

**Use of Deodorized Yellow Mustard Powder to Control *Escherichia coli* O157:H7 in  
Dry Cured Westphalian Ham**

**BY**

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## FOREWORD

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## TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENTS.....	iii
FOREWORD.....	iv
LIST OF TABLES.....	vii
ABSTRACT.....	viii
1.0 INTRODUCTION.....	1
2.0 LITERATURE REVIEW.....	6
2.1 Dry Cured Westphalian Ham.....	6
2.2 Production of Dry Cured Westphalian Ham.....	6
2.2.1 Dry Cured Westphalian Ham Manufacture.....	6
2.2.2 Development of Indigenous Microflora During Ripening.....	7
2.3 <i>Escherichia (E.) coli</i> O157:H7 in Fermented Meat Products.....	10
2.3.1 <i>E. coli</i> O157:H7, a Foodborne Pathogen.....	10
2.3.2 Pigs as Potential <i>E. coli</i> O157:H7 Reservoirs.....	11
2.3.3 <i>E. coli</i> O157:H7 Illness Outbreak Data and Survival in Dry Cured Meat Products.....	13
2.3.4 Regulations for the Control of <i>E. coli</i> O157:H7 in Fermented Meat Products.....	15
2.4 Mustard as a Natural Antimicrobial in Fermented Meat Products..	17
2.4.1 Mustard and its Antimicrobial Activity.....	17
2.4.2 Yellow Mustard Powder in Fermented Meat Products..	21
3.0 USE OF DEODORIZED YELLOW MUSTARD POWDER TO CONTROL <i>ESCHERICHIA COLI</i> O157:H7 IN DRY CURED WESTPHALIAN HAMS.....	26
3.1 Abstract.....	26
3.2 Introduction.....	27
3.3 Material and Methods.....	30
3.3.1 Preparation of <i>E. coli</i> O157:H7.....	30
3.3.2 Preparation of <i>S. carnosus</i> UM123M.....	31
3.3.3 Yellow Mustard Powder.....	32
3.3.4 Dry Cured Westphalian Ham Manufacture.....	33
3.3.5 Sampling and Analysis.....	35
3.3.6 Statistical Analysis.....	37
3.4 Results and Discussion.....	37
3.4.1 Physicochemical Changes During Dry Cured Westphalian Ham Ripening.....	37

3.4.2	Microbial Changes During Dry Cured Westphalian Ham Ripening.....	38
3.4.3	Effect of Deodorized Yellow Mustard Powder on <i>E. coli</i> O157:H7 Survival.....	40
3.5	Conclusion.....	42
4.0	GENERAL DISCUSSION.....	53
5.0	CONCLUSIONS.....	59
	REFERENCES.....	62

## LIST OF TABLES

TABLE	PAGE
Table 3.1. Changes in pH values of ripening dry cured Westphalian ham surfaces and interiors.....	44
Table 3.2. Changes in surface and interior water activities of ripening dry cured Westphalian hams.....	45
Table 3.3. Numbers of adventitious lactic acid bacteria (log cfu·g <sup>-1</sup> recovered on MRSS agar) from dry cured Westphalian ham surfaces and interiors during ripening .....	46
Table 3.4. Total numbers of aerobic mesophiles (log cfu·g <sup>-1</sup> recovered on TSA) from dry cured Westphalian ham surfaces and interiors during ripening....	47
Table 3.5. Numbers of adventitious yeast (log cfu·g <sup>-1</sup> recovered on PDA agar) from dry cured Westphalian ham surfaces and interiors during ripening .....	48
Table 3.6. Numbers of adventitious <i>Staphylococcus spp.</i> (log cfu·g <sup>-1</sup> recovered on MSA) from dry cured Westphalian ham surfaces and interiors during ripening (Trial 1).....	49
Table 3.7. Numbers of adventitious and inoculated <i>Staphylococcus spp.</i> (log cfu·g <sup>-1</sup> recovered on MSA) from dry cured Westphalian ham surfaces and interiors during ripening (Trial 2).....	50
Table 3.8. Numbers of <i>Escherichia coli</i> O157:H7 (log cfu·g <sup>-1</sup> recovered on VRBG and ctSMAC agar) from dry cured Westphalian ham surfaces and interiors during ripening (Trial 1).....	51
Table 3.9. Numbers of <i>Escherichia coli</i> O157:H7 (log cfu·g <sup>-1</sup> recovered on VRBG and ctSMAC agar) from dry cured Westphalian ham surfaces and interiors during ripening (Trial 2).....	52

## ABSTRACT

*Escherichia (E.) coli* O157:H7 survival in dry cured (uncooked) meat products leading to human illness outbreaks is an international problem. Their manufacture does not involve a heat kill step to ensure the destruction of the organism, and the adverse conditions created during processing may not be sufficient to prevent *E. coli* O157:H7 survival. Deodorized yellow mustard powder has antimicrobial properties from glucosinolate (sinalbin) hydrolysis catalyzed by the endogenous enzyme myrosinase, generating antimicrobial isothiocyanate (PHBIT). Previous work has shown that its addition during dry sausage manufacture was capable of eliminating the pathogen. In this study, its use for the same purpose was investigated during dry cured Westphalian ham production. Hams were inoculated with a  $7.5 \log \text{ cfu}\cdot\text{g}^{-1}$  cocktail of *E. coli* O157:H7, surface applied with 4% or 6% (w/w) deodorized yellow mustard powder, and monitored for *E. coli* O157:H7 survival during 80d ham maturation. One trial included the inoculation of *Staphylococcus (S.) carnosus*, a meat starter culture with myrosinase-like activity, onto the hams (after salt equilibration) to accelerate formation of antimicrobial isothiocyanate from mustard glucosinolate and help control the pathogen. In both trials, *E. coli* O157:H7 was reduced  $3 \log \text{ cfu}\cdot\text{g}^{-1}$  by 21d on hams treated with mustard powder, whereas only a  $1 \log \text{ cfu}\cdot\text{g}^{-1}$  reduction was found in the inoculated control which was not treated with mustard. By 45d, hams treated with mustard powder showed a reduction of  $>5 \log \text{ cfu}\cdot\text{g}^{-1}$  *E. coli* O157:H7, whereas it took 80d to for numbers in control hams to be similarly reduced. Since a 5 log kill of *E. coli* O157:H7 was achieved in control hams by the end of 80d, dry cured Westphalian ham manufacture would be considered capable of controlling the risk of *E. coli* O157:H7 survival by North American regulatory agencies. However, deodorized yellow mustard

powder at 4%, and to a greater extent at 6%, eliminated the pathogen at a significantly faster rate than the control during ham processing. Addition of the *S. carnosus* starter culture in trial 2 may have contributed to the maintenance of this effect through isothiocyanate formation. It also helped restore numbers of staphylococci, which were found to be sensitive to deodorized mustard powder.

## CHAPTER 1

### Introduction

Canada is the world's largest exporter and second largest producer of mustard (AAFC 2008), of which yellow is the largest type of mustard crop grown in the country. Yellow mustard, or *Sinapis alba*, belongs to the plant family *Brassicaceae*. Plants within this family contain compounds called glucosinolates, with sinalbin being the major glucosinolate in yellow mustard. Interestingly, the glucosinolate is held in a different compartment than the myrosinase enzyme (EC 3.2.1.147) in the mustard seed. Upon physical disruption to the tissues, myrosinase comes into contact with sinalbin and in the presence of water catalyzes its hydrolysis to produce *p*-hydroxybenzyl isothiocyanate (PHBIT) (Kawakishi and Muramatsu 1966, Delaquis and Mazza 1995, Ekanayake *et al.* 2006). Isothiocyanates have antimicrobial activity, in addition to being responsible for the hot mouthfeel of mustard.

The overpowering spiciness of non-deodorized (non-deheated) mustard has limited its use in food. However, the development of a deodorizing (deheating) process has lifted these limitations. By applying heat to the mustard, myrosinase is inactivated which prevents glucosinolate hydrolysis and isothiocyanate formation. Deodorized mustard has a bland flavour, and when added to other foods, causes no adverse flavour effects. In the meat industry today, deodorized yellow mustard powder is used as a binder, filler and emulsifier in cooked cured meats.

Interestingly, Luciano *et al.* (2011) found that deodorized mustard, even with myrosinase inactivated, is lethal against pathogens such as *E. coli* O157:H7. Further investigation led to the discovery that *E. coli* O157:H7, other pathogenic bacteria, and some meat starter cultures have myrosinase-like activity (Luciano *et al.* 2011). This

would explain why antimicrobial action is still observed in deodorized mustard, since isothiocyanates are still able to be generated from glucosinolates in the absence of plant myrosinase. It is suggested that the organism has activity similar to myrosinase because as it removes a glucose molecule from the glucosinolate for energy, it unknowingly hydrolyzes sinalbin to generate PHBIT and subsequently causes cell death. It is suggested that this only occurs during periods of low nutrient ability, since if other sources of energy are readily available the glucosinolate is not hydrolyzed by the pathogen (Muthukumarasamy *et al.*, 2004). Additionally, the microorganism needs to expend energy to release the glucose molecule from the glucosinolate, therefore this form of gaining energy seems to be a last resort resulting from nutrient competition from other microorganisms and adverse conditions created on the ham. It is also important to note that this myrosinase-like activity may not be the main purpose of the enzyme, but rather it may have another major function, with glucosinolate degradation occurring as a secondary function. Interestingly, of all the bacteria tested, *E. coli* O157:H7 had the fastest rate of glucosinolate degradation (507.9  $\mu\text{M}$  after 6d at 25°C) (Luciano *et al.* 2011). In comparison, the meat starter culture *Staphylococcus (S.) carnosus* UM123M had the second highest rate of glucosinolate hydrolysis (250.3  $\mu\text{M}$  after 6d at 25°C) (Luciano and Holley 2010; Luciano *et al.* 2011). Studies are now looking into the ability of deodorized mustard to provide antimicrobial activity in food products susceptible to pathogen contamination, such as *E. coli* O157:H7 in fermented meat products.

*E. coli* O157:H7 was first recognized as a pathogen in 1982 (Riley *et al.* 1983). Since then, beef products have been a major source of *E. coli* O157:H7 illness outbreaks, with cattle being documented as the major reservoir of the pathogen (Griffin and Tauxe 1991; Brandt *et al.* 1994; Armstrong *et al.* 1996; Buchanan and Doyle 1997; Tuttle *et*

*al.*, 1999). The first illness outbreak of *E. coli* O157:H7 from a fermented meat product (dry cured salami) occurred in the USA in 1994 (CDC 1995), and this was followed by two outbreaks in Canada in 1998 and 1999 (Williams *et al.* 2000; MacDonald *et al.* 2004). Immediately following these incidents, investigations into dry cured sausage manufacture revealed that viable cells survived the process and thus these products were at risk for *E. coli* O157:H7 contamination (Glass *et al.* 1992; Hinkens *et al.* 1996; Paton *et al.* 1996; Tilden *et al.* 1996; Calicioglu *et al.* 1997; Faith *et al.* 1998; Nissen *et al.* 1998). This led North American regulatory agencies to create mandatory guidelines for semi-dry and dry fermented sausage manufacture which require the validation of a 5 log reduction of *E. coli* O157:H7 (CFIA 2010).

Since beef products and cattle have been the focus in *E. coli* O157:H7 research, other animals have not been widely investigated as a source of contamination. Interestingly, it has been discovered that hogs can act as potential *E. coli* O157:H7 reservoirs, and in some cases at even higher levels than cattle (Borie *et al.*, 1997; Doane *et al.* 2007). Additionally, pork has been found in sausages linked to an *E. coli* O157:H7 illness outbreak (Paton *et al.* 1996) and though there is no documentation linking an outbreak to dry cured ham, these products are susceptible to *E. coli* O157:H7 contamination. Work has shown *E. coli* O157:H7 is able to survive dry cured ham manufacture when needle tenderized (Graumann *et al.* 2007).

Dry cured Westphalian ham, originally from the Westphalian region of Germany, is a raw meat product in which a whole pork muscle is preserved through an aging process. Manufacture takes approximately 3 months and requires the application of spices and curing agents (salt, nitrite, nitrate), smoke (from beech wood and juniper), the development of microflora, and extended drying periods. These long drying times

are important to reduce the water activity ( $a_w$ ) of the pork, which reduces the survival rate of *E. coli* O157:H7 (Sperber 1983). On the other hand, the growth of non-pathogenic microorganisms such as staphylococci and lactic acid bacteria (LAB) is desired as they are essential to the ripening of the ham (Lücke 1986). *Staphylococcus* is especially important for ham colour development. Native to this species are catalase enzymes which break down hydrogen peroxide ( $H_2O_2$ , generated as a byproduct of cellular metabolism) that can cause discoloration to the ham tissues as well as nitrate reductase enzymes which convert nitrate to nitrite and nitric oxide that binds with myoglobin to form the typical red colour (nitric oxide myoglobin) of dry cured ham. LAB, on the other hand, produce lactic acid through carbohydrate fermentation and slightly increase acidity of the ham. Through the combined hurdles of decreased  $a_w$  and pH, in conjunction with high salt conditions (5%), an environment is created in the ham that discourages the growth of undesired bacteria (Fadda *et al.* 2010). At the end of manufacture the result is a dense, dark fleshed, artisan style ham product that is ready-to-eat (RTE).

The manufacturing process of dry cured Westphalian ham, though original to Germany, is similar to that of dry cured hams made around the world such as Procuttio (Italy) and Serrano (Spain). However, they can differ with slight variations to their recipes including types and amounts of spices used, application of smoke, aging times, and cut of meat. The latter types of ham usually have longer ripening times, are typically not smoked, and encourage the growth of yeast and mould to develop a unique flavour (Hinrichsen and Pederson 1995; Flores *et al.* 1997). Nevertheless, all types of dry cured ham are susceptible to *E. coli* O157:H7 contamination since no lethal heat treatment

exists during manufacture, and adverse conditions created in the ham may not be sufficient to inhibit pathogen survival.

An alternative approach to address this issue would be the addition of antimicrobial agent(s) during fermented meat manufacture in an effort to control the pathogen and aid in achieving the mandatory 5 log kill. Deodorized yellow mustard powder has potential for this purpose, since it is already used in the meat industry (no new product would need to be introduced) and it is lethal against *E. coli* O157:H7. Furthermore, work has shown that the addition of 4% or 6% deodorized yellow mustard powder to dry sausage manufacture decreased *E. coli* O157:H7 by  $>5 \log \text{ cfu} \cdot \text{g}^{-1}$  at a faster rate than the control (Cordiero *et al.* 2011; Luciano *et al.* 2011).

The present work investigates the control of *E. coli* O157:H7 by deodorized yellow mustard powder on dry cured Westphalian hams, to see if its application could help dry cure manufacture achieve a 5 log reduction. Two trials were conducted in this study which involved *E. coli* O157:H7 inoculation onto the ham in addition to surface application of deodorized yellow mustard powder at 4% or 6%, and monitored over 80d ripening. The levels of mustard were chosen based on previous testing conducted in our pilot plant showing favourable control of *E. coli* O157:H7 in dry cured sausages. The second trial included the addition of the meat starter *S. carnosus* UM123M. Though meat starter cultures are not normally added to dry cured ham because they are felt to have little measurable effect (Sanchez-Molinero and Arnau 2008), in this case a starter culture was added to see if its myrosinase-like activity would help to control *E. coli* O157:H7 through accelerated PHBIT formation from mustard glucosinolate.

## CHAPTER 2

### Literature Review

#### 2.1 Dry cured Westphalian ham

Dry cured Westphalian ham is a fermented meat product that does not use a heat treatment during its manufacture. It is considered a delicacy and is premium priced since it is high in quality and has a lengthy production process. Westphalian dry cured ham, also known as “Westfälischer Schinken” in German, is traditionally made from acorn fed pigs in the Westphalian region of Germany. Since this product is uncooked, the original cut of raw pork goes through a series of production steps to ripen into an edible ham. The manufacturing process includes the application of curing agents (salt, nitrite, nitrate), fermentation periods, and long drying times (Lücke 1986). The application of smoke varies with type of dry cured ham produced, however Westphalian ham is unique in that it is specifically smoked over beech wood and juniper branches to give it a distinct flavour. The entire manufacturing process takes  $\geq 80$ d to complete, which results in a dense, dark coloured ham that is shelf stable at room temperature and is ready-to-eat (RTE). It is generally served cold, sliced paper thin, and has a salty, smoky flavour.

#### 2.2 Production of dry cured Westphalian ham

##### *2.2.1 Dry cured Westphalian ham manufacture*

The fresh pork to produce dry cured Westphalian ham is cut in a rectangular shape and weighs approximately 3.5kg. It is specifically made from pork thigh muscles, encompassing the leg outside muscle (*biceps femoris*), leg eye muscle (*semitendinosus*), and partial leg tip (including muscles from the *vastus lateralis*, *anterior rectus femoris*, *vastus intermedius* and *vastus medialis*). In addition, it is usually deboned and has a

layer of fat left along the bottom. The ham is produced through a series of controlled steps that ripen it throughout a 3 month period (in some recipes, Westphalian ham is aged up to 8 months). This process involves the addition of curing agents to the surface of the ham (salt, nitrite and nitrate) to promote water extraction and decrease water activity ( $a_w$ ), which is important to suppress the growth of undesired bacteria (Sperber 1983; McQuestin 2009). Long drying times further decrease the  $a_w$  of the ham, with periods of fermentation and smoking in between. Additionally, throughout the maturation process indigenous microflora develop on the ham surface, which are essential to its ripening. At the end of the dry cured Westphalian ham manufacturing period, the preservation of the ham is assured through the high salt conditions, the increase in acidity and the decrease in  $a_w$ , producing a shelf stable product (Lücke 1986).

### *2.2.2 Development of indigenous microflora during ripening*

The natural aging process of dry cured Westphalian ham depends on the development of indigenous organisms to produce a ham with the desired colour, flavour and texture (Lücke 1986). The initial composition of the microflora on fresh meat depends particularly on the raw materials, how the meat is handled, and the organisms in the surrounding environment. The interactions of these organisms during the ripening process is complex, and the factors that determine types and numbers of organisms that develop include temperature, pH,  $a_w$ , presence of  $O_2$  and additional agents present (salt, nitrite, nitrate and smoke) (Fadda *et al.* 2010).

The most important microorganisms in the manufacture of dry cured ham are lactic acid bacteria (LAB) and non-pathogenic *Staphylococcus*. The main genus of LAB that develop on the ham is *Lactobacillus*, and its species are salt tolerant, can grow at

refrigerated temperatures, and therefore are found to dominate the microflora during the first stage of manufacture (salt brine for ~3 weeks at 4°C).

After the salting stage, hams are removed from the brine and set on racks in controlled conditions (4°C and 70% relative humidity (RH) for ~ 2 wks). Salt equalization occurs throughout the pork muscle, water is drawn out to decrease  $a_w$ , and indigenous microflora continues to develop on the ham surface. Hams are subsequently placed on racks in a smokehouse where temperature and RH are raised to 33°C and 88%, respectively, for ~10d. These conditions encourage the fermentation and growth of desired bacteria, and smoke is added intermittently to prevent mould growth as well as add flavour. During this stage of manufacture, lactobacilli ferment carbohydrates and consequently produce lactic acid. As lactic acid accumulates, the pH of the ham decreases, which helps to preserve the product from undesired bacterial growth (Fadda *et al.* 2010). Though acidity only slightly rises in products made from whole pieces (dry cured ham) compared to products made from ground meat (sausages, salami), the lower pH works in conjunction with the other hurdles such as high salt and low  $a_w$  to preserve the product (Fadda *et al.* 2010; Lorenzo *et al.* 2010).

For the final stages of ham manufacture, hams are removed from the smokehouse and placed on racks in an environment-controlled unit (14°C and 75% RH for 5 wks). This promotes ham drying and decreases  $a_w$ , as well as allows indigenous microflora to further influence the organoleptic qualities of the ham such as flavour and texture development. During ripening, LAB are reduced in number and Gram-positive cocci such as *Staphylococcus* species eventually become dominant (Rodriguez *et al.* 1994; Lorenzo *et al.* 2010). LAB help to create the intended flavour and texture of a dry cured ham by acid production but alone are not sufficient to ensure good sensory quality and

colour formation. LAB are also unable produce catalase, an enzyme that breaks down hydrogen peroxide ( $H_2O_2$ ) generated as a by-product of cellular metabolism, which protects the ham from lipid oxidation, rancidity, and discoloration. The metabolic activity of catalase-positive bacteria, such as staphylococci, is therefore important for proper ham ripening. In addition, staphylococci are particularly important in contributing to the development and stabilization of the cured colour of dry cured ham. Endogenously, these bacteria contain nitrate reductase enzymes that convert nitrate to nitrite and allow formation of nitric oxide. Nitric oxide then combines with the myoglobin in the meat to form nitrosylmyoglobin, which produces the characteristic red colour (Gotterup *et al.* 2007). *Staphylococcus* also contain lipase which breaks down fat to create aroma and flavour during ripening (Lücke 1986; Rodriguez *et al.* 1996; Hugas and Monfort 1997). At the end of manufacture, Westphalian dry cured hams have a dense, soft texture with a salty, smoky flavour.

In comparison, Prosciutto di Parma from Italy and Serrano from Spain are also types of raw hams that are aged through traditional manufacturing processes. Italian Prosciutto is aged for a minimum of 18 months and this yields a ham with a deeper colour that has increased tenderness and is less salty in flavour than Westphalian ham. Spanish Serrano type ham is ripened for a minimum of 7 months and is intense in flavour and has a bright red tone to the flesh. However, though dry cured hams from different countries can vary widely in taste, aroma, texture and quality, they are all similar in that development of microflora is essential to ham ripening and that high salt (~5% salt brine) and decreased  $a_w$  ( $\leq 0.90$ ) are the main factors in ham preservation while pH is only slightly changed during manufacture (Hinrichsen and Pedersen 1995; Flores *et al.* 1997; Lorenzo *et al.* 2010; Campo and Sierra 2011).

The various groups of microorganisms described above are able to tolerate the adverse conditions created in dry cured ham since they have innate resistance to salt, acidity and low  $a_w$ . However, undesirable pathogenic and spoilage bacteria are mainly suppressed by this harsh environment. It is therefore important to note that indigenous microflora development on hams during manufacture is essential not only to help produce an organoleptically acceptable product, but also to aid in establishing a multi-barrier system that preserves the ham (Lücke 1986; Fadda *et al.* 2010; Lorenzo *et al.* 2010).

### **2.3 *Escherichia (E.) coli* O157:H7 in fermented meat products**

#### *2.3.1 E. coli* O157:H7, a foodborne pathogen

*E. coli* O157:H7 was first recognized as a foodborne pathogen in 1982 (Riley *et al.* 1983), and *E. coli* O157:H7 infections have since been reported with increasing frequency. *E. coli* O157:H7 is a serogroup of verotoxigenic/shigatoxigenic *E. coli* (VTEC/STEC) and is part of a subgroup of VTEC, enterohaemorrhagic *E. coli* (EHEC) (Kiranmayi *et al.* 2010). In most countries, *E. coli* O157:H7 is the predominant pathogenic VTEC strain of *E. coli* (Paton and Paton 1998). This organism is especially dangerous since it is highly infectious, with the ability to cause infection in humans at only 10 cells or less (Tilden *et al.* 1996; Buchanan and Doyle 2007; Kiranmayi *et al.* 2010). Symptoms of infection include bloody diarrhoea, abdominal cramping, and in severe cases anemia, profuse bleeding, haemolytic-uraemic syndrome (HUS), and kidney failure may develop (CDC 2010).

Though many foods have been implicated in *E. coli* O157:H7 illness outbreaks, foods of animal origin are considered the main source of *E. coli* O157:H7 infection in

humans (Yu and Chou 1998). Approximately 52% of recorded human disease outbreaks are associated with beef products (Griffin and Tauxe 1991), with the most documented and publicized source of *E. coli* O157:H7 contamination being undercooked ground beef (Brandt *et al.* 1994; Tuttle *et al.*, 1999). This has led to the general presumption that cattle are the primary reservoirs of this organism (Armstrong *et al.* 1996; Buchanan and Doyle 1997). However, further research in this area has shown that *E. coli* O157:H7 has been isolated from a variety of animals, including pigs, sheep, goats, horses and poultry. (Hancock *et al.* 2001; Doane *et al.* 2007).

### 2.3.2 Pigs as potential *E. coli* O157:H7 reservoirs

In general, most strains of *E. coli* are considered harmless and normally inhabit the intestines of healthy animals and humans. However, animals have the potential to act as a reservoir for *E. coli* O157:H7 by harbouring the pathogen without becoming ill. When an animal is slaughtered, intestinal contents or soiled hide that previously carried the pathogen can then contaminate the carcass (Armstrong *et al.* 1996; Hancock *et al.* 2001; Namvar and Warriner 2006). This is supported by evidence from a study by Gill and Jones (1998), who discovered two pig carcasses positive for *E. coli* O157:H7 of forty carcasses tested in a pork slaughtering plant. Moreover, if the meat harbouring the pathogen is used in the manufacture of meat products where there is no effective kill step during processing, contaminated products will be produced. Though contamination can occur at any stage of the manufacturing process, carcass contamination is the most common way *E. coli* O157:H7 ends up in meat products.

Research on the prevalence and occurrence of *E. coli* O157:H7 has primarily been focused on cattle and cattle farms since they have been regarded as the major

reservoir of this pathogen (Buchanan *et al.* 1997). Information is more limited on the presence of *E. coli* O157:H7 in other animals, but several studies have isolated the organism from a variety of animals (including pigs) (Chapman *et al.* 1997; Johnsen *et al.* 2001; Doane *et al.* 2007). Further reports have confirmed that the pathogen has been isolated from healthy pigs, not only in Canada but also around the world, including the United States, Japan, Germany, Sweden, and The Netherlands at rates of 2.0% (n=305), 1.4% (n=221), 7.5% (n=120), 0.08% (n=2446) and 1.4% (n=145), respectively (Beutin *et al.* 1993; Chapman *et al.* 1997; Heuvelink *et al.* 1999; Nakazawa *et al.* 1999; Eriksson *et al.* 2003; Feder *et al.* 2003). This suggests that pigs have the ability to act as a reservoir for the pathogen, though the prevalence in these studies was low compared to rates found in cattle.

Surprisingly, a few studies have found pigs to have an even higher prevalence of *E. coli* O157:H7 than cattle. Borie *et al.* (1997) analyzed for the presence of the pathogen in farm animals and found that a staggering 68.7% of swine were positive for EHEC, compared to only 28.7% of cattle. Likewise, Doane *et al.* (2007) found that 8.9% of pig samples were positive for *E. coli* O157:H7 compared to only 3.6% of cattle. Additionally, not only did the latter authors find a higher occurrence of *E. coli* O157:H7 on swine farms, but also found that the pathogen was persistently present throughout the year, unlike the cattle farms investigated where it was only present seasonally. Moreover, Cornick and Helgerson (2004) took a different approach in investigating the potential of pigs to harbour the pathogen by infecting pigs with *E. coli* O157:H7 (to allow shedding at  $\log 4 \text{ cfu}\cdot\text{g}^{-1}$ ) and examining whether transmission within the herd was possible. They found that the organism was in fact able to be transmitted from infected pigs to naive pigs, with half of the naive pigs exposed becoming infected (6/12). Their

findings suggested that swine do not have an innate resistance to *E. coli* O157:H7; the pathogen can colonize their intestinal tracts, and can be transmitted horizontally through the herd where it has the potential to be sustained in a population once introduced. The authors concluded that pigs could act as a reservoir for the organism. This topic warrants more study since pork has not been identified as an important source of human *E. coli* O157:H7 illness in developed countries.

### *2.3.2 E. coli O157:H7 illness outbreak data and survival in dry cured meat products*

Common belief that growth of harmful pathogens was suppressed under the conditions of fermented meat manufacture led to these products being regarded as safe. Though the manufacture of dry cured ham has been done for centuries, traditional and time-honoured food practices that may have been safe in the past may not be safe today (Buchanan and Doyle 1997). The first recorded outbreak of *E. coli* O157:H7 linked to a dry cured meat product (dry-cured salami) occurred in 1994 in the United States, specifically in Washington and North California, with 17 cases of infection (CDC 1995). In Canada, the first documented outbreak of this pathogen in a fermented meat product took place in British Columbia in 1998, likewise due to dry cured salami, with 39 cases reported (Williams *et al.* 2000). Furthermore, the largest outbreak of *E. coli* O157:H7 ever recorded in Canada occurred the next year, in 1999, also in British Columbia and also linked to dry cured salami (MacDonald *et al.* 2004). In this outbreak, 143 human cases were confirmed for the *E. coli* O157:H7 infection, with the high number of cases a result of the large amount of contaminated product and the broad market distribution.

The significance of *E. coli* O157:H7 as a foodborne pathogen received much attention as a result of the magnitude of this outbreak.

After the first outbreak of *E. coli* O157:H7 infection from fermented meat, the dry cure process was immediately investigated and research proved that the pathogen was capable of surviving the adverse conditions of dry cure manufacture (Glass *et al.* 1992; Hinkens *et al.* 1996; Paton *et al.* 1996; Tilden *et al.* 1996; Calicioglu *et al.* 1997; Faith *et al.* 1998; Nissen *et al.* 1998). Furthermore, *E. coli* O157:H7 had the ability to survive the hurdles of the dry cure process even when companies followed a CFIA-recognized Hazard Analysis and Critical Control Point (HACCP) plan that required regulated ranges of pH and  $a_w$  (MacDonald *et al.* 2004), and even when meat plants complied with regulations, exceeded sanitation requirements, maintained good separation of raw and finished products, and had a stable experienced work force (Tilden *et al.* 1996).

Though the preserving factors of high salt conditions, low pH and low  $a_w$  work together during the manufacturing process to preserve the product (Reynolds *et al.* 2001), when the impact of one of these factors is reduced, the effect of multiple barriers becomes less effective. Research has now shown that *E. coli* O157:H7 has the ability to adapt to acid environments, and acid adaptation of cells can result in the ability to survive longer in low pH foods (Glass *et al.* 1992). This has allowed *E. coli* O157:H7 to be one of the most dangerous pathogens in fermented meat products (Arnold and Kaspar 1995; Leyer *et al.* 1995; Duffy *et al.* 2000) and reinforces the importance of the initial quality of the raw product (Tilden *et al.* 1996). These findings led to significant changes in meat industry operations, with the creation of strict safety guidelines for the manufacturing processes of dry cured meats.

### 2.3.3 Regulations in place involving fermented meat products and *E. coli*

#### *O157:H7*

Measures were taken by government health officials since current standard dry cured meat processes could not be relied upon to produce products dependably free from *E. coli* O157:H7. In 1996, the United States Department of Agriculture (USDA) set guidelines that required all commercial producers of dry or semi-dry fermented sausages to implement one of the following safety options: achieve a 5 log kill of *E. coli* O157:H7 using a thermal process, develop a manufacturing process and validate it with a 5 log reduction in *E. coli* O157:H7, perform microbiological end-product testing of each lot and hold finished product until results are obtained, initiate a HACCP system that includes raw meat/batter testing and a process that achieves a 2 log reduction of *E. coli* O157:H7, or use an alternative manufacturing process that has scientifically demonstrated a 5 log reduction in *E. coli* O157:H7 (CFIA 2010). Canada did not implement these guidelines until years later, after the largest outbreak in Canada due to dry cured salami occurred in 1999 (MacDonald *et al.* 2004; CFIA 2010). Though all choices listed are viable options as they would ensure a safe product for consumers, several options in the above list are not as attractive as others to manufacturers for several reasons. The heat processing option would ensure elimination of the pathogen, but it would also destroy product integrity causing it to no longer resemble the original fermented meat product (Hinkens *et al.* 1996). Additionally, the batch and batter testing options are time consuming and are high in cost. Therefore, developing and validating a dry cure process that achieves a 5 log decrease of *E. coli* O157:H7 may be the most preferable option.

A vast amount of research in this area is focused on meat products that contain beef, therefore investigations into the validity of the dry cured manufacture of salami and sausages is well documented. In contrast, there is little information on the validation of the dry cured ham manufacturing process. Although no documents exist linking dry cured ham to an outbreak, research has shown that *E. coli* O157:H7 can survive this process as well (Graumann and Holley 2007). Interestingly, facilities manufacturing pork only products are not required to follow the same regulations as facilities that use beef ingredients that are required to validate *E. coli* O157:H7 absence from finished products (CFIA 2010).

Reynolds *et al.* (2001) conducted the first study on the ability of the dry cured ham process to sufficiently reduce *E. coli* O157:H7 to meet safety requirements for dry cured meats. Hams were inoculated with  $7 \log \text{ cfu} \cdot \text{g}^{-1}$  *E. coli* O157:H7 at the beginning of the process and ripened for 120d. By 69d, the pathogen decreased by  $5.5 \log \text{ cfu} \cdot \text{g}^{-1}$  and this validated the manufacturing process. Graumann and Holley (2008) also validated the Westphalian dry cured ham procedure, in which control hams were initially inoculated with  $7 \log \text{ cfu} \cdot \text{g}^{-1}$  *E. coli* O157:H7. The pathogen was reduced by  $5 \log \text{ cfu} \cdot \text{g}^{-1}$  at the end of the ripening period (80d) by the dry cure process. In another study by Portocarrero *et al.* (2002), it took 66d ripening to decrease *E. coli* O157:H7 to below the detection limit in country-cured hams initially inoculated with  $7-8 \log \text{ cfu} \cdot \text{g}^{-1}$  *E. coli* O157:H7. The hams made by Portocarrero *et al.* (2002) were held for a longer ripening period (234d) than the other studies, which resulted in the complete elimination of *E. coli* O157:H7 (the last viable cells were seen at 178d). The previous studies had ripening periods of 120d and 80d, respectively, and both found that *E. coli* O157:H7 cells were still viable after enrichment. This suggests that extended ripening times may be essential

in completely eliminating the pathogen from the product, though all results met the requirements of the USDA and CFIA guidelines for dry and semi-dry fermented meats.

Since dry cured hams have long production times, many manufacturers have looked for alternative means to speed up ham maturation. In 2007, Graumann and Holley tested the safety of a procedure for dry cured Westphalian hams that used a needle tenderizing treatment to shorten ripening time. Hams were initially inoculated with  $7 \log \text{ cfu}\cdot\text{g}^{-1}$  *E. coli* O157:H7, and following the needle tenderization step, their interiors were subsequently contaminated with  $4 \log \text{ cfu}\cdot\text{g}^{-1}$  *E. coli* O157:H7. After 112d of ripening, the organism was eliminated from the surface of the ham but was recovered internally at  $3.1 \log \text{ cfu}\cdot\text{g}^{-1}$ . This suggests that the dry cure Westphalian ham process may be successful in eliminating surface contamination of *E. coli* O157:H7, but using needle-tenderization to speed up the curing process is not recommended due to the possible survival of the pathogen inside of the ham.

Though the dry cured ham process was validated by these studies, more research is needed to confirm the ability of fermented ham manufacture to control *E. coli* O157:H7. Since the pathogen has such a dangerously low infectious dose in humans, different measures to help encourage elimination of the pathogen in fermented meats are being looked into. One alternative is to add antimicrobial agents during the dry cure process to help eliminate *E. coli* O157:H7 from these products.

## **2.4 Mustard as a natural antimicrobial in fermented meat products**

### *2.4.1 Mustard and its antimicrobial activity*

Canada is the world's second largest mustard producer, and exports more mustard than any other country in the world (AAFC 2008). The three major crops grown

are oriental (*Brassica juncea*), brown (*Brassica napis*) and yellow (*Sinapis alba*). Mustard (when deodorized) is primarily used in the meat industry as an emulsifier, a water-binding agent, and a filler in cooked cured meats. Without deodorizing, it is used as a flavouring agent to season various meat products such as hot dogs, bologna, sausages and salami. Mustard belongs to the plant family *Brassicaceae*, which naturally contains glucosinolate compounds. When glucosinolates are acted upon by the mustard's endogenous enzyme myrosinase (EC 3.2.1.147), antimicrobial isothiocyanates are generated (in the presence of water) (Ekanayake *et al.* 2006). This reaction, however, does not happen spontaneously as the mustard seed itself contains the glucosinolate in a separate compartment from that of the myrosinase enzyme. It is not until physical disruption of the cell walls occurs that myrosinase comes in contact with its substrate, catalyzing the hydrolysis of the glucosinolate and producing isothiocyanate (Kawakishi and Muramatsu 1966; Delaquis and Mazza 1995).

Isothiocyanates are responsible for the hot spicy flavour of mustard. Different isothiocyanates are present in different mustard types, each of which produces unique flavours. The glucosinolate sinigrin, dominant in oriental and brown mustards, yields volatile allyl isothiocyanate (AIT) which produces a sharp taste and pungent aroma. Yellow (white) mustard contains sinalbin as the major glucosinolate, which yields  $\rho$ -hydroxybenzyl isothiocyanate (PHBIT), responsible for the hot mouthfeel (Buskov *et al.* 2000). Interestingly, the isothiocyanate compound that gives mustard this flavour is also antimicrobial in nature. In the last few decades, mustard has been studied as a natural antimicrobial agent for use in food products. Focus has also been given to the use of mustard in fermented meats since there is no heat kill step to ensure pathogen elimination from these products (Chacon *et al.* 2006; Graumann and Holley 2008;

Luciano *et al.* 2011). Though the mechanism of action of isothiocyanates is still unknown, it is suggested that bacteria are inactivated through multi-targeted activity that causes membrane damage and enzymatic inhibition (Lin *et al.* 2000; Luciano and Holley 2009).

AIT has been reported to inhibit a variety of pathogens including *E. coli* O157:H7 (Kanemaru and Miyamoto 1990; Delaquis and Sholberg 1997; Nadarajah *et al.* 2005; Graumann and Holley 2009; Luciano and Holley 2009). Rhee *et al.* 2002 reported that *E. coli* O157:H7 at levels of  $6.5 \log \text{ cfu}\cdot\text{g}^{-1}$  was reduced to under  $0.3 \log \text{ cfu}\cdot\text{g}^{-1}$  after 7d at  $5^{\circ}\text{C}$  on agar with 10% Baltimore and Coleman mustard powder containing AIT. Luciano and Holley (2009) found that the minimum inhibitory concentrations of pure AIT against *E. coli* O157:H7 ( $7 \log \text{ cfu}\cdot\text{g}^{-1}$ ) varied with the pH of broth from  $50 \text{ ul}\cdot\text{l}^{-1}$  at pH 6.5 to  $500 \text{ ul}\cdot\text{l}^{-1}$  as pH increased to 8.5 during 48h, which suggests that AIT may be more antimicrobial in high acid foods. In addition, Delaquis and Sholberg (1997) found that  $1,500 \text{ ul}\cdot\text{l}^{-1}$  volatized AIT was required to produce the same outcome (reduction of  $7 \log \text{ cfu}/\text{cm}^2$ ) on neutral agar surfaces within 72h. On the other hand, Mayerhauser (2001) inoculated yellow mustard with *E. coli* O157:H7 at levels of  $6 \log \text{ cfu}\cdot\text{g}^{-1}$  and since no viable cells were seen after only 1h (at 5 and  $25^{\circ}\text{C}$ ), this was interpreted to mean that PHBIT was very effective for elimination of *E. coli* O157:H7 contamination. Comparatively, PHBIT was shown to reduce *E. coli* in neutral broth by over  $6 \log \text{ cfu}\cdot\text{g}^{-1}$  at levels of  $360 \text{ mg}\cdot\text{l}^{-1}$ , after 5d at  $6.5^{\circ}\text{C}$  (Ekanayake *et al.* 2006). Hence, the antimicrobial activity of isothiocyanates AIT from brown and oriental mustard and PHBIT from yellow and white mustard were comparable in *in vitro* studies.

Though *in vitro* studies may tell us how this microorganism reacts in a lab setting, it may not tell us how it will be affected by isothiocyanates in food, since food

composition can greatly affect an organism's ability to survive. Therefore, the antimicrobial properties of isothiocyanates have also been examined in fermented meat products for their potential to help the dry cured process achieve the mandatory 5 log decrease in *E. coli* O157:H7 (Chacon *et al.* 2006; Graumann and Holley 2008; Graumann and Holley 2009; Luciano *et al.* 2011; Cordiero *et al.* 2011). In a study by Chacon *et al.* (2006), AIT treatments of 500, 750 and 1000ppm were tested in dry fermented sausages inoculated with  $6.5 \log \text{ cfu} \cdot \text{g}^{-1}$  *E. coli* O157:H7. The authors found that AIT at 750 and 1000ppm reduced *E. coli* O157:H7 to undetectable levels after 21 and 16d, respectively, and in the 500ppm treatment *E. coli* O157:H7 was reduced by  $4.75 \log \text{ cfu} \cdot \text{g}^{-1}$  after day 28 of ripening. Though the control was only able to reduce the pathogen by  $2.4 \log \text{ cfu} \cdot \text{g}^{-1}$  by the end of the ripening period (45d), all AIT treatments completely inhibited *E. coli* O157:H7 by day 40. A few years later, Graumann and Holley (2009) tested AIT again for its potential to help the dry cured process eliminate the pathogen, this time in dry cured hams, and used treatments with less AIT since the panellists in the previous study found levels above 500ppm unacceptable (due to spiciness). Hams were inoculated with  $6.5\text{-}7.5 \log \text{ cfu} \cdot \text{g}^{-1}$  *E. coli* O157:H7 and treatments of 200, 300 and  $400 \mu\text{g} \cdot \text{kg}^{-1}$  microencapsulated AIT were applied, as well as a fourth treatment of  $60 \text{g} \cdot \text{kg}^{-1}$  hot yellow mustard powder. With the control only showing a  $2 \log \text{ cfu} \cdot \text{g}^{-1}$  reduction in 45d, it was impressive that treatments of 300 and  $400 \mu\text{g} \cdot \text{kg}^{-1}$  AIT and  $60 \text{g} \cdot \text{kg}^{-1}$  yellow mustard powder reduced the organism by over  $5 \log \text{ cfu} \cdot \text{g}^{-1}$  within the same period. Interestingly, the yellow mustard powder was even faster in reducing *E. coli* O157:H7 viability with a decrease of  $4 \log \text{ cfu} \cdot \text{g}^{-1}$  in only 21d.

However, though isothiocyanates have been demonstrated to have significant antimicrobial action against *E. coli* O157:H7, they are highly unstable and have been

found to last only a few hours before degrading into other non-antimicrobial products (Kawakishi and Muramatsu 1966; Buskov *et al.* 2000; Ekanayake *et al.* 2006). AIT is highly volatile, which makes it difficult to work with. Various forms of AIT, such as after microencapsulation, have been investigated to try and increase its manageability. Contrarily, research showed that yellow mustard in a powdered form stabilized PHBIT, making it easy to work with and thus very practical in an industry setting. Graumann and Holley (2008) examined both microencapsulated AIT as well as PHBIT from yellow mustard powder in fermented meats, and suggested that yellow mustard powder was the better alternative since AIT's volatility was a problem. Interestingly, the same authors also found that yellow mustard powder produced a quicker reduction in *E. coli* O157:H7, suggesting that PHBIT has more antimicrobial action. Therefore, the antimicrobial properties of yellow mustard powder are the focus of the next section.

#### *2.4.2 Yellow mustard powder in fermented meat products*

Yellow mustard powder has many applications in the meat industry. Non-deodorized (non-deheated or hot) yellow mustard powder has been widely used as a flavouring agent, with PHBIT causing a spicy hot mouthfeel and strong pungent odour. However, its spiciness also causes it to have limited use in food since consumers may not find this desirable (Ekanayake *et al.* 2006; Chacon *et al.* 2006). This limitation was overcome with the introduction of deodorized (deheated or cold) yellow mustard powder. Deodorized yellow mustard powder is produced by holding the mustard powder at high temperatures to inactivate the enzyme myrosinase (Brabban and Edwards 1994), with inactivation starting at 60°C (Van Eylen *et al.* 2006). The glucosinolates in the mustard powder are not affected by this process (Brabban and Edwards 1994), and since

they are left intact, isothiocyanates are not generated upon the addition of moisture. Deodorized yellow mustard powder therefore has a bland taste because PHBIT is not produced. It has been widely accepted and extensively used in the meat industry due to its excellent emulsifying, thickening, bulking properties and high water-binding capacity, and in addition its neutral flavour avoids causing adverse flavour effects to the product it is added to.

Deodorized yellow mustard powder is more stable than non-deodorized yellow mustard powder (where in moist conditions the myrosinase enzyme will rapidly initiate glucosinolate hydrolysis) and is therefore easier to work with. Interestingly, studies have shown that deodorized yellow mustard powder, even with myrosinase inactivated, still has antimicrobial properties. Moreover, research suggests that it is the microorganisms themselves that may have myrosinase-like activity. It is theorized that to obtain an energy source during periods of low nutrient availability, the microorganisms present hydrolyse the glucosinolate to remove a glucose molecule. However, in doing so PHBIT is released, which then destroys the microorganism in an ironic bactericidal, or self-destroying action (Luciano *et al.* 2011).

Brabban and Edwards (1994) and Luciano *et al.* (2011) noticed that some microorganisms use glucosinolates to a greater extent than others. Brabban and Edwards (1994) found that Gram positive bacteria degraded sinigrin and other natural glucosinolates found in rapemeal, with some *Staphylococcus* species having greater degradation rates. Sinigrin degradation occurred predominantly after other sources of glucose were almost exhausted, suggesting that glucose is preferentially utilized before sinigrin is hydrolyzed. Luciano *et al.* (2011) also demonstrated that *Staphylococcus* had one of the highest myrosinase-like activities of the fermented sausage starter cultures

tested. However, quite unexpectedly it was found that the pathogen *E. coli* O157:H7 had the highest rates of glucosinolate degradation and myrosinase-like activity of any of the 24 different starter cultures tested (Luciano *et al.* 2011). This suggests deodorized mustard powder could be an effective option to use in dry cured fermented meat products for control of *E. coli* O157:H7.

Graumann and Holley (2009) showed that use of 6% (w/w) non-deodorized yellow mustard powder on dry cured hams inoculated with  $7 \log \text{cfu}\cdot\text{g}^{-1}$  *E. coli* O157:H7 reduced the pathogen by  $4 \log \text{cfu}\cdot\text{g}^{-1}$  in 21d, and it was further reduced to below the detection limit at 45d. The same authors (Graumann and Holley 2008) also investigated the effect of 6% deodorized mustard powder, as well as 2, 4, and 6% non-deodorized yellow mustard powder, in dry fermented sausages inoculated with  $7 \log \text{cfu}\cdot\text{g}^{-1}$  *E. coli* O157:H7. Non-deodorized mustard powder at 2, 4 and 6% resulted in significant reductions of 3.4, 4.4 and  $6.9 \log \text{cfu}\cdot\text{g}^{-1}$ , respectively, in 30d. When the drying period was extended to 36 and 48d, the pathogen was reduced by over  $5 \log \text{cfu}\cdot\text{g}^{-1}$  with both 2 and 4% non-deodorized yellow mustard powder. More importantly, the 6% deodorized yellow mustard powder provided the most rapid reduction of *E. coli* O157:H7, yielding undetectable levels ( $<0.20 \log \text{cfu}\cdot\text{g}^{-1}$ ) after 24 d. Similarly, Cordeiro *et al.* (2011) examined the effects of both deodorized and non-deodorized yellow mustard powder in dry cured sausages, but treatments included deodorized and non-deodorized yellow mustard powder mixes (50/50). Dry cured sausages were inoculated with  $7 \log \text{cfu}\cdot\text{g}^{-1}$  *E. coli* O157:H7 with 2 or 4% yellow mustard powder added (including a deodorized only treatment (by autoclave) and a deodorized + non-deodorized yellow mustard powder mix). The 2% treatments did not reduce the pathogen by the mandatory 5 log reduction during ripening, but the 4% deodorized yellow mustard powder, and 4% mixed yellow

mustard powder did achieve this decrease at 35 d, with the control treatment yielding only a 2 log reduction by day 42 at the end of the ripening period.

Luciano *et al.* (2011) also investigated the effect of yellow mustard powder in dry fermented sausages but further examined the cause of the bactericidal activity. Dry fermented sausage batter was inoculated with  $6.5 \log \text{ cfu} \cdot \text{g}^{-1}$  *E. coli* O157:H7 and two starter cultures, *Staphylococcus carnosus* UM123M and *Pediococcus pentosaceus* UM121P (which were previously found to be active in degrading glucosinolates). Treatments of 6% non-deodorized, deodorized (by dry thermal treatment) and deodorized (by autoclave) yellow mustard powder were used and after 31 and 38d, results showed that over a  $5 \log \text{ cfu} \cdot \text{g}^{-1}$  decrease in *E. coli* O157:H7 occurred with non-deodorized and deodorized (by dry thermal treatment) yellow mustard powder, respectively, which was not achieved with the control. Interestingly, the deodorized (by autoclave) yellow mustard powder caused the most rapid action against *E. coli* O157:H7, yielding  $>5 \log \text{ cfu} \cdot \text{g}^{-1}$  reduction in 18d. The authors theorized that this could have been due to the formation or release of antimicrobial substances such as phenolic compounds during the autoclave treatment of the yellow mustard powder. Since the starter cultures had little effect on the results, the antimicrobial effect of the deodorized mustard powder was attributed to the bactericidal activity of *E. coli* O157:H7.

There is increasing demand by consumers for all-natural products that are minimally processed. Though the biopreservation of dry cured ham is a natural process, it may not be sufficient to produce a pathogen-free product. With the addition of yellow mustard powder, dry cured sausage processes have been able to ensure the 5 log kill of *E. coli* O157:H7 in accordance with the CFIA and USDA mandatory requirements.

Therefore, deodorized yellow mustard powder is an attractive alternative for use as an added antimicrobial in fermented meat products.

## CHAPTER 3

### Use of deodorized yellow mustard powder to control *Escherichia coli* O157:H7 on dry cured Westphalian ham

#### 3.1 Abstract

Dry cured (uncooked) hams with low water activity and  $\text{pH} \geq 5.6$  seem likely food vehicles for *E. coli* O157:H7. In previous work isothiocyanates produced from mustard glucosinolates by bacterial myrosinase-like activity converted deodorized mustard into a potent antimicrobial in dry sausage. In this study its value in controlling *E. coli* O157:H7 survival in Westphalian ham was investigated. Hams were surface inoculated with a  $7.5 \log \text{cfu} \cdot \text{g}^{-1}$  cocktail of *E. coli* O157:H7, followed by surface application of 4% or 6% (w/w) deodorized yellow mustard powder, then monitored during 80d ham manufacture for *E. coli* O157:H7 survival. In one trial to accelerate formation of isothiocyanate, a *Staphylococcus (S.) carnosus* meat starter culture was added to hams at 45d (after salt equilibration). At 21d, *E. coli* O157:H7 was reduced by  $3 \log \text{cfu} \cdot \text{g}^{-1}$  on hams treated with mustard powder compared to only a  $1 \log \text{cfu} \cdot \text{g}^{-1}$  reduction in the control. The addition of mustard powder resulted in a reduction of  $>5 \log \text{cfu} \cdot \text{g}^{-1}$  *E. coli* O157:H7 by 45d, whereas it took 80d for numbers in control hams to be similarly reduced. Although the commercial process resulted in a  $5 \log \text{cfu} \cdot \text{g}^{-1}$  reduction of *E. coli* O157:H7 in 80d, 4% or 6% deodorized mustard accelerated this reduction and the *S. carnosus* starter culture may have contributed to the maintenance of this effect.

### 3.2 Introduction

Dry cured Westphalian ham is an artisanal uncooked, ready-to-eat (RTE) meat product originally from the Westphalian region of Germany. It is made from fresh whole pork muscle preserved by aging with salt, cure agents and smoke followed by drying. The process can take up to 8 months and involves fermentation during which an indigenous microflora containing lactic acid bacteria (LAB) and staphylococci develops (Lücke 1986). Though the preservation of meat by fermentation has been done for centuries, problems regarding fermented meat safety occur and are reported more frequently with dry sausages than dry cured ham.

Foodborne illness outbreaks caused by *E. coli* O157:H7 in dry cured sausage were first reported 12 years after this organism was discovered to be foodborne in 1982 (Riley *et al.* 1983). Investigation has established that the preservation process used during dry sausage fermentation is inadequate to eliminate *E. coli* O157:H7 if present, since it tolerates the high salt, low pH, and low water activity ( $a_w$ ) generated (Glass *et al.* 1992; Hinkens *et al.* 1996; Paton *et al.* 1996; Tilden *et al.* 1996; Calicioglu *et al.* 1997; Faith *et al.* 1998; Nissen and Holck 1998). If internalized in dry cured ham by injection, the organism can survive in the ripened product (Graumann and Holley 2007). *E. coli* O157:H7 is especially dangerous since it has an infectious dose of  $\leq 10$  cells (Kiranmayi *et al.* 2010). Symptoms following infection include bloody diarrhoea, abdominal cramping, and in about 5% of cases, haemolytic-uraemic syndrome (HUS) (CDC 2010).

The first outbreak of *E. coli* O157:H7 from consumption of dry cured salami occurred in 1994 in the US (CDC 1995). The USDA implemented strict guidelines to ensure a 5 log kill of *E. coli* O157:H7 in all dry and semi-dry uncooked fermented sausages containing beef ingredients (USDA 2005). The CFIA adopted these regulations

after Canada's first documented illness outbreak from fermented sausages (1998, British Columbia) which was followed by another outbreak in 1999 (the largest *E. coli* O157:H7 outbreak recorded in Canada), also due to dry cured salami (CFIA 2010; Williams *et al.* 2000; MacDonald *et al.* 2004).

Cattle have been a major focus of *E. coli* O157:H7 investigations, as they are regarded as the primary reservoirs of the pathogen. Approximately 52% of *E. coli* O157:H7 outbreaks are associated with beef products, with undercooked ground beef most often the cause (Griffin and Tauxe 1991; Brandt *et al.* 1994; Tuttle *et al.* 1999). However, hogs have been found to be potential *E. coli* O157:H7 reservoirs, and more alarmingly, in some cases the pathogen prevalence has been observed to be higher in pigs (Borie *et al.* 1997; Doane *et al.* 2007). Though there is no documentation linking dry cured ham with an outbreak of *E. coli* O157:H7, pork in dry cured salami has been linked to an outbreak (Paton *et al.* 1996). However, facilities processing only pork are not required to follow the same guidelines for *E. coli* O157:H7 control as those manufacturing products that contain beef (CFIA 2010).

To improve the safety of raw dry cured products, the addition of other agents during manufacture to control *E. coli* O157:H7 is of interest. One alternative with promise is mustard, which has natural antimicrobial properties. All plants in the *Brassicaceae* family contain glucosinolates as secondary metabolites, and yellow mustard (produced in largest amounts in Canada) contains the glucosinolate sinalbin. Upon physical damage of the plant tissue, hydrolysis of sinalbin is catalyzed by the endogenous enzyme myrosinase (EC 3.2.1.147) in the presence of moisture to produce the antimicrobial p-hydroxybenzyl isothiocyanate (PHBIT) (Kawakishi and Muramatsu 1966; Delaquis and Mazza 1995; Ekanayake *et al.* 2006). The mechanism of PHBIT

antimicrobial action is uncertain, but it may inhibit essential enzymes and cause membrane damage (Lin *et al.* 2000).

PHBIT is not only antimicrobial, it is also responsible for the hot, spicy characteristics of yellow mustard (Buskov *et al.* 2000). Since this compound can overpower other flavours, the use of yellow mustard as a food ingredient has been limited. However, the optional inactivation of myrosinase by thermal treatment to deodorize mustard has broadened the range of food products where it can be used as a neutral-flavoured, high protein ingredient. Deodorized (deheated) mustard is extensively used as a binder-extender in cooked meat products, contributing to texture without flavour impact. However, glucosinolates are still present. Interestingly, studies have shown that even with myrosinase inactivated, deodorized or deheated yellow mustard powder was lethal to *E. coli* O157:H7 (Graumann and Holley 2008), which suggested that sinalbin was somehow hydrolyzed to produce PHBIT.

Recently it was shown that *E. coli* O157:H7, other pathogens and some meat starter cultures have myrosinase-like activity which converted glucosinolates sinalbin and sinigrin into antimicrobial isothiocyanates (Luciano *et al.* 2011, unpublished, this laboratory). In theory, by hydrolyzing the glucosinolate in mustard, the myrosinase positive organisms can assimilate energy from the resulting glucose molecule, though this exposes the organisms to the lethal effects of the isothiocyanate PHBIT in the case of yellow mustard. However, since glucosinolate is not a readily available food source and requires energy to extract a glucose molecule from the glucosinolate, it seems as though glycosinolate hydrolysis from bacterial enzymes only occurs when all other available sources of energy are exhausted. Other sources of energy may not be available due to competition for nutrients from other bacterial species or the adverse conditions

created on the meat during dry cure manufacture. Glucosinolate hydrolysis of deodorized mustard was not observed on fresh meat inoculated with *E. coli* O157:H7, likely because the pathogen was able to obtain nutrients from the meat, which resulted in no isothiocyanate formation from mustard and thus no pathogen reduction due to isothiocyanate challenge (Muthukumarasamy *et al.*, 2004). Of further interest, it was found that of the microorganisms tested, *E. coli* O157:H7 had the greatest capacity for glucosinolate degradation, while the meat starter culture *S. carnosus* UM123M showed the second greatest rate of glucosinolate hydrolysis (250.3  $\mu\text{M}$  for *S. carnosus* UM123M versus 507.9 for  $\mu\text{M}$  *E. coli* O157:H7, after 6d at 25°C) (Luciano and Holley 2010; Luciano *et al.* 2011).

Work has shown that yellow mustard was dependably effective in eliminating *E. coli* O157:H7 from dry cured meats (Graumann and Holley 2008 & 2009, Cordiero *et al.* 2011; Luciano *et al.* 2011). The purpose of the present study was to investigate whether the surface application of deodorized yellow mustard powder to dry cured Westphalian ham at levels of 4% and 6% would consistently prevent *E. coli* O157:H7 survival. The study was also designed to determine whether the starter culture *S. carnosus* UM123M could contribute toward the elimination of *E. coli* O157:H7 from ham in the presence of deodorized mustard by increasing the total rate of glucosinolate and providing a continuous source of PHBIT.

### **3.3 Materials and methods**

#### *3.3.1 Preparation of E. coli O157:H7*

A five strain mixture of *E. coli* O157: H7 was prepared for inoculation of dry cured Westphalian hams. Strains of *E. coli* O157:H7 (non-pathogenic, human isolates)

included 00:3581, 02:0304, 02:0628, 02:0627, and 02:1840, and were supplied by Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency, Canadian Center for Human and Animal Health, Winnipeg, MB, Canada. Strains were revived from frozen stock and streaked on tryptic soy agar (TSA; Oxoid Ltd, Basingstoke, UK) twice and incubated at 37°C for 24h. All strains were grown separately by transferring one colony of each strain to a tube of 10ml tryptic soy broth (TSB; Oxoid Ltd.) and grown overnight at 37°C. From each of the 5 tubes, 100µl was transferred into 1L TSB separately (5L total) and grown overnight at 37°C. The optical density (OD) (600<sub>nm</sub>) of each strain was measured in a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Cambridge, UK) to check bacterial growth. When culture OD<sub>600nm</sub> reached an absorbance (A) of 1.3-1.5, it was centrifuged at 1643 x g at 4°C for 10 min (Sorvall Instruments RC-5C, DuPont, Newton, CT, USA), washed with 0.1% (w/v) peptone water (Fisher Scientific, Fair Lawn, NJ, USA), and recentrifuged as before. Finally, cultures were resuspended in peptone water and combined in a large pail to generate 5L of a 5 strain *E. coli* O157:H7 mixture sufficient to yield a level of 7-7.5 log cfu·g<sup>-1</sup> on the ham surfaces. This inoculation level was chosen to accommodate a >5 log cfu·g<sup>-1</sup> decrease of the pathogen during the experiments. For inoculation, hams were dipped into the 5L pail containing the *E. coli* O157:H7 cocktail, held for 4s to facilitate transfer, removed and held over the pail for an additional 4s as excess inoculum dripped off. Hams were then processed according to the dry cured Westphalian ham manufacture described in section 3.3.4.

### 3.3.2 Preparation of *S. carnosus* UM123M

*S. carnosus* UM123M (isolated from a commercial meat starter culture mixture Lactacel 115, Microlife Technics, Sarasota, FL, USA) was revived from a frozen stock

culture and transferred to TSA (Oxoid Ltd.) twice and incubated at 37°C for 24h. One representative colony was transferred to 10ml TSB (Oxoid Ltd.) and grown overnight at 37°C, from which 300ul was then transferred into 3.5L TSB (Oxoid Ltd.). On the day of inoculation, OD (600<sub>nm</sub>) was measured and when an OD<sub>600nm</sub> of A=1.3-1.5 was reached it was centrifuged at 1643 x g and 4°C for 10 min, washed with 0.1% peptone water and recentrifuged as before. The culture was then resuspended with 0.1% peptone water to a volume of 3.5L, which was inoculated onto the hams at day 45 of trial 2. The delayed inoculation of *S. carnosus* avoided an exposure to high initial NaCl concentrations resulting from surface rubbing of salt and cure, unlike *E. coli* O157:H7 inoculation at 0d which subjected the pathogen to all stages of dry cured Westphalian ham manufacture. Therefore, to support *S. carnosus* survival, its addition took place after salt had equilibrated in the tissue at 4°C and as the hams were moved to 14°C. The hams were inoculated by dipping a thick cloth (Cheesecloth wipes; Fisher Scientific) into the *S. carnosus* culture and wrapping it around the surface of the ham. The cloth was left on the ham overnight and removed the next day to facilitate transfer. This method was used as an alternative to dipping the ham itself in the inoculum, because it was thought that the number of *E. coli* O157:H7 remaining could be reduced by washing off into the inoculum.

### 3.3.3 Yellow mustard powder

Commercial non-deodorized yellow mustard powder from *Sinapis alba* (Newly Weds Foods, Edmonton, AB, Canada) was deodorized by autoclaving at 115°C for 15 min. To confirm that myrosinase had been inactivated, a 5g sample of the autoclaved yellow mustard powder was transferred to 250ml of distilled water, stirred continuously

for 1 h, and screened for the presence of sinalbin with high performance liquid chromatography (HPLC; Waters 2695, Waters Corp., Milford, MA, USA) according to the method of Luciano and Holley (2009). When HPLC showed that sinalbin levels were stable the mustard powder was used in the tests (the initial sinalbin concentration of deodorized mustard powder was 1648 ppm). A single thermal treatment was sufficient to inactivate myrosinase. Treatments of 4% ( $40\text{g}\cdot\text{kg}^{-1}$ ) and 6% ( $60\text{g}\cdot\text{kg}^{-1}$ ) mustard powder were applied evenly to the surface of the pork.

#### *3.3.4 Dry cured Westphalian ham manufacture*

The Westphalian dry cured ham manufacturing process took 80 days to complete and involved 4 main stages of manufacture: salt “brining”, “burning” (where salt equalization, cure reactions and drying occurred), fermentation/smoking, and “resting” which further promoted drying and curing. Two trials were conducted in this study, with 4 treatments per trial. Treatment 1 was an uninoculated control (0% mustard powder and no *E. coli* O157:H7 inoculation). Treatment 2 was an *E. coli* O157:H7 inoculated control, and treatments 3 and 4 were inoculated with *E. coli* O157:H7 and had either 4% or 6% deodorized yellow mustard powder added to the ham surface, respectively.

Forty-eight pork thighs were used per trial, with 12 hams in each of the 4 treatments. Fresh pork, obtained from Miller’s Meats (Winnipeg, MB, Canada), were cut to commercial specifications (155mm x 265mm x 100mm, Piller’s Sausages and Delicatessens, Waterloo, ON, Canada) and weighed approximately 3.5kg each. For treatment 1, 12 pork pieces were surface rubbed with curing agents ( $41\text{g}\cdot\text{kg}^{-1}$  salt,  $6\text{g}\cdot\text{kg}^{-1}$  nitrite/nitrate and  $7\text{g}\cdot\text{kg}^{-1}$  spices - Piller’s Sausages and Delicatessens) and separate treatments were placed together horizontally in a large plastic tub (45 x 55 x 30cm)

which was pressed by a second weighted tub and sealed with a lid. Pork in treatments 2, 3 and 4 was inoculated by dipping the meat for 4s in 5L of the *E. coli* O157:H7 mixture, removed and held for 4s to allow excess inoculum to drip off ham surface, then surface applied with salt/cure and 0%, 4% or 6% deodorized yellow mustard powder before the meat was pressed in tubs. All 4 treatments were placed in a 3m x3m x2m cold room and held at 4°C for 3 weeks under a force of 50g/cm<sup>2</sup>. During this stage, the salt and the pressure promoted water extraction, created a salt brine (~5% salt) and decreased the water activity ( $a_w$ ) of the pork muscle. During salt equilibration LAB are able to multiply at 4°C and high salt concentrations, and later contribute to the ham organoleptic properties.

At the “burning” stage, hams were removed from the brine and placed horizontally on racks in the cold room at 4°C and a relative humidity (RH) of 70% for 2 weeks. This allowed for continued salt equalization to occur and promoted drying. Then hams were placed in a computer controlled smokehouse (Titan, Maurer AG, Reichenau, Germany) for 10d to allow fermentation at 33°C and 88% RH. At this stage, LAB ferment available carbohydrates and produce lactic acid, which slightly lowers the pH. Additionally, *Staphylococcus spp.* are able to grow during this period and contribute to colour stability by catalase production and to safety by reducing NO<sub>3</sub> to NO<sub>2</sub>. Smoke from burning beech wood chips was also applied intermittently to control mould growth and promote flavour development.

At the next stage of manufacture, “resting”, hams were removed from the smokehouse and placed back onto racks in the cold room at 14°C and 75% RH for 5 weeks. This allowed further curing and drying to take place and promoted *Staphylococcus* growth. During this stage, hams were smoked once per week for 15 min

to control mould growth and by day 80, dry cured Westphalian ham manufacture was complete.

### 3.3.5 Sampling and analysis

Throughout ham ripening, levels of inoculated *E. coli* O157:H7, as well as the indigenous microflora (*Staphylococcus*, aerobic mesophiles, LAB, and yeast), and pH plus  $a_w$  were monitored at the ham surface and interior. At day 0 and after the completion of each stage during the manufacturing process (day 21, 35, 45, 62 and 80), 2 hams per treatment were analyzed. At sampling, 25g meat tissue was removed aseptically from the surface ( $\leq 2$ mm thick) and interior ( $\geq 2$ cm deep) of the ham. The surface samples were retrieved by cutting off tissue with a knife on random parts of the ham surface until 25g was achieved. To retrieve interior ham samples, the surface tissue of the ham was cut off with a knife and the new exposed surface was torched with a flame to kill any further surface bacteria that could contaminate the interior tissues. A core borer (3.5cm diameter) was then pushed through the ham and the resulting sample had each end cut off to provide an interior sample with a final weight of 25g. All surface and interior ham samples were placed in a stomacher bag (Filtrabag; VWR, Edmonton, AB, Canada), diluted with 225ml 0.1% peptone water (Fisher Scientific) and homogenized 4 min with a stomacher (BagMixer 400, Intersciences Inc., Markham, ON, Canada). Serial dilutions were plated using an Autoplate 400 spiral plater (Spiral Biotech, Norwood, CT, USA).

Additionally, it was uncertain as to whether *E. coli* O157:H7 could survive in the seam or fold between muscles of the ham after ripening. To explore this further, collagen sheets were used to detect seam contamination. During meat processing,

collagen is commonly used in the form of casings to provide sausage shape, and single sheets are used to partially wrap dry cured hams to help achieve uniform shape. In this experiment, small collagen sheets (10mm x 10mm x 0.5mm) were cut from larger sheets (30mm x 30mm x 0.5mm) (Naturin GmbH & Co., Baden-Württemberg, Germany), and inserted onto the surface seam between ham muscles for all treatments (for inoculated hams, sheets were inserted after inoculation) and left there throughout ham manufacture. At 80d, each collagen sheet (n=8, weighing ~10g) was removed from the seam, placed in a stomacher bag, diluted with 90ml 0.1% peptone water and homogenized 4 min with the stomacher. Serial dilutions were plated using the Autoplate 400 spiral plater.

Violet red bile glucose agar, VRBG (VRB; Oxoid Ltd., with 10% (w/v) D-glucose, Sigma Chemical Co., St. Louis, MO, USA), as well as Sorbitol MacConkey Agar (ctSMAC; Difco, Sparks, MD, USA with cefixime tellurite supplement, Oxoid Ltd.) were used to recover *E. coli* O157:H7 and were incubated at 35°C for 48h. Mannitol Salt Agar (MSA; Oxoid Ltd.) was used to recover *Staphylococcus*, and Tryptic Soy Agar (TSA; Oxoid Ltd.) was used to recover total aerobic mesophiles, which were both incubated at 35°C for 48h. Potato Dextrose Agar (PDA; Oxoid Ltd.), incubated at 21°C for 72h, was used to recover yeast while LAB were enumerated on deMan, Rogosa, and Sharpe Agar (MRSS; MRS, Oxoid Ltd., with the addition of 10% (w/v) potassium sorbate as a yeast inhibitor, Sigma) and was incubated at 21°C for 5d anaerobically (AnaeroPack system; Mitsubishi Gas Chemical America Co., Sparks, MD, USA). After incubation, colonies were counted on a colony counter (Quebec Darkfield, Reichert Analytical Instruments, New York, USA). Representative bacterial colonies from all media were monitored by performing catalase, oxidase and Gram reaction tests, as well as observing cell morphology. Furthermore, the identity of *E. coli* O157:H7 from

ctSMAC and VRBG agars was confirmed using the API Identification system (API 20E; BioMerieux, St. Laurent, QC, Canada), while *Staphylococcus spp.* from MSA, and yeast species from PDA were identified with API Staph and API AUX, respectively. When *E. coli* O157:H7 numbers were below the detection limit ( $0.8 \log \text{cfu}\cdot\text{g}^{-1}$ ), the sample was enriched in TSB, incubated at 35°C for 24h, and immunomagnetic separation (IMS; Dynabeads, Dynal Biotech, Oslo, Norway) was performed to detect viable cells. In addition, pH (pH probe; PH56-SS, IQ Scientific Instruments Inc. Carlsbad, CA, USA) and  $a_w$  values (Novasina Water Activity-Sprint machine; Axion AG, Pfaffikon, Switzerland) were recorded for all samples.

### 3.3.6 Statistical Analysis

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

## 3.4 Results and Discussion

### 3.4.1 Physicochemical changes during dry cured Westphalian ham ripening

Physicochemical changes were monitored during dry cured ham trials. Initially, both the surface and interior pH values of the fresh pork pieces averaged 5.87 which indicated that the meat used was fresh and of good quality (Table 3.1). By day 80, the pH of the surface and interior samples were on average 5.80 and 5.90, respectively, with no significant differences among treatments ( $P = 0.05$ ). This small change in pH is normal for a fermented whole muscle meat product in contrast with that of sausage which is  $\leq 5.30$  after fermentation (Lorenzo *et al.* 2010). These lower pH values are not usually

attained with dry cured hams, however the initial lengthy low temperature salt equilibration is normally effective when combined with slight fermentation and subsequent drying to yield safe dry cured ham (Fadda *et al.* 2010). In contrast, the low pH usually attained within 2 to 3d during dry sausage manufacture has proven to be incapable of preventing the survival of *E. coli* O157:H7, which is not surprising since it has been found to survive in environments with pH of 3.4 (Leyer *et al.* 1995).

Initial  $a_w$  values of 0.999 were also typical of fresh meat, and by day 80, the  $a_w$  of the ham ranged from 0.844 to 0.914 in both surface and interior samples. No significant differences were seen between surface and interior samples or among treatments (Table 3.2). These  $a_w$  values were slightly lower than commercial dry cured ham, but similar results were also obtained by others (Graumann and Holley 2009). It is worth noting that  $a_w$  values  $<0.950$  are able to prevent *E. coli* O157:H7 growth (Sperber 1983). Results from the present study indicated that deodorized yellow mustard powder had minimal effects on the physicochemical values of the dry cured Westphalian ham samples.

#### 3.4.2 Microbial changes during dry cured Westphalian ham ripening

Microbial analysis of both trials yielded similar profiles of LAB, mesophilic aerobes, and yeast levels on the surface and interior of dry cured Westphalian hams. For both trials, LAB levels were  $\sim 5.5 \log \text{cfu}\cdot\text{g}^{-1}$  initially on the surface of the fresh pork, which on average increased throughout the ripening period to  $\sim 7.75 \log \text{cfu}\cdot\text{g}^{-1}$  by the end of day 80, with no significant difference among treatments (Table 3.3). Total aerobic mesophiles in uninoculated meat initially averaged  $5.9 \log \text{cfu}\cdot\text{g}^{-1}$ , and reached maximum numbers averaging  $7.6 \log \text{cfu}\cdot\text{g}^{-1}$  in all treatments by day 35, thereafter remaining at that level with no significant differences among treatments. The penetration

of *Staphylococcus*, total aerobic mesophiles and LAB to the interior of the muscle was observed day 62 and beyond (Tables 3.3 and 3.4). Average initial yeast levels of 3 log cfu·g<sup>-1</sup> increased by the end of manufacturing to 5 log cfu·g<sup>-1</sup>, with no significant differences among treatments, although their numbers were lower on mustard treated hams. Unlike bacteria, the yeast did not penetrate the muscle tissues (Table 3.5). The dominant yeast species identified in both trials were *Cryptococcus albidus* and *Candida zeylanoides* (identified using the API identification system, Biomerieux) which are common in dry cured ham (Asefa *et al.* 2009).

*Staphylococcus* results varied among treatments and differences were related to the use of mustard. For trial 1, levels initially averaged 3.58 log cfu·g<sup>-1</sup> for all treatments, and by the end of ripening reached between 8.46 and 8.72 log cfu·g<sup>-1</sup> in uninoculated and *E. coli* O157:H7 inoculated controls, respectively, but were 3.54 and 3.89 log cfu·g<sup>-1</sup> in 4% and 6% mustard powder treatments, respectively (Table 3.6). In contrast, during trial 2 the addition of *S. carnosus* at day 45 yielded greater recoveries of staphylococci from this point to the end of the study where levels were 7.5 log cfu·g<sup>-1</sup> in all treatments, and were not significantly different from each other (Table 3.7). Dominant species identified on all hams during both trials were *S. saprophyticus* and *S. xylosus*, which are commonly found on dry cured hams (Lücke 1986, Vilar *et al.* 2000) as well as *S. carnosus* after its addition at day 45 in trial 2 identified by the API identification system (Biomerieux). Additionally, some of the interior ham samples at 62d and 80d had LAB, aerobic mesophiles, and *Staphylococcus* species present at levels of up to 4 log cfu·g<sup>-1</sup> (Tables 3.3, 3.4, 3.6 & 3.7), which is typical for dry cured hams (Lücke 1986). Moreover, no *E. coli* O157:H7 was recovered from any of the interior ham samples, even after enrichment procedures. The reduction of staphylococci by 4%

and 6% mustard treatments during trial 1 was notable because they and LAB play technologically important roles during ripening of dry cured ham pieces larger than 1kg. The addition of starter cultures during dry cured ham manufacture is not normal practice, and when tested their use was found to be of limited value (Sanchez-Molinero and Arnaud 2008). In the present work *S. carnosus* addition re-established the staphylococci population depressed by mustard powder. A similar reduction in numbers of staphylococci by mustard had been reported in dry sausages (Luciano *et al.* 2011) and this was likely due to their greater sensitivity toward isothiocyanates than that of the LAB (Luciano and Holley 2010, Luciano *et al.* 2011). The *S. carnosus* strain used in the present work had 2-fold greater capacity to hydrolyze sinalbin than other staphylococci strains tested (Luciano *et al.* 2011).

#### 3.4.3 Effect of deodorized yellow mustard powder on *E. coli* O157:H7 survival (Treatment)

Overall, deodorized yellow mustard powder showed promise for the control of *E. coli* O157:H7 at levels of 4% and 6%. However, *E. coli* O157:H7 appeared to recover from isothiocyanate challenge in the first, but not the second trial. In the first trial, *E. coli* O157:H7 numbers were below detection limits on ctSMAC agar at day 45 in 4% and 6% mustard treatments, but by day 80, an increase was seen (0.80 and 2.46 log cfu·g<sup>-1</sup>, respectively) (Table 3.8). Similarly, on VRBG agar, 6% mustard treatment decreased the pathogen to below the detection limit at day 45, but by day 80 numbers increased to 2.42 log cfu·g<sup>-1</sup>. *E. coli* O157:H7 in the 4% mustard treatment showed a less pronounced increase (0.50 log cfu·g<sup>-1</sup>) during this same time period.

Addition of the *S. carnosus* starter may have had a positive effect on the control of the pathogen during trial 2, since in both 4% and 6% mustard treatments *E. coli* O157:H7 remained inhibited at day 80 (Table 3.9). In the 6% mustard treatment, pathogen numbers fell below the detection limit at day 45 on ctSMAC agar and at day 62 on VRBG agar, where they remained. In the 4% mustard powder treatment, *E. coli* O157:H7 reached the detection limit by day 80.

In order to better understand differences in the *E. coli* O157:H7 response to mustard during the two trials, mustard powder was removed from the surface of the hams at day 80 and tested for sinalbin using HPLC. Results indicated that the deodorized yellow mustard powder from trial 1 no longer contained sinalbin. However, sinalbin was still present in the deodorized yellow mustard powder from trial 2 (75ppm and 123ppm in 4% and 6% mustard treatments, respectively), which may be why repair of *E. coli* O157:H7 was not observed during this trial followed by growth of repaired cells and increased recoveries in *E. coli* O157:H7 population. It is possible that sinalbin was consumed during trial 1 because of the higher number of *E. coli* O157:H7 cells inoculated on the hams. It had been planned to inoculate the hams with 7-7.5 log cfu·g<sup>-1</sup> *E. coli* O157:H7, but final numbers were 1 log higher in trial 1 than desired. Since *E. coli* O157:H7 has capacity to degrade glucosinolates, it may have contributed to premature depletion of sinalbin, enabling survivors to recover from injury inflicted (Cordiero *et al.* 2011) and be detected. It should be noted that PHBIT formed would be unstable and transiently present, given the physicochemical properties of the ripening hams.

Though *E. coli* O157:H7 numbers were below the detection limit on ham samples treated with mustard powder, the pathogen was still recovered from all surface

samples after enrichment. In addition, collagen sheet samples (n=8) from the seam between ham muscles yielded *E. coli* O157:H7 numbers below the detection limit, but the pathogen was recovered through enrichment (results not shown). Data from uninoculated controls, as well as from the other interior tissue samples for all treatments, are not shown as *E. coli* O157:H7 was absent even after enrichment procedures. It may be of concern that *E. coli* O157:H7 may contaminate surfaces of muscles that are folded during ham fabrication after bone removal and essentially become mechanically internalized. Once internalized, *E. coli* O157:H7 seems to have added protection as it has been shown to better survive the ham ripening process (Graumann and Holley 2007).

### **3.5 Conclusion**

*E. coli* O157:H7 survival in dry cured meat products is an international safety concern. Deodorized yellow mustard powder at 4% or 6% was able to reduce *E. coli* O157:H7 on dry cured Westphalian hams to a greater extent and at a faster rate than a commercial dry cure manufacturing process without mustard. During the first trial, hurdles used to prevent *E. coli* O157:H7 survival were overwhelmed by the very large inocula used and sinalbin was likely depleted early from the mustard powder by bacterial myrosinase-like activity. However, the consistent repression of *E. coli* O157:H7 in the second trial seemed due in part to residual sinalbin in mustard, even at day 80. Since the manufacture of dry cured hams is similar internationally, use of deodorized mustard to control *E. coli* O157:H7 could be widely adopted. It is a viable option for the safe commercial manufacture of fermented meats, by ensuring a 5 log reduction of *E. coli* O157:H7 viability. In addition, deodorized yellow mustard powder

is stable, easily applied, all natural, is a common ingredient in the meat industry where it is used as a filler, binder and emulsifier in cooked processed meats, and with its neutral flavour, can be used in fermented meat products without adverse effects on taste. Further research is warranted to evaluate the need for use of the *S. carnosus* starter culture during dry cured ham manufacture to restore numbers of staphylococci inhibited after glucosinolate hydrolysis (Luciano *et al.* 2011). Visual inspection did not detect colour differences among treatments.

**Table 3.1.** Changes in pH values<sup>a</sup> of ripening dry cured Westphalian ham surfaces and interiors

Day	Uninoculated Control		Inoculated Control <sup>b</sup>		4% Mustard Powder <sup>b</sup>		6% Mustard Powder <sup>b</sup>	
	Surface	Interior	Surface	Interior	Surface	Interior	Surface	Interior
0	5.87±0.30a	5.92±0.11a	5.83±0.19a	5.84±0.06a	5.88±0.10a	5.87±0.18a	5.91±0.18a	5.89±0.19a
21	5.80±0.21a	6.09±0.19a	5.75±0.25a	6.09±0.18a	5.95±0.11a	6.08±0.17a	5.76±0.11a	5.86±0.11a
35	5.98±0.22a	6.16±0.10a	6.09±0.11a	6.05±0.21a	5.76±0.28a	5.92±0.26a	5.71±0.23a	5.95±0.20a
45	6.01±0.22ab	6.15±0.24a	5.80±0.12ab	5.92±0.15ab	5.71±0.13b	5.97±0.14ab	5.77±0.15ab	5.95±0.15ab
62	6.13±0.17a	6.19±0.18a	5.92±0.25a	5.99±0.29a	5.79±0.20a	5.85±0.18a	5.73±0.21a	5.83±0.09a
80	5.92±0.21ab	6.05±0.20a	5.87±0.24ab	6.00±0.16ab	5.83±0.20ab	5.84±0.15ab	5.62±0.12b	5.79±0.09ab

<sup>a</sup>Values represent the mean ± SD of 2 trials replicated 2 times (n=4). Numbers followed by different letters (a-b) within the same row are significantly different ( $P < 0.05$ ).

<sup>b</sup>Samples were inoculated at day 0 with *E. coli* O157:H7.

**Table 3.2.** Changes in surface and interior water activities<sup>a</sup> of ripening dry cured Westphalian ham

Day	Uninoculated Control		Inoculated Control <sup>b</sup>		4% Mustard Powder <sup>b</sup>		6% Mustard Powder <sup>b</sup>	
	Surface	Interior	Surface	Interior	Surface	Interior	Surface	Interior
0	0.999±0.000a	0.999±0.000a	0.999±0.000a	0.999±0.000a	0.999±0.000a	0.999±0.000a	0.999±0.000a	0.999±0.000a
21	0.949±0.018a	0.979±0.019a	0.959±0.029a	0.982±0.018a	0.949±0.030a	0.980±0.004a	0.960±0.023a	0.978±0.007a
35	0.978±0.010ab	0.988±0.008a	0.972±0.023abc	0.988±0.006a	0.961±0.007bc	0.976±0.002abc	0.949±0.010c	0.980±0.013ab
45	0.933±0.020ab	0.966±0.008a	0.919±0.026b	0.958±0.009a	0.936±0.020ab	0.959±0.009a	0.930±0.016ab	0.956±0.012ab
62	0.851±0.014d	0.924±0.012ab	0.874±0.028bcd	0.936±0.019a	0.872±0.014cd	0.934±0.017a	0.865±0.031cd	0.906±0.032abc
80	0.844±0.030a	0.914±0.027a	0.850±0.037a	0.895±0.028a	0.836±0.052a	0.881±0.040a	0.846±0.046a	0.886±0.039a

<sup>a</sup>Values represent the mean ± SD of 2 trials replicated 2 times (n=4). Numbers followed by different letters (a-d) within the same row are significantly different ( $P < 0.05$ ).

<sup>b</sup>Samples were inoculated at day 0 with *E. coli* O157:H7.

**Table 3.3.** Numbers of adventitious lactic acid bacteria (log cfu·g<sup>-1a</sup> recovered on MRSS<sup>b</sup> agar) from dry cured Westphalian ham surfaces and interiors during ripening

Day	Uninoculated Control		Inoculated Control <sup>c</sup>		4% Mustard Powder <sup>c</sup>		6% Mustard Powder <sup>c</sup>	
	Surface	Interior	Surface	Interior	Surface	Interior	Surface	Interior
0	5.50±0.42a	0.00b	5.70±0.71a	0.00b	5.62±0.81a	0.00b	5.52±0.80a	0.00b
21	7.56±1.01a	0.00b	7.48±1.13a	0.00b	6.64±1.17a	0.00b	6.86±0.98a	0.00b
35	8.56±0.13a	0.00b	8.27±0.49a	0.00b	7.95±0.35a	0.00b	8.24±0.41a	0.00b
45	8.10±0.52a	0.00b	7.84±0.26a	0.00b	7.77±0.62a	0.00b	7.50±0.54a	0.00b
62	7.01±0.64a	1.33±0.94c	7.20±0.43a	3.84±0.95b	7.13±1.48a	3.01±1.06bc	6.17±0.87a	3.09±0.26bc
80	8.08±0.30a	2.58±1.20b	8.20±0.61a	4.09±1.16b	7.63±0.76a	3.66±0.64b	7.05±0.98a	3.72±1.27b

<sup>a</sup>Values represent the mean ± SD of 2 trials replicated 2 times (n=4). Numbers followed by different letters (a-c) within the same row are significantly different ( $P < 0.05$ ).

<sup>b</sup> deMan, Rogosa, Sharpe Agar supplemented with sorbic acid at 10g·L<sup>-1</sup> to inhibit the growth of yeast (MRSS). Plated samples were incubated anaerobically at 21°C for 5d.

<sup>c</sup>Samples were inoculated at day 0 with *E. coli* O157:H7.

**Table 3.4.** Total numbers of aerobic mesophiles (log cfu·g<sup>-1a</sup> recovered on TSA<sup>b</sup>) from dry cured Westphalian ham surfaces and interiors during ripening

Day	Uninoculated Control		Inoculated Control		4% Mustard Powder		6% Mustard Powder	
	Surface	Interior	Surface	Interior	Surface	Interior	Surface	Interior
0	5.86±0.37a	0.00b	7.64±1.31a	0.00b	7.93±1.65a	0.00b	7.44±1.34a	0.00b
21	7.41±0.38a	0.00b	7.57±0.41a	0.00b	6.60±1.08a	0.00b	6.77±0.60a	0.00b
35	8.05±0.57a	0.00b	7.46±0.61a	0.00b	7.55±0.50a	0.00b	7.46±0.87a	0.00b
45	7.94±0.66a	0.00b	7.61±0.43a	0.00b	7.46±0.64a	0.00b	7.31±0.70a	0.00b
62	8.05±0.56a	3.44±0.54c	8.21±0.54a	4.60±0.34bc	6.99±1.73a	3.34±0.86c	6.30±0.85ab	2.67±0.35c
80	8.16±0.34a	3.06±0.81b	8.07±0.82a	3.77±1.43b	7.30±1.04a	3.46±0.49b	6.55±0.97a	3.41±1.47b

<sup>a</sup> Values represent the mean ± SD of 2 trials replicated 2 times (n=4). Numbers followed by different letters (a-c) within the same row are significantly different ( $P < 0.05$ ).

<sup>b</sup> Tryptic Soy Agar (TSA). Plated samples were incubated at 35°C for 48h.

<sup>c</sup> Samples were inoculated at day 0 with *E. coli* O157:H7.

**Table 3.5.** Numbers of adventitious yeast (log cfu·g<sup>-1a</sup> recovered on PDA<sup>b</sup>) from dry cured Westphalian ham surfaces and interiors during ripening

Day	Uninoculated Control		Inoculated Control <sup>c</sup>		4% Mustard Powder <sup>c</sup>		6% Mustard Powder <sup>c</sup>	
	Surface	Interior	Surface	Interior	Surface	Interior	Surface	Interior
0	3.47±0.78a	0.00b	3.10±0.72a	0.00b	3.21±0.29a	0.00b	3.09±0.48a	0.00b
21	2.28±0.39ab	0.00c	2.51±0.24a	0.00c	1.12±1.30bc	0.00c	0.33±0.65c	0.00c
35	4.13±0.75a	0.00b	4.04±0.65a	0.00b	2.17±1.89ab	0.00b	1.51±1.74b	0.00b
45	3.17±2.34ab	0.00b	4.06±0.53a	0.00b	2.56±2.01ab	0.00b	2.34±2.70ab	0.00b
62	4.60±0.63a	0.00b	4.41±0.71a	0.00b	2.46±2.84ab	0.00b	0.65±1.30b	0.00b
80	5.31±0.64a	0.00b	4.80±0.43a	0.00b	3.08±2.14ab	0.00b	2.56±2.96ab	0.00b

<sup>a</sup>Values represent the mean ± SD of 2 trials replicated 2 times (n=4). Numbers followed by different letters (a-c) within the same row are significantly different ( $P < 0.05$ ).

<sup>b</sup>Potato Dextrose Agar acidified with L-tartaric acid (PDA). Plated samples were incubated at 21°C for 72h.

<sup>c</sup>Samples were inoculated at day 0 with *E. coli* O157:H7.

**Table 3.6.** Numbers of adventitious *Staphylococcus spp.* (log cfu·g<sup>-1a</sup> recovered on MSA<sup>b</sup>) from dry cured Westphalian ham surfaces and interiors during ripening (Trial 1)

Day	Uninoculated Control		Inoculated Control <sup>c</sup>		4% Mustard Powder <sup>c</sup>		6% Mustard Powder <sup>c</sup>	
	Surface	Interior	Surface	Interior	Surface	Interior	Surface	Interior
0	3.49±0.02a	0.00b	3.57±0.18a	0.00b	3.62±0.04a	0.00b	3.64±0.20a	0.00b
21	4.47±0.16ab	0.00c	4.91±0.43a	0.00c	4.25±0.62ab	0.00c	3.67±0.01b	0.00c
35	4.61±0.28a	0.00b	5.11±0.19a	0.00b	1.00±1.41b	0.00b	1.00±1.41b	0.00b
45	7.52±0.00a	0.00b	7.45±0.47a	0.00b	5.34±2.34a	0.00b	1.00±1.41b	0.00b
62	7.95±0.11a	3.89±0.32b	8.01±0.30a	4.14±0.00b	4.05±1.80b	2.80±0.17b	2.87±0.39b	2.59±0.25b
80	8.46±0.11ab	2.86±0.73c	8.72±0.11a	4.54±0.09c	3.54±2.18c	1.42±2.00c	3.89±1.39bc	2.48±0.47c

<sup>a</sup>Values represent the mean ± SD of 1 trial replicated 2 times (n=2). Numbers followed by different letters (a-c) within the same row are significantly different ( $P < 0.05$ ).

<sup>b</sup>Mannitol Salt Agar (MSA). Plated samples were incubated at 35°C for 48h.

<sup>c</sup>Samples were inoculated at day 0 with *E. coli* O157:H7.

**Table 3.7.** Numbers of adventitious and inoculated *Staphylococcus spp.* (log cfu·g<sup>-1a</sup> recovered on MSA<sup>b</sup>) from dry cured Westphalian ham surfaces and interiors during ripening (Trial 2)

Day	Uninoculated Control		Inoculated Control <sup>c</sup>		4% Mustard Powder <sup>c</sup>		6% Mustard Powder <sup>c</sup>	
	Surface	Interior	Surface	Interior	Surface	Interior	Surface	Interior
0	5.07±0.18a	0.00c	4.27±0.05b	0.00c	4.49±0.13b	0.00c	4.54±0.11b	0.00c
21	4.17±0.24a	0.00a	3.54±0.76a	0.00a	3.98±0.28a	0.00a	3.56±0.81a	0.00a
35	6.27±0.33a	0.00c	6.33±0.15a	0.00c	4.98±0.98ab	0.00c	4.41±0.25b	0.00c
45 <sup>d</sup>	7.14±0.18a	0.00b	6.92±0.69a	0.00b	6.02±0.37a	0.00b	6.70±0.89a	0.00b
62	7.64±0.28ab	3.16±0.23bcd	7.89±0.04a	4.56±0.10d	7.72±1.42a	4.11±0.85cd	6.71±1.42abc	3.34±0.11d
80	7.67±0.14ab	2.24±0.34c	7.65±0.05ab	3.58±2.23bc	8.05±0.90a	4.05±0.09abc	6.71±1.57ab	2.48±0.67c

<sup>a</sup>Values represent the mean ± SD of 1 trial replicated 2 times (n=2). Numbers followed by different letters (a-d) within the same row are significantly different ( $P < 0.05$ ).

<sup>b</sup>Mannitol Salt Agar (MSA). Plated samples were incubated at 35°C for 48h.

<sup>c</sup>Samples were inoculated at day 0 with *E. coli* O157:H7.

<sup>d</sup>Samples were inoculated at day 45 with *S. carnosus* UM123M meat starter culture.

**Table 3.8.** Numbers of *Escherichia coli* O157:H7 (log cfu·g<sup>-1a</sup> recovered on VRBG<sup>b</sup> and ctSMAC<sup>c</sup> agar) from dry cured Westphalian ham surfaces during ripening (Trial 1)

Day	Inoculated Control <sup>d</sup>		4% Mustard Powder <sup>d</sup>		6% Mustard Powder <sup>d</sup>	
	VRBG	ctSMAC	VRBG	ctSMAC	VRBG	ctSMAC
0	8.54±0.34 a	8.75±0.31A	8.29±0.28 a	8.37±0.15 A	8.18±0.59 a	8.25±0.75 A
21	5.35±0.06 a	5.64±0.05 A	5.09±0.69 a	4.74±0.20 B	2.42±0.60 b	2.78±0.97 C
35	4.68±0.01 a	4.57±0.11 A	3.45±0.63 b	3.31±0.75 AB	3.21±0.08 b	3.20±0.35 B
45	3.62±0.72 a	3.47±0.66 A	<0.80 <sup>e</sup> b	1.00±1.41 AB	<0.80 <sup>e</sup> b	<0.80 <sup>e</sup> B
62	2.59±1.39 a	2.70±1.13 A	1.87±2.64 a	1.81±2.56 A	<0.80 <sup>e</sup> a	<0.80 <sup>e</sup> A
80	3.24±1.21 a	3.19±1.32 A	0.80±1.13 b	1.54±0.34 A	2.46±0.28 b	2.42±0.16 A

<sup>a</sup>Values represent the mean ± SD of 1 trial replicated 2 times (n=2). Numbers followed by different letters (a-b for VRBG and A-C for ctSMAC) within the same row and using the same agar are significantly different ( $P < 0.05$ ).

<sup>b</sup>Violet Red Bile Agar supplemented with 10g·L<sup>-1</sup> D-glucose (VRBG). Plated samples were incubated at 35°C for 48h.

<sup>c</sup>Sorbitol MacConkey Agar supplemented with cefixime-tellurite (ctSMAC). Plated samples were incubated at 35°C for 48h.

<sup>d</sup>Samples were inoculated at day 0 with *E. coli* O157:H7.

<sup>e</sup>Positive for *E. coli* O157:H7 following enrichment and immunomagnetic separation.

**Table 3.9.** Numbers of *Escherichia coli* O157:H7 (log cfu·g<sup>-1a</sup> recovered on VRBG<sup>b</sup> and ctSMAC<sup>c</sup> agar) from dry cured Westphalian ham surfaces during ripening (Trial 2)

Day	Inoculated Control <sup>d</sup>		4% Mustard Powder <sup>d</sup>		6% Mustard Powder <sup>d</sup>	
	VRBG	ctSMAC	VRBG	ctSMAC	VRBG	ctSMAC
0	6.33±0.06a	6.44±0.08A	6.26±0.21a	6.28±0.06A	6.29±0.08a	6.29±0.13A
21	5.51±0.37a	5.59±0.00A	3.66±0.76b	3.43±1.11B	3.62±0.66b	3.64±0.62AB
35	3.54±0.12a	3.42±0.48A	3.20±0.30a	3.09±0.41A	2.78±1.24a	2.71±1.15A
45	1.60±0.42a	0.80±1.13A	1.78±0.00a	0.65±0.92A	1.54±0.34a	<0.80 <sup>e</sup> A
62	2.57±0.22a	1.97±0.26A	1.55±2.19a	1.41±1.99A	<0.80 <sup>e</sup> a	<0.80 <sup>e</sup> A
80	1.95±0.07a	1.04±1.47A	<0.80 <sup>e</sup> b	<0.80 <sup>e</sup> A	<0.80 <sup>e</sup> b	<0.80 <sup>e</sup> A

<sup>a</sup>Values represent the mean ± SD of 1 trial replicated 2 times (n=2). Numbers followed by different letters (a-b for VRBG and A-B for ctSMAC) within the same row and using the same agar are significantly different ( $P < 0.05$ ).

<sup>b</sup>Violet Red Bile Agar supplemented with 10g·L<sup>-1</sup> D-glucose (VRBG). Plated samples were incubated at 35°C for 48h.

<sup>c</sup>Sorbitol MacConkey Agar supplemented with cefixime-tellurite (ctSMAC). Plated samples were incubated at 35°C for 48h.

<sup>d</sup>Samples were inoculated at day 0 with *E. coli* O157:H7.

<sup>e</sup>Positive for *E. coli* O157:H7 following enrichment and immunomagnetic separation.

## CHAPTER 4

### General Discussion

For all treatments in both trials, ham surface and interior were monitored throughout 80d ripening for physicochemical (pH and  $a_w$ ) and microbial changes. Initially, both the surface and interior pH values of fresh pork averaged 5.87 (Table 3.1). In comparison, the average pH of fresh meat is 5.90, which indicates the meat used during experimentation was fresh and of good quality. By the end of 80d manufacture, the pH of surface and center samples were on average 5.81 and 5.92, respectively, with no significant differences seen between treatments. These results are typical of dry cured ham as it is normal for a whole muscle fermented product to have only slight change in pH during manufacture, with the main factors influencing safety of dry cured ham being high salt concentrations (~5%) and low  $a_w$  (Hinrichsen and Pedersen 1995, Lorenzo *et al.* 2010).

Initial ham surface and interior  $a_w$  values were 0.999, and with a final dry cured ham value averaging 0.844, there were no significant differences between treatments or surface and center samples (Table 3.2). The  $a_w$  results from this study were characteristic of a dry cured ham product, and were similar to previous work (Graumann and Holley 2009). At this  $a_w$  (<0.930), growth of *E. coli* O157:H7 is discouraged (Sperber 1983). Fortunately, deodorized yellow mustard powder had no significant effect on ham  $a_w$  values and in addition, visual inspection of the ham flesh showed no colour differences between treatments.

During both trials, microbiological analysis yielded no significant ( $P=0.05$ ) effect from mustard treatment on the development of indigenous LAB, total mesophilic aerobes and yeast. Initially, LAB levels averaged  $5.59 \log \text{cfu}\cdot\text{g}^{-1}$  on fresh pork which increased

by 80d to  $7.74 \log \text{cfu}\cdot\text{g}^{-1}$ , with no significant difference between treatments (Table 3.3). By 80d there was only a 1 log difference between the mustard treatments and control. These results are positive since they show that mustard does not greatly affect the LAB, which are important because they contribute to ham preservation and flavour development (Lücke 1986). Total mesophilic aerobe levels averaged  $5.86 \log \text{cfu}\cdot\text{g}^{-1}$  during the initial analysis before *E. coli* O157:H7 inoculation and were  $7.14 \log \text{cfu}\cdot\text{g}^{-1}$  after inoculation (Table 3.4). The pathogen would have been recovered on this medium after ham inoculation. Aerobic mesophiles recovered in the uninoculated control reflects adventitious microflora development on the ham during ripening, with organisms being identified as LAB, *Staphylococcus* spp. and yeast. By 80d, numbers averaged  $7.52 \log \text{cfu}\cdot\text{g}^{-1}$ , with no significant differences between treatments. Yeast levels averaged  $3.22 \log \text{cfu}\cdot\text{g}^{-1}$  initially, and rose slightly by 80d to  $4.80 \log \text{cfu}\cdot\text{g}^{-1}$  in the absence of mustard. When mustard was used yeast numbers were  $\geq 1 \log \text{cfu}\cdot\text{g}^{-1}$  lower but the difference was not significant (Table 3.5). Though yeast growth is desired in some fermented meat products for flavour and texture development, yeast are not involved nor desired in dry cured Westphalian ham manufacture. Yeast at the levels found here were considered to be acceptable (Graumann and Holley 2009). Dominant yeast species identified in both trials were *Cryptococcus albidus* and *Candida zeylanoides*, which are common in dry cured ham (Asefa *et al.* 2009).

Unlike the LAB, indigenous *Staphylococcus* showed sensitivity to mustard treatment. In trial 1, *Staphylococcus* levels were initially  $4 \log \text{cfu}\cdot\text{g}^{-1}$ , and by the end of ripening reached between  $8.46$  and  $8.72 \log \text{cfu}\cdot\text{g}^{-1}$  in uninoculated and *E. coli* O157:H7 inoculated controls, respectively, but were  $3.54$  and  $3.89 \log \text{cfu}\cdot\text{g}^{-1}$  in 4% and 6% mustard powder treatments, respectively (Table 3.6). In trial 2, the addition of *S.*

*carneus* at 45d yielded greater recoveries of staphylococci from this point to the end of the study, where levels were not significantly different from each other and averaged 7.5 log cfu·g<sup>-1</sup> in all treatments (Table 3.7). Dominant species identified on all hams during both trials were *S. saprophyticus* and *S. xylosus*, which are commonly found on dry cured hams (Lücke 1986, Vilar *et al.* 2000) as well as *S. carneus* after its addition at 45d in trial 2. Furthermore, *S. carneus* inoculation replenished the staphylococci population on the ham previously inhibited by PHBIT to ~7.40 log cfu·g<sup>-1</sup> by 80d in both mustard treatments. *Staphylococcus* sensitivity to mustard isothiocyanate has been reported in previous work (Graumann and Holley 2009, Luciano *et al.* 2011)

During this investigation, some interior ham samples contained LAB, aerobic mesophiles, and *Staphylococcus* species at levels of up to 4 log cfu·g<sup>-1</sup> at ≥62d (Tables 3.3, 3.4, 3.6 and 3.7). However, it is not uncommon to find these species in the interior of the ham tissue. Good quality dry cured hams usually have microorganisms within the tissue, but numbers are not normally large (≤6 log cfu·g<sup>-1</sup>) (Lücke 1986).

Deodorized yellow mustard powder showed promise for the control of *E. coli* O157:H7 at levels of 4% and 6%, though 6% showed stronger inhibition of the pathogen. During trial 1, both VRBG and ctSMAC agar used to recover the pathogen yielded similar results (Table 3.8). By 21d, the 6% mustard treatment significantly (P=0.05) reduced the pathogen by 3 log whereas 4% mustard had no significant effect on pathogen reduction at that time. Thereafter, both 4% and 6% mustard treatments had significantly lower numbers of *E. coli* O157:H7 than the control.

Likewise, during trial 2 both VRBG and ctSMAC agars yielded similar *E. coli* O157:H7 recoveries (Table 3.9). By 21d, 4% and 6% mustard reduced the pathogen 2 log lower than the control, however this was not significantly different. By 80d, both 4%

and 6% treatments reduced *E. coli* O157:H7 below the detection limit, unlike the control ( $\sim 1.95 \log \text{cfu}\cdot\text{g}^{-1}$ ), though this was again not statistically significant.

Differences in *E. coli* O157:H7 numbers were observed between trials at 80d. It is likely that repair of *E. coli* O157:H7 injury in trial 1, due to isothiocyanate challenge, contributed to this difference. However, increased recoveries could also be attributed to *E. coli* O157:H7 survival in ham surface folds or muscle seams and having added protection from PHBIT exposure. Though the mechanism of action of PHBIT is unknown, cell death is caused by enzyme inhibition and cell membrane damage. Therefore, injury repair would involve the pathogen's ability to recover from challenges leading up to this outcome. Resistance of *E. coli* O157:H7 to mustard isothiocyanate has yet to be reported, making it is less likely that cells were no longer producing the myrosinase-like enzyme to become resistant to further inhibition.

In the first trial, *E. coli* O157:H7 numbers were near or below the detection limit at day 45 in 4% and 6% mustard treatments, but by 80d, numbers increased to 1.54 and 2.46  $\log \text{cfu}\cdot\text{g}^{-1}$ , respectively. To better understand this result, deodorized yellow mustard powder was removed from the surface of the ham at 80d (by scraping the ham surface with a knife) and assayed for sinalbin using HPLC. In the trial 1 mustard sample, there was no sinalbin present which indicated that sinalbin had been exhausted by day 80 from the mustard. In comparison, both 4% and 6% deodorized yellow mustard powder from trial 2 still had sinalbin present at 80d (75ppm and 123ppm, respectively) which is possibly why an increase in *E. coli* O157:H7 numbers (injury repair) was not observed. It is most likely that the larger *E. coli* O157:H7 inoculum used during the first trial resulted in accelerated sinalbin degradation. Even though *S. carnosus* was inoculated on the hams during trial 2, its lower glucosinolate degradation rate did not

have as big of an impact on sinalbin hydrolysis as the high *E. coli* O157:H7 population in trial 1.

Both trials reached below the detection limit of *E. coli* recovery at 45d, and this was consistent with previous work where 6% non-deodorized yellow mustard powder was used to control the pathogen in dry cured ham (Graumann and Holley 2009). It is evident from prior work and this present study that either type of mustard powder ( $\pm$ myrosinase) is effective in reducing the viability of *E. coli* O157:H7 substantially faster than naturally occurs in its absence. In addition, there are advantages in using deheated mustard that include the ability to safely handle this type of mustard during ham manufacture in the presence of moisture and its faster action against *E. coli* O157:H7. It is important to note that at the end of ripening even though the pathogen was below the detection limit on dry cured ham surfaces treated with mustard and in the seam between muscles, viable cells were still recovered after enrichment. Similarly, previous studies investigating *E. coli* O157:H7 survival on dry cured hams (ripening periods of 120d and 80d, respectively) showed that *E. coli* O157:H7 cells were still viable after enrichment (Reynolds *et al.* 2001, Graumann and Holley 2009). Interestingly, dry cured ham manufacture involving extended drying periods (>234d) completely eliminated the pathogen from the product, and analysis revealed that no viable cells were found after enrichment (Portocarrero *et al.* 2002). Risk associated with potential survival of *E. coli* O157:H7 during dry cured ham manufacture may be reduced by extension of drying intervals, however this increases processing cost. Unfortunately, the use of needle tenderization to accelerate ham manufacture has been proven unsafe since *E. coli* O157:H7 cells were internalized in ham tissue, and this promoted pathogen survival (Graumann and Holley 2007).

The use of starter cultures during sausage fermentation cannot successfully guarantee a 5 log reduction of *E. coli* O157:H7 (Hinkens et al. 1996, Erkkila *et al.* 2000, Lahti *et al.* 2001), even though meat pH quickly reaches  $\leq 5.3$ . Dry cured ham is vulnerable because low pH is not a substantial hurdle against pathogen survival and starter culture use is not normal practice (Sanches-Molinero and Arnaud 2008). The present work investigated the addition of *S. carnosus* to hams in the second trial to determine whether its myrosinase-like activity could help in the control of *E. coli* O157:H7. The strain chosen had the greatest capacity to hydrolyze sinalbin among starters available and the culture may have played a role in the maintenance of pathogen inhibition through prevention of injury recovery by *E. coli* O157:H7. It was clear that the addition of *S. carnosus* restored the staphylococci population which had been reduced by the mustard treatment. Staphylococci sensitivity to mustard isothiocyanate has been reported in dry sausages (Luciano *et al.* 2011, Luciano and Holley 2010), although the significance of low numbers of these organisms in mustard-treated hams in terms of ham colour and texture is uncertain.

## CHAPTER 5

### Conclusions

Results from the present study support previous work suggesting that yellow mustard powder is lethal to *E. coli* O157:H7 in fermented meat products. As importantly, the ability of mustard glucosinolate to serve as a precursor of a microbially-generated antimicrobial was demonstrated in dry cured ham manufactured to commercial specifications. During dry cured Westphalian ham manufacture, deodorized yellow mustard powder at 4% and to a greater extent 6% decreased *E. coli* O157:H7 numbers at a faster rate than the control. By 45d, 4% and 6% mustard treatment reduced the pathogen by >5 log, whereas it took 80d for the control to reach similar numbers. The initial exposure during 21d salt brine treatment seemed to be the most lethal during mustard treatment where a 3 log reduction of *E. coli* O157:H7 occurred. Since a 5 log reduction legally validates a fermented meat process as being capable of producing a product free from *E. coli* O157:H7 contamination, both dry cured Westphalian ham trials were compliant at 80d. Though deodorized yellow mustard powder was lethal toward *E. coli* O157:H7, it showed no adverse effects on physicochemical properties of the ham during manufacture ( $a_w$ , pH) or to the indigenous microflora essential to ham ripening, with the exception of some sensitivity observed by *Staphylococcus* and yeast after 21d. The effect of deodorized yellow mustard powder at levels of 4% and 6% on the taste of dry cured ham was not tested during this project, however since the mustard is deodorized it no longer retains the strong spicy flavour and pungent odour that is characteristic of hot yellow mustard powder and therefore its flavour effect on the ham is likely minimal.

*S. carnosus* meat starter culture may have contributed toward maintaining inhibitory conditions in mustard-treated ham, since no injury repair of *E. coli* O157:H7 cells was observed in trial 2. In addition, *S. carnosus* inoculation restored numbers of staphylococci inhibited after mustard glucosinolate hydrolysis and PHBIT formation.

Deodorized yellow mustard powder is an ideal antimicrobial agent to apply to dry cured meats since during manufacture the decrease in  $a_w$  (and to a lesser extent pH), and high salt (~5%) concentrations create an adverse environment that reduces *E. coli* O157:H7 survival and the pathogen is confronted with limited access to glucose from meat tissue. In the presence of mustard as *E. coli* O157:H7 removes a glucose molecule from mustard glucosinolate, the lethal antimicrobial PHBIT is produced.

There are many benefits to using deodorized yellow mustard powder as an antimicrobial agent in dry cured meat manufacture. The meat industry would not need approval for a new ingredient as mustard is already used as a binder and a filler for cooked cured meats. When deodorized it has a bland, neutral taste which will not affect flavours of products to which it is added. Additionally, it is all-natural, so its addition would satisfy consumer demand for natural and preservative-free food products. The use of mustard as a powder provides a stable source of normally instable isothiocyanates which is safe and convenient to handle.

Dry cured Westphalian ham, as well as other types of dry cured hams produced around the world, could benefit from the antimicrobial properties of deodorized yellow mustard powder. Since dry cured meats have a high risk of *E. coli* O157:H7 contamination and do not have a heat kill step during manufacture, the use of an antimicrobial agent generated by the target bacterium itself would contribute to risk reduction. This research highlights the importance of creating and validating food

manufacturing processes that ensure safe, pathogen-free food is produced. Though other safety options for validating dry cured ham manufacture include end product testing (CFIA 2010), in reality, end product testing does not ensure product safety when the manufacturing process itself is not validated. A contaminated product is not always found during end product testing, where the frequency of pathogen occurrence is commonly <0.1% of samples. A process that ensures pathogen destruction would always ensure a safe product. We should be moving towards validating our food manufacturing processes, rather than allowing foods to be produced without built-in assurance of safety. Results from the present study will contribute to improved product safety and are immediately applicable in the meat industry internationally.

Further research is warranted to evaluate the addition of the *S. carnosus* starter culture during dry cured ham manufacture to replace staphylococci reduced by mustard treatment. This may be examined by quantifying colour and texture differences that may occur following starter culture use. Also, investigations are needed to determine factors influencing the production of myrosinase-like activity of staphylococci involved in the formation of PHBIT from deodorized yellow mustard to control *E. coli* O157:H7 on dry cured ham. The occurrence of myrosinase-like activity in a broader range of organisms should be established and factors influencing the extent of its production characterized. And finally, the use of deodorized yellow mustard powder in conjunction with extended dry cured ham drying times should be explored to ensure complete elimination of *E. coli* O157:H7 from the ham surface even after enrichment.

## REFERENCES

- Agriculture and Agri-Food Canada. 2008. The case for Canadian mustard. Available online (2011): <http://www.ats-sea.agr.gc.ca/pro/4512-eng.htm>
- Armstrong, G. L., Holligsworth, J. and Morris, J.G. 1996. Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. *Epidemiol. Rev.* 18:29-51.
- Arnold, K.W. and Kaspar, C.W. 1995. Starvation- and stationary-phase-induced acid tolerance in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 61:2037-2039.
- Asefa, D.T., Moreto, T., Gjerde, R.O., Langsrud, S., Kure, C.F., Sidhu, M.S., Nesbakken, T. and Skaar, I. 2009. Yeast diversity and dynamics in the production processes of Norwegian dry-cured meat products. *Int. J. Food Microbiol.* 133:135-140.
- Beutin, L., Geier, D., Streinruck, H., Zimmerman, S., and Sheutz, F. 1993. Prevalence and some properties of verotoxin (shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. *J. Clin. Microbiol.* 31:2483-2488.
- Borie, C., Monreal, Z., Sanchez, M.L., Martinez, J., Arellano, C. and Prado, V. 1997. Prevalence and characterization of enterohaemorrhagic *Escherichia coli* isolated from healthy cattle and pigs slaughtered in Santiago, Chile. *Arch. Med. Vet.* 29:205-212.
- Brabban, A.D. and Edwards, C. 1994. Isolation of glucosinolate degrading microorganisms and their potential for reducing the glucosinolate content of rapemeal. *FEMS Microbiol. Lett.* 119:83-88.
- Brandt, J.R., Fouser, L.S., Watkins, S.L., Zelikovic, I., Tarr, P.I., Nazarstewart, V. and Avner, E.D. 1994. *Escherichia coli* O157:H7-associated haemolytic-uremic syndrome after ingestion of contaminated hamburgers. *J. Pediatr.* 125:519-526.
- Buchanan, R.L. and Bagi, L.K. 1994. Expansion of response surface models for the growth of *Escherichia coli* O157:H7 to include sodium nitrite as a variable. *Int. J. Food Microbiol.* 23:317-332.
- Buchanan, R.L. and Doyle, M.P. 1997. Foodborne disease significance of *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli*. *Food Technol.* 51:69-76.
- Buskov, S. Hasselstrom, J., Olsen, C.E., Sorensen, H., Sorensen, J.C. and Sorensen, S. 2000. Supercritical fluid chromatography as a method of analysis for the determination of 4-hydroxybenzylglucosinolate degradation products. *J. Biochem. Bioph. Meth.* 43:157-174.
- Calicioglu M., Faith, N.G., Buege, D.R. and Luchansky, J.B. 1997. Viability of *Escherichia coli* O157:H7 in fermented semidry low-temperature-cooked beef summer sausage. *J. Food Protect.* 60:1158-1162.

Campo, M.M. and Sierra, I. 2011. Fatty acid composition of selected varieties of Spanish dry-cured ham. Surveys from 1995 and 2007. Span. J. Agric. Res. 9: 66-73.

Canadian Food Inspection Agency (CFIA), Government of Canada. 2010. Meat hygiene manual of procedures: processing and handling techniques – fermented meat products. Available online (2011): <http://www.inspection.gc.ca/english/fssa/meavia/man/ch4/4-11-4-6e.shtml#a4-11-4-6>

Center for Disease Control and Prevention (CDC). 1995. *Escherichia coli* O157:H7 outbreak linked to commercially distributed dry-cured salami – Washington and California, 1994. Morb. Mortal. Wkly. Rep. 44:157-160.

Center for Disease Control and Prevention (CDC). 2010. Foodborne Illnesses: general information. Available online (2011): [http://www.cdc.gov/ncidod/dbmd/diseaseinfo/foodborneinfections\\_g.htm#mostcommon](http://www.cdc.gov/ncidod/dbmd/diseaseinfo/foodborneinfections_g.htm#mostcommon)

Chacon, P.A., Buffo, R.A. and Holley, R.A. 2006. Inhibitory effects of microencapsulated allyl isothiocyanate (AIT) against *Escherichia coli* O157:H7 in refrigerated, nitrogen packed, finely chopped beef. Int. J. Food Microbiol. 107: 231-237.

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

Chapman, P.A., Siddons, C.A., Gerdan Malo, A.T. and Harkin, M.A. 1997. A 1-year study of *Escherichia coli* O157:H7 in cattle, sheep, pigs and poultry. Epidemiol. Infect. 119:245-250.

Cordiero, R., Luciano, F. and Holley, R.A. 2011. Injury of *Escherichia coli* O157:H7 by mustard powder during dry sausage manufacture and the value of CT-SMAC plating over thin agar layer recovery. Int. J. Food Microbiol. (Submitted)

Cornick, N.A. and Helgerson, A.F. 2004. Transmission and infectious dose of *Escherichia coli* O157:H7 in swine. Appl. Environ. Microbiol. 70:5331-5335.

Delaquis, P.J. and Mazza, G. 1995. Antimicrobial properties of isothiocyanates in food preservation. Food Technol. 49:73-84.

Delaquis, P. and Sholberg, P. 1997. Antimicrobial activity of gaseous allyl isothiocyanate. J. Food Protect. 60:943-947.

Doane, C.A., Richards, H.A., Mount, J.R., Golden, D.A. and Draughon, F.A. 2007. Occurrence of *Escherichia coli* O157:H7 in diverse farm environments. J. Food Protect. 70:6-10.

- Ekanayake, A., Kester, J.J., Li, J.J., Zehentbauer, G.N., Bunke, P.R. and Zent, J.B. 2006. Isoguard<sup>TM</sup>: a natural anti-microbial agent derived from white mustard seed. *Acta Hort.* 709:101-108.
- Eriksson, E., Nerbrink, E., Aspan, E.B.A. and Gunnarsson, A. 2003. Verocytotoxin-producing *Escherichia coli* O157:H7 in the Swedish pig population. *Vet. Rec.* 152:712-717.
- Erkkila, S., Venalainen, M., Hielm, S., Petaja, E., Puolanne, E., and Mattila-Sandholm, T. 2000. Survival of *Escherichia coli* O157:H7 in dry sausage fermented by probiotic lactic acid bacteria. *J. Sci. Food Agric.* 80:2101-2104.
- Fadda, S., Lopez, C. and Vignolo, G. 2010. Role of lactic acid bacteria during meat conditioning and fermentation: peptides generated as sensorial and hygienic biomarkers. *Meat Sci.* 86:66-79.
- Faith, N.G., Parniere, N., Larson, T., Lorang, T.D., Kaspar, C.W. and Luchansky, J.B. 1998. Viability of *Escherichia coli* O157:H7 in salami following conditioning of batter, fermentation and drying of sticks, and storage of slices. *J. Food Protect.* 61: 377-382.
- Feder, I., Wallace, F.M., Gray, J.T., Fratamico, P., Fedorka-Cray, P.J., Pearce, R.A., Call, J.E., Perrine, R. and Luchansky, J.B. 2003. Isolation of *Escherichia coli* O157:H7 from intact colon fecal samples of swine. *Emerg. Infect. Dis.* 9:380-383.
- Flores, M., Ingram, D., Bett, K., Toldra, F., and Spanier, A. 1997. Sensory characteristics of Spanish Serrano dry cured hams. *J. Sens. Stud.* 12: 169-179.
- Gill, C.O. and Jones, T. 1998. Control of contamination of pig carcasses by *Escherichia coli* from their mouths. *Int. J. Food Microbiol.* 44:43-48.
- Glass, K.A., Loeffelholz, J.M., Ford, P.J. and Doyle, M.P. 1992. Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. *Appl. Environ. Microbiol.* 58:2513-2516.
- Gotterup, J., Olsen, K., Knochel, S., Tjener, K., Stahnke, L. and Moller, J.K.S. 2007. Relationship between nitrate/nitrite reductase activities in meat associated with staphylococci and nitrosylmyoglobin formation in a cured meat model system. *Int. J. Food Microbiol.* 120:303-310.
- Graumann, G.H. and Holley, R.A. 2007. Survival of *Escherichia coli* O157:H7 in needle-tenderized dry cured Westphalian ham. *Int. J. Food Microbiol.* 118:173-179.
- Graumann, G.H. and Holley, R.A. 2008. Inhibition of *Escherichia coli* O157:H7 in ripening dry fermented sausage by ground yellow mustard. *J. Food. Protect.* 71:486-493.

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

Griffin, P.M. and Tauxe, R.V. 1991. The epidemiology of infections caused by *Escherichia coli* O157, other enterohaemorrhagic *E. coli*, and the associated haemolytic uraemic syndrome. *Epidemiol. Rev.* 13:60-98.

Hancock, D., Besser, T., Lejeune, J., Davis, M. and Rice, D. 2001. The control of VTEC in the animal reservoir. *Int. J. Food Microbiol.* 66:71-78.

Heuvelink, A.E., Zwartkruis-Nahuis, J.T.M., Beumer, R.R. and De Boer, E. 1999. Occurrence and survival of verocytotoxins-producing *Escherichia coli* O157 in meats obtained from retail outlets in the Netherlands. *J. Food Protect.* 62:1115-1122.

Heuvelink, A.E., Zwartkruis-Nahuis, J.T.M., Van den Biggelaar, F.L.A.M., Van Leeuwen, W.J. and De Boer, E. 1999. Isolation and characterization of verocytotoxin-producing *Escherichia coli* O157:H7 from slaughter pigs and poultry. *Int. J. Food Microbiol.* 52:67-75.

Hinkens, J.C., Faith, N.G., Lorang, T.D., Bailey, P., Bueye, D., Kaspar, D. and Luchansky, J.B. 1996. Validation of pepperoni processes for control of *Escherichia coli* O157:H7. *J. Food Protect.* 59:1260-1266.

Hinrichsen, L.L., and Pedersen, S.B. 1995. Relationship among flavour, volatile compounds, chemical-changes, and microflora in Italian-type dry-cured ham during processing. *J. Agric. Food Chem.* 43: 2932-2940.

Hugas, M. and Monfort, J.M. 1997. Bacterial starter cultures for meat fermentation. *Food Chem.* 59:547-554.

Johnsen, G., Wasteson, Y., Heir, E., Berget, O.I. and Herikstad, H. 2001. *Escherichia coli* O157:H7 in faeces from cattle, sheep and pigs in the southwest part of Norway during 1998 and 1999. *Int. J. Food Microbiol.* 65:193-200.

Kanemaru, K. and Miyamoto, T. 1990. Inhibitory effects on the growth of several bacteria by brown mustard and allyl isothiocyanate. *Nippon Shoku-hin Kogyo Gakkaishi.* 37:823-829.

Kawakishi, S. and Muramatsu, K. 1966. Studies on the decomposition of sinalbin. Part 1. The decomposition products of sinalbin. *Agr. Biol. Chem.* 30:688-692.

Kiranmayi, C.B., Krishnaiah, N. and Mallika, E.N. 2010. *Escherichia coli* O157:H7 – an emerging pathogen in foods of animal origin. *Vet. World.* 3:382-389.

Lahti, E., Johansson, T., Honkanen-Buzalski, T., Hill, P. and Nurmi, E. 2001. Survival and detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* during the

manufacture of dry sausage using two different starter cultures. *Food Microbiol.* 18:75-85.

Leyer, G.J., Wang, L.L. and Johnson, E.A. 1995. Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. *Appl. Environ. Microbiol.* 61:3094-3100.

Lin, C.M., Preston, J.F. and Wei, C.I. 2000. Antibacterial mechanism of allyl isothiocyanate. *J. Food Protect.* 63:727-734.

Lorenzo, J.M., Fontan, M.C.G., Cachaldora, A., Franco, I. and Carballo, J. 2010. Study of the lactic acid bacteria throughout the manufacture of dry-cured lacon (a Spanish traditional meat product). Effect of some additives. *Food Microbiol.* 27:229-235.

Luciano, F.B. and Holley, R.A. 2009. Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7. *Int. J. Food. Microbiol.* 131:240-245.

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

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

Lücke, F.K. 1986. Microbiological processes in the manufacture of dry sausage and raw ham. *Fleischwirtsch.* 66:1505-1509.

MacDonald, D.M., Fyfe, M., Paccagnella, A., Trinidad, A., Louie, K., and Patrick, D. 2004. *Escherichia coli* O157:H7 outbreak linked to salami, British Columbia, Canada, 1999. *Epidemiol. Infect.* 132:283-289.

Mayerhauser, C.M. 2001. Survival of enterohemorrhagic *Escherichia coli* O157:H7 in retail mustard. *J. Food Protect.* 64:783-787.

McQuestin, O.J., Shadbolt, C.T. and Ross, T. 2009. Quantification of the relative effects of temperature, pH and water activity on inactivation of *Escherichia coli* in fermented meat by meta-analysis. *Appl. Environ. Microb.* 75:6963-6972.

Muthukumarasamy, P., Han, J.H., Holley, R.A. 2004. Lethal effects of non-deheated (hot) mustard flour on *E. coli* O157:H7 in refrigerated nitrogen packed ground beef. Presented at the 50th International Congress of Meat Science and Technology, Helsinki, Finland.

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

- Nakazawa, M., Akiba, M. and Sameshima, T. 1999. Swine as a potential reservoir of shiga toxin-producing *Escherichia coli* O157:H7 in Japan. *Emerg. Infect. Dis.* 5:Nov-Dec.
- Namvar, A. and Warriner, K. 2006. Application of enterobacterial repetitive intergenic consensus-polymerase chain reaction to trace the fate of generic *Escherichia coli* within a high capacity pork slaughter line. *Int. J. Food Microbiol.* 108:155-163.
- Nissen, H. And Holck, A. 1998. Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Kentucky in Norwegian fermented, dry sausage. *Food Microbiol.* 15:273-279.
- Paton, A.W., Ratcliff, R.M., Doyle, R.M., Seymore-Murray, J., Davos, D., Lanser, J.A. and Paton, J.C. 1996. Molecular microbiological investigation of an outbreak of haemolytic-uremic syndrome caused by dry fermented sausage contaminated with shiga-like toxin-producing *Escherichia coli*. *J. Clin. Microbiol.* 34: 1622-1627.
- Paton, J.C. and Paton, A.W. 1998. Pathogenesis and diagnosis of Shiga producing *Escherichia coli* infections. *Clin. Microbiol. Rev.* 11:450-479.
- Portocarrero, S.M., Newman, M. and Mikel, B. 2002. Reduction of *Listeria monocytogenes*, *Salmonella* spp., and *Escherichia coli* O157:H7 during processing of country-cured hams. *J. Food Sci.* 67:1892-1898.
- Reynolds, A.E., Harrison, M.A., Rose-Morrow, R. and Lyon, C.E. 2001. Validation of dry cured ham process for control of pathogens. *J. Food Sci.* 66:1373-1379.
- Rhee, M., Dougherty, R.H. and Kang, D. 2002. Combined effects of mustard flour, acetic acid, and salt against *Escherichia coli* O157:H7 stored at 5 and 22°C. *J. Food Protect.* 65:1632-1636.
- Riley, L.W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A., and Cohen, M.L. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N. Engl. J. Med.* 308:681-685.
- Rodriguez, M., Nunez, F., Corboda, J.J., Sanabria, C., Bermudez, E. and Asensio, M.A. 1994. Characterization of *Staphylococcus* spp. and *Micrococcus* spp. isolated from Iberian ham throughout the ripening process. *Int. J. Food Microbiol.* 24:329-335.
- Rodriguez, M., Nunez, F., Cordoba, J.J., Bermudez, E. and Asensio, M.A. 1996. Gram-positive catalase-positive cocci from dry cured Iberian ham and their enterotoxigenic potential. *Appl. Environ. Microbiol.* 62:1897-1902.

Sanchez-Molinero, F. and Arnau, J. 2008. Effect of the inoculation of a starter culture and vacuum packaging (during resting stage) on the appearance and some microbiological and physiochemical parameters of dry cured ham. *Meat Sci.* 79:29-38.

Sperber, W.H. 1983. Influence of water activity on foodborne bacteria – a review. *J. Food Protect.* 46:142-150.

Tilden, J., Young, W., McNamara, A., Custer, A., Boesel, B., Lambert-Fair, M.A., Majkowski, J., Vugia, D., Werner, S.B., Hollingsworth, J. and Morris, J.G. 1996. A new route of transmission for *Escherichia coli*: infection from dry fermented salami. *Am. J. Public Health.* 86:1142-1145.

Tuttle, J., Gomez, T., Doyle, M. P., Wells, J.G., Zhao, T., Tauxe, R.V. and Griffin, P.M. 1999. Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiol. Infect.* 122:185-192.

U.S. Department of Agriculture (USDA). Food Safety and Inspection Service (FSIS). 2005. Microbiology – shelf stable dried meats. Available online (2011): [http://www.fsis.usda.gov/PDF/FSRE\\_SS\\_5MicrobiologyDried.pdf](http://www.fsis.usda.gov/PDF/FSRE_SS_5MicrobiologyDried.pdf)

Van Eylen, D., Indrawati, Hendrickx, M. and Van Loey, A. 2006. Temperature and pressure stability of mustard seed (*Sinapis alba* L.) myrosinase. *Food Chem.* 97:263-271.

Vilar, I., Garcia-Fontan, M.C., Prieto, B., Tornadijo, M.E. and Carballo, J. 2000. A survey on the microbiological changes during the manufacture of dry-cured lacon, a Spanish traditional meat product. *J. Appl. Microbiol.* 89:1018-1026.

Williams, R.C., Isaacs, S., Decou, M.L., Richardson, E.A., Buffett, M.C., Slinger, R.W., Brodsky, M.H., Ceibin, B.W., Ellis, A., Hockin, J. and the *E. coli* O157:H7 Working Group. 2000. Illness outbreak associated with *Escherichia coli* O157:H7 in Genoa salami. *Can. Med. Assoc. J.* 162:1409-1413.

Yu, C. and Chou, C. 1998. Fate of *Escherichia coli* O157:H7 in Chinese-style sausage subjected to different packaging and storage conditions. *J. Sci. Food Agric.* 78:486-490.