). *S. carnosus* and other coagulase-negative cocci produce proteolytic and lipolytic enzymes which enhance the characteristic flavor and aroma of sausages. More importantly, they maintain the desirable red color by the production of nitrate reductase which reduces nitrate to nitrite, enabling formation of the pigment nitrosomyoglobin. These organisms are also involved in inhibition of lipid oxidation (Talon et al. 1999). In the present and other work, it was found that *S. carnosus* was acid sensitive and the fast growth of *P. pentosaceus* caused rapid reductions in pH, which affected the viability of *S. carnosus* to some extent. This result was similar to the report by Papamanoli et al. (2002). In mustard-treated sausages, variation in starter culture numbers was observed and there was concern that this may have been due to myrosinase hydrolytic activity in mustard. However, changes in bacterial numbers observed here have not been shown in other work

to affect sausage quality. With the absence of plant myrosinase action in sausage trial 6, *P. pentosaceus* numbers increased by 1 log CFU/g during sausage ripening, suggesting mustards offered extra nutrients. *S. carnosus*, however, showed a slight decrease due to its acid sensitivity (Table 5-7). In addition, when slight or residual myrosinase activity was present in mustard in trial 7, the viability of *S. carnosus* was reduced by about 2 log CFU/g (Table 5-8). It seemed that the production of the antimicrobial isothiocyanate not only inhibited *E. coli* O157:H7 in dry fermented sausages, it also had a moderate inhibitory effect on the viability of *S. carnosus*.

The RP-HPLC myrosinase assay and residual myrosinase measurement

It was surprising to find that 6 % (w/w) deodorized yellow mustard had significant antimicrobial activity against *E. coli* O157:H7, in view of previous work done with microencapsulated AITC and hot mustard (Chacon et al. 2006, Graumann and Holley 2008, Luciano et al. 2011, Nilson and Holley 2012, Cordeiro et al. 2013). Work from some of these studies showed that bacterial myrosinase-like activity could hydrolyze glucosinolate substrates and inhibit a variety of bacteria *in vitro* which produced the enzyme (Luciano and Holley 2010, Herzallah et al. 2011). It was thought this occurred because of the attempt by the organisms to acquire glucose from the glucosinolate, yielding isothiocyanate which was lethal to the producing bacteria. The order of this antimicrobial action from greatest to least was *E. coli* O157:H7 > *S. carnosus* > *P. pentosaceus* (Luciano et al. 2011). Thus, the antimicrobial activity of deodorized mustard or the formation of isothiocyanate was attributed to the myrosinase-like activity of *E. coli*

O157:H7 itself and the starter cultures (Luciano et al. 2011, Nilson and Holley 2012, Cordeiro et al. 2013).

However, the present work showed *E. coli* O157:H7 elimination rates caused by deodorized mustards were variable. In the present work it was noticed that those deodorized mustards containing high antimicrobial activity had a light yellowish appearance, whereas a dark brown color was shown in those mustards with poor antimicrobial activity. This suggested that differences in the extent of thermal treatment may have occurred and influenced the *E. coli* O157:H7 elimination rates observed in dry fermented sausages. An examination of the method previously used for confirmation of myrosinase inactivation suggested that the method used may not have been rigorous enough to detect residual myrosinase activity in some deodorized mustards because the assay was not conducted for more than 3 h after heat treatment.

To confirm this possibility, myrosinase was extracted from heat-treated mustards by ammonium sulfate precipitation and then added to glucosinolate substrate at known concentrations. The absence of myrosinase activity, or its reduced activity in mustard after autoclave treatment, was confirmed by changes in glucosinolate concentration in the substrate at 6 sample intervals.

Results showed that residual myrosinase activity was still present in deodorized mustards showing a light yellowish color. After quantification of myrosinase activity using a calibration curve in conjunction with protein determination, it was found that mustards treated in the autoclave at 115^{o}C for 15 min where a 2 cm thick layer of material was used,

occasionally contained 10 to 20-fold reduced levels of residual myrosinase activity, compared to levels present before heat treatment (Table 4-1).

Additionally, from the RP-HPLC myrosinase assay, it was found that glucosinolate degradation caused by residual or slight myrosinase activity was linear for at least 40 h, after which the rate tended to decline and level off. Residual glucosinolate was still recoverable at 120 h.

Several reports have shown that a first order kinetic model was applicable for myrosinase action on glucosinolates. Thus, it is reasonable to consider that glucosinolate degradation caused by myrosinase tended to decline with decreased glucosinolate content in the substrate (Ludikhuyze et al. 1999, Van Eylen et al. 2006, Ghawi et al. 2012).

The present work suggested the deodorization method used previously (Luciano et al. 2011, Nilson and Holley 2012, Cordeiro et al. 2013) was not consistently able to cause complete inactivation of myrosinase when the mustard layer was 2 cm thick during autoclave treatment. After the layer of mustard was reduced to 1 cm, the same thermal treatment eliminated residual myrosinase (Figure 5-1). Although this was not fully documented, it was apparent that the two autoclaves used, model SV-120 scientific prevacuum sterilizer and LV-250 laboratory steam sterilizer, both from STERIS (Mentor, OH, USA), required different periods to complete the 115^{o}C, 15 min cycle. Differences in warm-up and come-down during operation, when internal temperatures of the autoclaves were > 60^{o}C may have affected the extent of myrosinase inactivation and caused variation in residual myrosinase present in different batches. Because the moisture content in all

mustard samples was low (< 8 %, w/w), it was considered that differences in moisture did not contribute to differences in myrosinase inactivation, but may have enhanced its thermal stability.

In summary, use of the RP-HPLC myrosinase assay demonstrated the existence of residual myrosinase in mustard when it was deodorized in a 2 cm thick layer at 115^{o}C for 15 min. The initial antimicrobial activity of deodorized mustard against *E. coli* O157:H7 during sausage ripening was dependent upon this residual enzyme activity. It also showed that glucosinolate could still be recovered from mustard during 120 h of exposure to heat-treated enzyme extract. It is suspected that in earlier work, tests done to confirm myrosinase action did not compare the glucosinolate concentration in the reaction mixture at extended time (Luciano et al. 2011, Nilson and Holley 2012) and that this residual myrosinase activity may not have been detected.

Characterization of high antimicrobial activity in mustards against *E. coli* O157:H7 during dry fermented sausage manufacture

In the present work, the dry fermented sausage manufacturing method used reduced the number of *E. coli* O157:H7 from 2 log to 3.5 log CFU/g during 30 d or 42 d of ripening, respectively. This result was consistent with previous reports (Chacon et al. 2006, Graumann and Holley 2008, Luciano et al. 2011), and further indicated the need for an alternative processing step to ensure a 5 log reduction of *E. coli* O157:H7 in the dry fermented sausage.

To explore the potential ability of mustard to satisfy this need, the present work tested 5 yellow and 4 oriental mustard samples deodorized by autoclave treatment. An inconsistent outcome towards *E. coli* O157:H7 reduction was observed and this was shown to have occurred because of residual myrosinase in the deodorized mustard. Because residual myrosinase activity was difficult to control by autoclave treatment, fully deodorized mustard was used independently, or mixed with a small proportion of the same type of hot mustard to yield mixtures which contained predictable increments of myrosinase activity (trials 6 and 7).

In trial 6, mustards were fully deodorized in a 1 cm layer and the absence of myrosinase was verified by RP-HPLC myrosinase assay. At 6 % (w/w) in sausages, all mustard samples that previously had high antimicrobial activity (Figure 4-3, Figure 4-4 and Figure 4-5), at this concentration no longer reduced *E. coli* O157:H7 viability as effectively. At least 42 d ripening was required to cause a 5 log reduction (Table 5-1). In contrast, when the same types of mustards contained residual myrosinase, it took only 18 to 24 d of sausage ripening to yield a 5 log CFU/g reduction of *E. coli* O157:H7.

In trial 7, hot mustards were mixed at 2.5 % and 5 % (w/w) with fully deodorized mustards to produce mustard mixtures (yellow and oriental) with slight myrosinase activity. When added to sausage batter at 4 % (w/w), these mixtures yielded 0.1 and 0.2 % (w/w) hot mustard. It was found that these yellow mustard mixtures were as effective in reducing the pathogen as 6 % (w/w) mustard in the preliminary work, causing ≥ 5 log CFU/g reduction of *E. coli* O157:H7 in 18 d. In contrast, with 4 % (w/w) partially

deodorized yellow mustard, deliberately prepared by the autoclave treatment of material in a 2 cm thick layer, 24 d was needed to achieve the same result (Table 5-2). However, it is notable that the mustard mixture containing the greater proportion of hot mustard (5 %) did not eliminate the pathogen faster than the mixture containing 2.5 % (w/w) hot mustard. This suggested that the lower concentration of hot mustard supplied sufficient myrosinase to optimize glucosinolate degradation and that even lower proportions might suitably eliminate the pathogen.

However, with oriental mustard, neither 4 % (w/w) mixtures nor the partially deodorized treatment (autoclave) achieved a 5 log reduction within 36 d of ripening (Table 5-2). This result was unexpected even though oriental mustards were shown to have less antimicrobial activity than yellow mustards in preliminary work (Figure 4-4 and Figure 4-5). Since oriental mustards contained less glucosinolate than the yellow mustards, their use in the sausages at the same level meant that sausages treated with oriental mustard contained lower glucosinolate levels than those treated with yellow mustard. Because myrosinase follows first order kinetics, differences in substrate concentration will affect the myrosinase activity and the conversion of glucosinolate. Additionally, since the glucosinolates in these mustards differ, the isothiocyanates produced by myrosinase (AITC and ρHBIT in oriental and yellow, respectively) which have different stability (ρHBIT is highly unstable) are likely to have different antimicrobial efficacy in sausages. This has not been tested. However, there has been some *in vitro* work which showed that glucosinolates and their hydrolysis products

differed in their biological effects (Griffiths et al. 1998). Luciano and Holley (2011) found that AITC was more effective than ρHBIT against *E. coli* O157:H7 in Luria broth. With minimum bactericidal concentrations of 1.06 mM (AITC) and 1.48 mM (ρHBIT), these agents inhibited growth at 0.26 mM and 0.59 mM, respectively.

From sausage trials 6 and 7, results suggested that although bacterial myrosinase-like activity in fully deodorized yellow mustards used at 6 % (w/w) in sausages against *E. coli* O157:H7 contributed to the ≥ 5 log CFU/g reduction in 42 d observed with samples DYM and #106 (Table 5-1), small or residual plant myrosinase present in the hot/deodorized yellow mustard mixtures and partially deodorized yellow mustard #106, when used at 4 % (w/w) in sausages, yielded a ≥ 5 log CFU/g reduction of *E. coli* O157:H7 by 18-24 d (Table 5-2). Since oriental mustard treatments failed to reduce the number of *E. coli* O157:H7 by 5 log CFU/g within 36 d, other as yet undetermined factors may have influenced this result. It is worth noting that in a comparison of antimicrobial action of mustard extracts (yellow and oriental) and pure sinigrin against *Listeria monocytogenes* contaminating bologna packaged in plastic film containing the extracts or sinigrin, oriental mustard extract eliminated the pathogen, but the yellow mustard extract as well as pure sinigrin did not (Lara-Lledo et al. 2012).

The total phenolic content (TPC) of yellow mustard was significantly higher than oriental mustard and may have contributed to the overall antimicrobial effects observed. However, when the endogenous myrosinase activity was eliminated from trial 6 samples,

the phenolic compounds in yellow mustards were not able to control the pathogen. It appeared that TPC may have contributed only additively to the antimicrobial activity.

The ultimate goal of present work was to control *E. coli* O157:H7 in dry fermented sausage using mustard glucosinolate at concentrations of mustard at or below 4 % (w/w) to reduce organoleptic changes. The present work showed that if a small proportion of hot mustard were added to deodorized mustard, the overall antimicrobial effects with yellow mustard were substantially improved and the target 5 log CFU/g reduction of *E. coli* O157:H7 could be achieved dependably within 24 d.

Additionally, it is possible that there are benefits in terms of antimicrobial efficacy in sausage by gradual generation of isothiocyanates because of their volatility and instability (Bailey et al. 1961, Lewis and Papavizas 1971) in aqueous media and food matrices. It is believed, in antimicrobial food packaging systems, that controlling AITC release can promote its antimicrobial activity by reducing its volatility rate (Fujita et al. 1999, Plackett et al. 2007, Vega-Lugo and Lim 2009). Results from the present work with yellow cultivars suggest that 0.1 % and 0.2 % hot mustard (w/w) in sausages containing 4 % mustard (w/w) can be as antimicrobial as when 6 % hot mustard (w/w) is added to the sausages.

CHAPTER 7

7. GENERAL CONCLUSION

Several studies have shown the ability of deodorized yellow mustard to reduce *E. coli* O157:H7 by ≥ 5 log CFU/g during ripening of dry fermented meats (Luciano et al. 2011, Nilson and Holley 2012, Cordeiro et al. 2013). However, in the present work it was found that when a 2 cm thick layer of mustard was used during deodorization by autoclave treatment at 115^{o}C for 15 min, there was periodic failure to completely inactivate the endogenous myrosinase. This residual myrosinase activity then caused glucosinolate degradation and contributed to the antimicrobial activity when formulated in dry fermented meats.

When yellow mustard was partially deodorized or when fully deodorized yellow mustard contained 0.1-0.2 % hot mustard and then used at 4 % (w/w) in dry fermented sausages, these preparations eliminated ≥ 5 log CFU/g *E. coli* O157:H7 within 24 d of ripening. In contrast, when fully deodorized yellow mustards were used, it took at least 42 d to achieve the same antimicrobial result. It is highly likely that incomplete inactivation of myrosinase contributed to variable antimicrobial performance of mustard in previous work when used in dry fermented sausages to control *E. coli* O157:H7. The inability to demonstrate the same effect with oriental mustard indicates that there are other factors which have yet to be determined which influence antimicrobial activities of oriental mustard.

It is possible that oriental mustard did not perform as well in sausages as yellow mustard because it contained lower levels of glucosinolate(s). The total phenolic content (TPC) differences in yellow and oriental mustards may also have influenced results. The 5 yellow mustards tested showed significantly higher TPC than the 4 types of oriental mustards analyzed. Nonetheless, the reasons for the different antimicrobial performance of oriental mustard should be better understood.

The current study has shown that 4 % (w/w) yellow mustard treatments containing myrosinase activity from 0.1 or 0.2 % hot mustard were as antimicrobial as 6 % yellow mustard in sausages.

Further work should explore the optimal addition of hot mustard in mixtures with deodorized mustard. It may be possible to reduce levels of both hot/deodorized components to < 0.1 and 4 % (w/w) in sausages, respectively, and still dependably achieve ≥ 5 log CFU/g reductions.

It is also evident that the antimicrobial potency of AITC and ρHBIT against *E. coli* O157:H7 in sausages should be better characterized. The thermal stability of plant myrosinase should be studied further to provide guidance on minimal treatments of mustard powder and flour necessary to completely inactivate myrosinase.

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APPENDIX I

Table 1: pH changes in sausage during ripening in trial 1.

Day	Treatments				
	Control	3 % DYM	3.5 % DYM	4 % DYM	6 % DYM
0	5.95±0.06^{a}	5.99±0.05^{a}	6.03±0.05^{a}	6.05±0.06^{a}	5.97±0.04^{a}
6	4.86±0.08^{a}	4.65±0.05^{b}	4.62±0.03^{b}	4.64±0.02^{b}	4.60±0.01^{b}
12	4.70±0.02^{a}	4.55±0.02^{b}	4.55±0.02^{b}	4.56±0.02^{b}	4.50±0.02^{b}
18	4.66±0.05^{a}	4.47±0.03^{b}	4.50±0.01^{b}	4.50±0.02^{b}	4.46±0.02^{b}
24	4.66±0.06^{a}	4.45±0.01^{b}	4.46±0.01^{b}	4.49±0.03^{b}	4.45±0.03^{b}
30	4.65±0.03^{a}	4.47±0.03^{b}	4.45±0.02^{b}	4.44±0.14^{b}	4.48±0.12^{b}

Values are mean of two trials replicated twice (mean ± SD, n=4).
Different letters indicate a significant difference ($P < 0.05$) within the same row.

Table 2: a_w changes in sausage during ripening in trial 1.

Day	Treatments				
	Control	3 % DYM	3.5 % DYM	4 % DYM	6 % DYM
0	0.945±0.015^{a}	0.957±0.002^{a}	0.960±0.002^{a}	0.960±0.003^{a}	0.953±0.006^{a}
6	0.930±0.001^{a}	0.919±0.001^{a}	0.929±0.004^{a}	0.925±0.004^{a}	0.930±0.002^{a}
12	0.905±0.001^{a}	0.901±0.003^{a}	0.902±0.007^{a}	0.912±0.001^{a}	0.914±0.001^{a}
18	0.876±0.008^{a}	0.873±0.003^{a}	0.879±0.002^{a}	0.877±0.005^{a}	0.881±0.004^{a}
24	0.850±0.001^{a}	0.854±0.006^{a}	0.848±0.005^{a}	0.856±0.006^{a}	0.857±0.001^{a}
30	0.804±0.013^{b}	0.829±0.004^{ab}	0.820±0.008^{ab}	0.821±0.008^{ab}	0.845±0.002^{a}

Values are mean of two trials replicated twice (mean ± SD, n=4).
Different letters indicate a significant difference ($P < 0.05$) within the same row.

Table 3: pH changes in sausage during ripening in trial 2.

Day	Treatments				
	Control	3 % DOM	3.5 % DOM	4 % DOM	6 % DOM
0	5.93±0.02^{a}	5.96±0.03^{a}	5.93±0.03^{a}	5.98±0.03^{a}	5.97±0.04^{a}
6	4.76±0.01^{a}	4.50±0.03^{b}	4.45±0.02^{b}	4.49±0.02^{b}	4.42±0.04^{b}
12	4.72±0.03^{a}	4.51±0.03^{b}	4.51±0.03^{b}	4.45±0.04^{b}	4.45±0.06^{b}
18	4.63±0.15^{a}	4.58±0.10^{a}	4.52±0.02^{a}	4.53±0.03^{a}	4.42±0.02^{a}
24	4.77±0.09^{a}	4.49±0.13^{b}	4.45±0.09^{b}	4.55±0.01^{ab}	4.53±0.01^{ab}
30	4.75±0.01^{a}	4.56±0.02^{b}	4.57±0.06^{b}	4.55±0.07^{b}	4.48±0.08^{b}

Values are mean of two trials replicated twice (mean ± SD, n=4).
Different letters indicate a significant difference ($P < 0.05$) within the same row.

Table 4: a_w changes in sausage during ripening in trial 2.

Day	Treatments				
	Control	3 % DOM	3.5 % DOM	4 % DOM	6 % DOM
0	0.965±0.002^{a}	0.962±0.001^{a}	0.960±0.002^{a}	0.956±0.002^{a}	0.957±0.001^{a}
6	0.924±0.002^{a}	0.927±0.001^{a}	0.928±0.004^{a}	0.930±0.001^{a}	0.925±0.004^{a}
12	0.898±0.005^{a}	0.893±0.007^{b}	0.896±0.003^{b}	0.890±0.001^{b}	0.895±0.004^{b}
18	0.855±0.005^{a}	0.858±0.001^{a}	0.862±0.007^{a}	0.869±0.001^{a}	0.855±0.002^{a}
24	0.814±0.003^{b}	0.843±0.002^{ab}	0.833±0.005^{ab}	0.807±0.002^{ab}	0.848±0.012^{a}
30	0.797±0.002^{b}	0.801±0.003^{b}	0.804±0.003^{b}	0.796±0.003^{b}	0.820±0.003^{a}

Values are mean of two trials replicated twice (mean ± SD, n=4).
Different letters indicate a significant difference ($P < 0.05$) within the same row.
Intermittent failures occurred in the drying chamber during the last two sampling intervals (24 d and 30 d), which may be the reason for the unusual lower values of a_w.

Table 5: pH changes in sausage during ripening in trial 3.

Day	Treatments				
	Control	6 % CDP	6 % YM	6 % DYM	6 % DOM
0	6.02±0.01^{c}	6.11±0.06^{ab}	6.05±0.01^{bc}	6.16±0.02^{a}	6.07±0.03^{bc}
6	4.78±0.06^{a}	4.58±0.13^{ab}	4.58±0.05^{ab}	4.57±0.06^{b}	4.53±0.04^{b}
12	4.76±0.05^{a}	4.56±0.02^{b}	4.58±0.03^{b}	4.63±0.04^{b}	4.55±0.01^{b}
18	4.80±0.05^{a}	4.58±0.03^{b}	4.58±0.03^{b}	4.69±0.03^{ab}	4.57±0.04^{b}
24	4.78±0.04^{a}	4.52±0.01^{b}	4.54±0.03^{b}	4.57±0.03^{b}	4.49±0.03^{b}
30	4.79±0.01^{a}	4.51±0.01^{c}	4.52±0.02^{c}	4.59±0.03^{b}	4.52±0.01^{c}
36	4.70±0.01^{a}	4.48±0.01^{d}	4.49±0.01^{d}	4.61±0.01^{b}	4.55±0.01^{c}
42	4.73±0.01^{a}	4.56±0.03^{b}	4.53±0.02^{bc}	4.53±0.03^{bc}	4.52±0.01^{c}

Values are mean of two trials replicated twice (mean ± SD, n=4).
Different letters indicate a significant difference ($P < 0.05$) within the same row.

Table 6: a_w changes in sausage during ripening in trial 3.

Day	Treatments				
	Control	6 % CDP	6 % YM	6 % DYM	6 % DOM
0	0.953±0.001^{a}	0.953±0.001^{a}	0.958±0.001^{a}	0.953±0.002^{a}	0.953±0.003^{a}
6	0.915±0.001^{b}	0.924±0.003^{a}	0.927±0.005^{a}	0.922±0.002^{ab}	0.922±0.004^{ab}
12	0.903±0.006^{a}	0.906±0.003^{a}	0.907±0.005^{a}	0.890±0.004^{a}	0.902±0.008^{a}
18	0.876±0.005^{a}	0.884±0.004^{a}	0.880±0.005^{a}	0.876±0.004^{a}	0.875±0.008^{a}
24	0.853±0.003^{a}	0.855±0.011^{a}	0.860±0.006^{a}	0.856±0.007^{a}	0.864±0.005^{a}
30	0.810±0.012^{b}	0.842±0.004^{a}	0.838±0.002^{a}	0.830±0.003^{a}	0.842±0.003^{a}
36	0.785±0.001^{b}	0.812±0.004^{a}	0.810±0.008^{a}	0.807±0.001^{a}	0.816±0.003^{a}
42	0.785±0.003^{c}	0.816±0.003^{ab}	0.815±0.002^{ab}	0.826±0.003^{a}	0.804±0.001^{b}

Values are mean of two trials replicated twice (mean ± SD, n=4).
Different letters indicate a significant difference ($P < 0.05$) within the same row.

Table 7: pH changes in sausage during ripening in trial 4.

Day	Treatments				
	Control	6 % CDP	6 % YM	6 % DYM	6 % DOM
0	5.89±0.03^{a}	5.85±0.04^{ab}	5.76±0.02^{c}	5.80±0.01^{bc}	5.91±0.03^{a}
6	4.70±0.01^{a}	4.60±0.01^{b}	4.56±0.04^{c}	4.56±0.01^{c}	4.52±0.01^{d}
12	4.70±0.02^{a}	4.50±0.01^{b}	4.51±0.01^{b}	4.57±0.04^{c}	4.50±0.01^{c}
18	4.71±0.02^{a}	4.58±0.01^{bc}	4.57±0.01^{c}	4.59±0.02^{b}	4.51±0.01^{d}
24	4.77±0.02^{a}	4.51±0.02^{b}	4.43±0.03^{c}	4.40±0.03^{c}	4.36±0.01^{d}
30	4.66±0.02^{a}	4.53±0.01^{b}	4.47±0.01^{c}	4.47±0.01^{c}	4.40±0.02^{d}
36	4.60±0.01^{a}	4.48±0.01^{b}	4.50±0.02^{b}	4.47±0.01^{b}	4.40±0.02^{c}
42	4.62±0.02^{a}	4.46±0.01^{b}	4.53±0.02^{b}	4.51±0.01^{b}	4.43±0.01^{c}

Values are mean of two trials replicated twice (mean ± SD, n=4).
Different letters indicate a significant difference ($P < 0.05$) within the same row.

Table 8: a_w changes in sausage during ripening in trial 4.

Day	Treatments				
	Control	6 % CDP	6 % YM	6 % DYM	6 % DOM
0	0.959±0.005^{a}	0.955±0.004^{b}	0.956±0.013^{ab}	0.956±0.007^{ab}	0.955±0.003^{b}
6	0.931±0.002^{a}	0.930±0.003^{a}	0.928±0.002^{ab}	0.926±0.004^{b}	0.931±0.009^{a}
12	0.901±0.011^{b}	0.902±0.013^{b}	0.912±0.004^{a}	0.890±0.006^{c}	0.904±0.004^{b}
18	0.872±0.007^{a}	0.877±0.004^{a}	0.876±0.002^{a}	0.872±0.008^{a}	0.881±0.013^{a}
24	0.847±0.013^{c}	0.855±0.005^{a}	0.834±0.011^{d}	0.850±0.003^{bc}	0.852±0.012^{ab}
30	0.823±0.006^{c}	0.830±0.003^{b}	0.831±0.003^{b}	0.831±0.008^{b}	0.842±0.012^{a}
36	0.783±0.003^{d}	0.817±0.008^{a}	0.792±0.007^{c}	0.802±0.014^{b}	0.801±0.008^{b}
42	0.778±0.012^{c}	0.802±0.007^{a}	0.784±0.005^{b}	0.784±0.005^{b}	0.784±0.001^{b}

Values are mean of two trials replicated twice (mean ± SD, n=4).
Different letters indicate a significant difference ($P < 0.05$) within the same row.

Table 9: pH changes in sausage during ripening in trial 5.

Day	Treatments				
	Control	6 % #106	6 % #201	6 % #107	6 % #202
0	5.75±0.05^{a}	5.75±0.01^{a}	5.76±0.14^{a}	5.78±0.01^{a}	5.81±0.07^{a}
6	4.63±0.01^{a}	4.46±0.02^{b}	4.49±0.02^{b}	4.42±0.01^{c}	4.48±0.02^{b}
12	4.65±0.02^{a}	4.44±0.01^{c}	4.48±0.02^{bc}	4.48±0.04^{bc}	4.51±0.03^{b}
18	4.65±0.01^{a}	4.47±0.01^{c}	4.49±0.01^{b}	4.45±0.02^{c}	4.45±0.01^{c}
24	4.62±0.02^{a}	4.46±0.02^{b}	4.45±0.03^{b}	4.46±0.02^{b}	4.46±0.01^{b}
30	4.66±0.02^{a}	4.54±0.01^{b}	4.54±0.02^{b}	4.55±0.01^{b}	4.52±0.02^{b}
36	4.71±0.02^{a}	4.53±0.01^{bc}	4.55±0.02^{b}	4.52±0.01^{b}	4.42±0.02^{c}

Values are mean of two trials replicated twice (mean ± SD, n=4).
Different letters indicate a significant difference ($P < 0.05$) within the same row.

Table 10: a_w changes in sausage during ripening in trial 5.

Day	Treatments				
	Control	6 % #106	6 % #201	6 % #107	6 % #202
0	0.956±0.012^{ab}	0.959±0.005^{a}	0.957±0.011^{ab}	0.955±0.007^{b}	0.956±0.006^{ab}
6	0.933±0.005^{ab}	0.929±0.016^{cd}	0.931±0.003^{bc}	0.926±0.017^{d}	0.936±0.003^{a}
12	0.911±0.002^{b}	0.911±0.007^{b}	0.915±0.007^{a}	0.905±0.012^{c}	0.907±0.008^{c}
18	0.882±0.007^{b}	0.895±0.001^{a}	0.865±0.003^{d}	0.872±0.005^{c}	0.884±0.005^{b}
24	0.847±0.013^{c}	0.855±0.005^{a}	0.834±0.011^{d}	0.850±0.003^{bc}	0.854±0.005^{c}
30	0.825±0.006^{c}	0.838±0.011^{a}	0.841±0.003^{a}	0.831±0.013^{b}	0.833±0.011^{b}
36	0.805±0.014^{b}	0.825±0.012^{a}	0.818±0.006^{a}	0.812±0.006^{a}	0.812±0.004^{a}

Values are mean of two trials replicated twice (mean ± SD, n=4).
Different letters indicate a significant difference ($P < 0.05$) within the same row.