Effects of Caliber Size and Fat Level on the Inactivation of *E. coli* O157:H7 and *Salmonella enterica* Serovars in Dry Fermented Sausage

by

James DeSouza

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ABSTRACT

EFFECTS OF CALIBER SIZE AND FAT LEVEL ON THE INACTIVATION OF E. COLI O157:H7 AND SALMONELLA ENTERICA SEROVARS IN DRY FERMENTED SAUSAGES

James DeSouza
University of Guelph, 2016

Advisor: Professor S. Barbut

The effects of caliber size and fat level on the inactivation of E. coli O157:H7 and Salmonella enterica serovars in dry fermented sausage (DFS) were examined. Sausages batters of two fat levels (low, 9.67%; high, 18.46% w/w) were inoculated with a Salmonella enterica serovars or E. coli O157:H7 five strain cocktail and stuffed into small, medium, and large (32, 55, 80 mm) caliber casings. The sausages were fermented and dried for eight weeks, following conventional commercial procedures and monitored for changes in pathogen counts, pH, a_w, and moisture:protein ratio (M:Pr). A significantly (P<0.05) faster reduction of S. enterica serovars was seen in comparison to E. coli O157:H7 in all sausages. There was no significant (P>0.05) difference in the reduction of S. enterica serovars between the six sausage types, while high fat and larger caliber sizes required longer drying in order to achieve a 5 log reduction of E. coli O157:H7.
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<td>Canadian Food Inspection Agency</td>
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<td>Centers for Disease Control and Prevention</td>
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<td>Colony Forming Unit</td>
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<td>Dry Fermented Sausage</td>
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<tr>
<td>Gluconate Delta Lactone</td>
<td>GDL</td>
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<tr>
<td>Guelph Research and Development Center</td>
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<td>Hazard Analysis and Critical Control Points</td>
<td>HACCP</td>
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<td>Lactic Acid Bacteria</td>
<td>LAB</td>
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<tr>
<td>Moisture : Protein ratio</td>
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<td>Ready to Eat</td>
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<td>Relative Humidity</td>
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<td>Trypticase Soy Agar</td>
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<td>Trypticase Soy Broth</td>
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<td>Verotoxin Producing <em>E. coli</em></td>
<td>VTEC</td>
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<tr>
<td>Water Activity</td>
<td>$a_w$</td>
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Xylose Lactose Tergitol 4 Agar  
XLT 4
Chapter 1  Introduction

Fermentation is one of the oldest methods of food preservation and has been used for thousands of years. Today preservation is still an important reason for fermenting foods but flavour and variety are also important (Farkas, 2007). There are numerous types of fermented foods originating from countries around the world. Well-known fermented foods include dairy products such as yogurt (fermented milk), vegetable products such as sauerkraut (fermented cabbage), and alcoholic beverages such as wine and beer (Chilton, Burton, & Reid, 2015). In terms of fermented meats, there is a diverse range of products, in which raw ground meat is preserved by processes such as fermentation and drying. The typical sausage making process combines minced meat, fat, salt, sodium nitrite, sugar, herbs/spices and a starter culture containing lactic acid bacteria (LAB). These sausage products are manufactured without any heat inactivation treatment before consumption, and, therefore, the inhibition of contaminating pathogens relies upon the collective effects of a relatively low pH, and reduced water activity ($a_w$), conveyed ingredients such as starter cultures, curing salts, and spices, as well as the fermentation and drying processes (Bacus, 1986). Today, dry fermented sausage (DFS) products are considered ready to eat products (RTE) even without the use of a heat treatment (Health Canada, 2000). The products are dried until 25%-50% of the moisture removed leaving a final moisture protein ratio (MP:$r$) no larger than 1.9:1 and a final pH no higher than 5.3, (Hwang et al., 2009). However, after a chain of unrelated foodborne illness outbreaks linked to DFS beginning in 1994, and affecting various countries around the world, it was discovered that pathogenic Escherichia coli (E. coli O157:H7) and Salmonella enterica serovars (S. enterica serovars) can contaminate and survive in fermented meats at levels sufficient to cause serious illness (European Commission, 2003). These pathogenic strains can enter the product through
contaminated raw meat or ingredients, as well as equipment, or post-processing contamination (Hwang et al., 2009).

1.1 Introduction to Processing of Dry Fermented Sausages

Dry fermented sausages (DFS) are prepared by grinding meat into uniform particle sizes; depending on the fat ratio there can be 2 to 3 different particle sizes in a sausage. After grinding, a starter culture, salt, curing salt, and spices are added to the ground meat creating a batter. The batter is mixed and stuffed into casings composed of either natural intestines (typically pork intestine), or synthetic casings such as cellulose or manufactured collagen-based. Typically a LAB strain is used as a starter culture, breaking down sugars producing lactic acid upon fermentation. The slow release of lactic acid in turn reduces the pH (Barbuti & Parolari, 2002) resulting in better binding compared to acidified sausages. Another method to reduce the pH is direct acidification; instead of using a starter culture, manufacturers can directly acidify the product by adding an encapsulated acid (e.g. lactic acid, citric acid, or Glucono Delta Lactone (GDL)). The quick drop in pH results in early denaturation of proteins preventing the formation of a matrix in the product. Direct acid (e.g. lactic and citric) causes a fast protein denaturation which leads to clumping and moisture release causing the resultant product to loss its typically smooth texture (Barbut, 2005). This would produce an organoleptically displeasing product with a soft grain-like texture and pungent taste (Barbut, 2006). While encapsulated lactic acid and citric acid can provide a fairly similar texture to that of LAB, encapsulation material and processing time are important factors to consider with respect to overall product texture and organoleptic properties. Another alternative to LAB is the addition of GDL, as it provides a slow acidification in DFS products, but if too much time is taken before the batter is stuffed into casings, the acidified meat particles will crumble making for a brittle product with noticeable fat separation (Barbut, 2005). Apart from acid production, LAB also contribute unique compounds
such as lipases, and protease which add to the product’s flavour profile (Barbut, 2006), and bacteriocins (a natural antimicrobial agent) (Barbut, 2005; Metaxopoulos, Genigeorgis, Fanelli, Franti, & Cosma, 1981). After fermentation (i.e., several days) the sausages are dried under a regulated relative humidity (RH) and temperature until the $a_w$ is reduced. DFS are commonly not heated above 26 °C and the final moisture content can range from 25%-45% depending on the product (Bacus, 1986). Two types of salts that are added to DFS are regular table salt (NaCl) and curing salt (NaNO$_2$). Salt is added not only for flavour but also as a preservative, as it can help lower the $a_w$ which prevents the growth of spoilage microorganisms (at $a_w<0.80$ about 20% salt would be needed which is not the case in DFS product). Salt also plays a major role in texture formation in fermented meat products. Overall, it solubilizes proteins which increases binding properties and creates a protein coat around the fat particles in the meat batter to help stabilize them (Holck et al., 2011). Nitrites present in the curing salts are antimicrobial, and they help to inhibit the growth of $C. botulinum$ (i.e., produces botulinum – a fatal neurotoxin) (Riordan et al., 1998). Nitrites also help to maintain the red/pink pigment in processed meat even after the final product is fermented and dried by, creating a stable bond to myoglobin molecules, thus substituting for the oxygen that would typically be removed during the process. $C. botulinum$ has such a low infectious dose if found in DFS, thus it is imperative to not only inhibit growth but inactivate the pathogen as well (Holck et al., 2011).

The objective of this study was to evaluate the effects of caliber size (sausage diameter) and fat level on the inactivation of $E. coli$ O157:H7 and $S. enterica$ serovars in DFS during the fermentation and drying processes.
Chapter 2  Literature Review

2.1  Dry Fermented Sausages Associated Pathogens (*E. coli* O157:H7 and *Salmonella enterica* serovars)  

There are two main species of *Salmonella*; *S. enterica* and *S. bongori*, and of the two species *S. enterica* serovars are the cause of most *Salmonella* infections in both humans and animals. *S. enterica* serovars are a Gram-negative bacteria belonging to the *Enterobacteriaceae* family, and is the leading strain of reported foodborne illness and deaths in the United States within the last decade (Thomas et al., 2013). There are 2610 different serotypes of the *Salmonella enterica* subspecies, with *Salmonella Typhimurium* and *Salmonella Heidelberg* having the most significant impact on human health (PHAC, 2009). *Salmonella* can be infectious at doses ranging from 1,000 to 100,000 cells depending on the strain, as well as the host. Common symptoms of *Salmonella* infection include diarrhea, fevers, and abdominal pain as quickly as 12 to 72 hrs post infection, with symptoms lasting anywhere from 4 to 7 days before recovery. There have been reports of *Salmonella* causing death in humans when the host is a part of the susceptible group (i.e. young children, elderly, immune compromised, or pregnant women). As the infection is able to spread from the human intestines into the blood stream, this in turn can be distributed into lymph nodes, spleen, liver, and/or the gall bladder (PHAC, 2009). Being heat-sensitive *S. enterica* is most often inactivated during the cooking process. With DFS products not going through a heating process, this can be of concern from a food safety standpoint.

*Salmonella* can be found not only in animals but humans as well, as they are commonly found in the intestines (PHAC, 2009). *Salmonella* is naturally found in chickens, beef, and pigs. Most cases of positive *Salmonella* results are reported to be sourced from these meats as they are a healthy carrier. With 5.4% of broilers, 8.7% in turkey, 0.7% pigs, 0.6% eggs 0.2% bovine
being sources from these meats. For this reason, slaughter and processing equipment must be inspected and cleaned thoroughly in order to avoid cross contamination (Team, 2013).

*E. coli* is a rod shaped Gram-negative bacteria commonly associated with beef cattle, sheep, and pigs as they are naturally occurring healthy carriers of the bacteria. Although most strains are non-pathogenic, some strains have been shown to cause harm to humans. One pathotype of *E. coli* in particular of recent great concern has been shiga toxin producing *E. coli* (STEC). STEC produce a shiga toxin which is the cause of infection to humans. The ability of STEC to contaminate foods is a risk as the infectious dose ranges from 10 to 100 cells (Feng, Jinneman, Scheutz, & Monday, 2011; Paton & Paton, 1998). Of the carrier animals, 10 to 25% of beef cattle have been found to carry STEC (Padola & Etcheverría, 2014). In 2014, the Centers for Disease Control and Prevention (CDC) estimated that STEC is causing 265,000 illnesses annually in the United States (CDC, 2015). Additionally *E. coli* O157:H7 has been estimated to cause more than 134,000 infections every year in Canada (PHAC, 2013). Therefore, the ingestion of contaminated beef is of concern when foods are not handled or cooked properly. In DFS, *E. coli* O157:H7 has been suggested to be the most resistant serotype under the standard conditions of production (Teunis, Takumi, & Shinagawa, 2004). Although heat can be effectively used to destroy pathogens in food products including DFS, heat treatment of dry fermented sausages can cause undesirable changes in colour (dark red to light brown), fat separation and leakage, as well as undesirable flavour changes. These are all issues of concern as this makes for not only an organoleptically displeasing product but aesthetically displeasing too (G. Duffy et al., 1999). Other than the heat treatment option, Health Canada provides a mandatory guideline for controlling *E. coli* O157:H7 in DFS products, which includes five potential options manufacturers can follow. The first option a) is the inclusion of a heat process.
during the manufacturing of the sausage. The heat process used must be recognized by Health Canada where minimal internal temperatures of the product must be no lower than 54.4 °C to 62.8 °C at 121 min to 4 min respectively (specific recognized times and temperatures are present on Health Canada’s website). The second option b) is the use of a manufacturing process that has been scientifically validated to achieve a 5 log reduction of *E. coli* O157:H7 such as a combination of fermentation, heating, and holding/drying time. The third option c) is what Health Canada assumes manufacturers are choosing when none of the other four options is being used. In this case, microbiological end-product testing must be done on each production lot provided with sampled product lots being held until lab results are proven to be negative. The fourth option d) is the implementation of a Hazard Analysis and Critical Control Points (HACCP) system at the manufacturing site. The HACCP plan must also include the testing of raw meat and batter as well as use a scientifically validated manufacturing process such as fermentation, heating, and holding/drying in order to achieve a minimum 2 log reduction. The fifth and last option e) is to use a scientifically validated alternative manufacturing process that is proven to inactivate *E. coli* O157:H7. If using this option, the manufacturer’s process must go through an evaluation process set by Health Canada to determine if it is a viable option. Health Canada has put these options in place not only for *E. coli* O157:H7 but for *S. enterica* as well. As studies show survival of both pathogens under standard DFS production conditions.

### 2.2 Review of Validation Studies

*E. coli* O157:H7 was examined in several different DFS products manufactured under different processing parameters (Apaydin, Ceylan, & Kaya, 2009; Dalzini et al., 2015; L. Duffy & Vanderlinde, 2000; Ellajosyula et al., 1998; Heir et al., 2010; Hwang et al., 2009; Naim, Messier, Saucier, & Piette, 2003). In these studies, there was a lack of consistency in the results between the studies, as log reductions ranged from 0.67 to 4.7, and no 5 log reduction was
achieved. From the literature reviewed, an important note is the variation of DFS products made in the studies. Since each product was processed differently and had an end product consisting of a different proximate analysis, a question of whether or not these products can be compared in terms of pathogen reduction is of concern. A study by Riordan et al., (1998) demonstrated a log reduction of *E. coli* O157:H7 ranging from 0.67 to 4.79 in a pepperoni product with NaCl and nitrite levels ranging from 2.5 to 4.8% and 100 to 400ppm, respectively. It is important to note that the upper level of nitrite used would be too high to be used in a product for consumption under government regulations. Duffy & Vanderlinde (2000) observed a 4.65 log reduction of *E. coli* O157:H7 when fermentation temperatures were raised to as high as 42°C from 21°C. This raised the fermentation temperature well above that of a typical commercially processed DFS where optimal temperature for the growth of starter cultures are 20 to 30°C (Heir et al., 2010). Hwang et al., (2009) demonstrated a 4.4 log reduction of *E. coli* O157:H7 and *Salmonella Typhimurium* in a 25 mm Soudjouk DFS (Turkish style), these products were fermented for 3 to 5 days and dried for 3 to 7 days, thus a shorter process was used in comparison to other DFS products where drying times can last for months, depending on the product. The final a\textsubscript{w} of the Soudjouk was on the upper scale ranging from 0.86 to 0.92 with a final pH of 4.6 to 5.2. It is also important to note that there is a lack of studies pertaining to *Salmonella* in such fermented products, although *S. enterica* is another virulent pathogen that can sometimes be found in DFS products. Of the studies analyzing the inactivation of *S. enterica* in DFS Nightingale, Thippareddi, Phebus, Marsden, & Nutsch, (2006) reported a 3.6 to 4.5 log CFU/g reduction in Italian style salami, after the product was dried to an MP\textsubscript{r} from 1.4:1 to 1.9:1.
2.3 Current Antimicrobial Factors

Current antimicrobial hurdles (ingredients and processing) that are being used in DFS products to inhibit pathogen growth and to reduce survival include ingredients such as salt, nitrite, starter cultures, and various fermentation/drying processing parameters (Pond, Wood, Mumin, Barbut, & Griffiths, 2001). Studies have altered the ratios of these antimicrobial ingredients in formulations, but results show that a 5 log reduction was not achieved without the suggestion that organoleptic properties could be negatively impacted (G. Duffy et al., 1999). Although the added salts do lower the $a_w$ of DFS, acid adapted $S$. enterica were studied and were found to have an increased resistance to heat in the presence of salt (Leyer & Johnson, 1993). $E$. coli has also been shown to gain a developed resistance to osmotic stress by accumulating potassium glutamate to counteract the change in turgor pressure present. This is one of the two factors that play a role in acid tolerance (Gauthier, Flatau, Le Rudulier, Clement, & Combarro, 1991). The solutes that are produced by the pathogenic bacteria do not alter the function of intracellular components, yet they allow the cell to survive the change in turgor pressure via osmoregulation. The second factor is the ability to restore and stabilize the membrane’s lipid bilayer. This is done by changing the ratio of anionic and zwitterionic (positive and negative electrical charges) membrane lipids (negatively charged and neutral with positively and negatively charged phospholipids, respectively). With an increase in anionic phospholipids (diphosphatidylglycerol and phosphatidylglycerol) and a decrease in zwitterionic compounds (phosphatidylethanolamine), the membrane lipid bilayer is stabilized preventing any changes in pressure as a result from the low $a_w$ (Beales, 2004). It can be noted, that apart from the role of decreasing the $a_w$ of DFS, the salt content (at relative concentrations) had no direct effect on the inhibition of $S$. enterica (Coroller, Jeuge, Couvert, Christeans, & Ellouze, 2015).
Starter cultures play an important role not only in terms of product taste, colour, and cohesion, but antimicrobial properties as well. The production of lactic acid by the LAB starter cultures is effective in lowering the pH to inhibit pathogenic growth (Bacus, 1986). Therefore, choosing a viable starter culture is an important aspect to ensure pathogen reduction. An efficient starter culture is able to quickly produce lactic acid, thus inhibiting pathogenic growth during early stages of the process while the $a_w$ is still of relative concern. Having a lower pH early on in the drying process prevents the growth of pathogens until the water activity drops from $\sim$0.99 to $\sim$0.96 during fermentation, and to $\sim$ 0.90 after drying. Upon drying, the pH gradually rises again (from $\sim$4.5 to $\sim$5.0) as a result of proteolysis and surface flora activity (Hwang et al., 2009). Thus pathogens that are able to survive the change in pH during fermentation are then succumbing to the low $a_w$ of the final product. The CFIA has regulations in place to ensure that all products are safe to consume. If the $a_w$ is not checked, the final pH of the product must be $\leq$4.6 or, on the contrary, if the pH is not checked the final $a_w$ must be $\leq$0.85. If both the $a_w$ and pH are measured in the final product, the pH and $a_w$ can be as high as $\leq$5.3 and $\leq$0.90, respectively (Health Canada, 2000). DFS products can also be acidified, meaning instead of a starter culture, acid is added directly to the batter. An issue that comes with acidification is the need to use encapsulated acids, as directly adding the acid would cause the pH to drop too quickly in comparison to the slow release of acids that would be produced by lactic acid bacteria (Barbut, 2005). Some strains of LAB can also produce significant amounts of bacteriocins as a by-product of fermentation (Zacharof & Lovitt, 2012). Bacteriocins are able to disrupt surface membrane layers, and prevent the uptake of various compounds which are strain-specific to the bacteriocin being produced. Bacteriocins can be used as antimicrobial agents in food in two ways; they can be added directly as a specific purified bacteriocin (e.g. nisin) or specific strains of starter
cultures can be used to produce bacteriocins. *Pediococcus* acidilactici is a strain that is often used in DFS products for its bacteriocin producing capabilities as it produces the bacteriocin PAC1.0 (Zacharof & Lovitt, 2012). Using lab strains specific for producing bacteriocins as a starter culture has been studied in the production of DFS products. Campanini et al., (1993) studied *Lactobacillus* plantarum used as a starter culture and compared with a non bacteriocin producing mutant *L. plantarum* strain. They reported no significant difference in the reduction of *L. monocytogenes* between the two different cultures used. In terms of sensory aspects and product composition, there was no difference noted in the previous study as well as in a study done by Coffey et al., (1998) comparing high bacteriocin producing strains of LAB against typical sausage starter cultures. There are 4 classes of bacteriocins produced by LAB; i) lantibiotics (nisin), ii) small heat stable peptides, iii) large heat labile proteins, and iv) bacteriocins requiring carbohydrates or lipids. All four of these classes are composed of bacteriocins that are hydrophobic or amphilic and are composed of 20 to 60 amino acid residues (Zacharof & Lovitt, 2012). Lantibiotics can be further broken down into two categories (Type A and B). Type A are positively charged elongated peptides whose mode of antimicrobial function is to create pores in bacterial membranes. While Type B lantibiotics are globular peptides with a negative or neutral charge. These lantibiotics can inhibit specific enzymes as an antimicrobial mode of action. Small heat-stable peptides contain the largest number of bacteriocins of the four classes and are effective at reducing *L. monocytogenes* (Jack et al., 1996). Most research has focused on the first two classes while the third class is not widely studied and of lesser interest to food scientists and the fourth class has not been widely studied due to the bacteriocin dependency on carbohydrates or lipids in order to be active but more research needs to be done in this area for further evaluation.
A reduction of *E. coli* O157:H7 and *S. enterica* could also be achieved by post primary processing parameters such as heating, freezing, and altered storage conditions and times of finished meat products (Rode et al., 2012). Health Canada’s suggested heating time-temperature combinations range from 54.5°C for 121 min to 6 °C for 12 min, and have been shown to have effective results to inhibit *E. coli* O157:H7 (Health Canada, 2000). However, changes in product appearance were noted at high temperatures. Heating the product at 43°C followed by freezing and storage for a month showed positive inhibition results, but again products that have been heated to temperatures that high have resulted in significant changes in organoleptic properties (L. Duffy & Vanderlinde, 2000). During the freezing process sharp ice crystals are formed, the rigid crystals rupture protein structure causing a change in the products texture.

### 2.4 Effect of Fat Level on Pathogen Inactivation

Apart from the chemical composition of DFS having an effect on the growth of bacteria, an often overlooked aspect is the effect of the food structure on microbial growth. Several studies were done to solely determine an antimicrobial compound’s effect on the inactivation of bacteria *in vitro* as opposed to *in vivo*. A factor that can be further studied is the effect of fat levels on microbial inactivation. Moisture content of a DFS can vary based on fat levels, as there is water present in the lean raw meat. Thus, the fat to lean mean ratio can affect the initial moisture of a product, which can potentially alter the growth patterns of any pathogens present. As fat composition and particle size are essential to the meat batter, there is potential for the batter to contain pockets of free water where pathogens can thrive. Previous studies that examined the effect of fat on the inactivation of pathogens are limited in respect to DFS. To our knowledge,
the studies that have been conducted have been done using various types of DFS each with different processing parameters and varying final composition.

Included in the studies examined, the use of saturated fat replacements (i.e. olive oil and grape seed oil) in different meat systems was explored. Results showed greater antimicrobial activation on samples containing oils as opposed to the control samples which contained animal fats (Jung et al., 2012), although DFS were not used in that study. Another research interest pertaining to fat levels is the favorable environment of reduced fat products on *E. coli* O157:H7 and *S. enterica* as they are typically higher in moisture in comparison to full-fat products. Due to insufficient research literature in regards to the effects on microbial growth in low fat DFS products, further research needs to be undertaken to fully comprehend its effects. Although numerous studies have shown that fat has the ability to protect pathogenic growth, specifically *E. coli* O157:H7 from inactivation in ground beef, (Ahmed et al., 1995; Line et al., 1991), studies applying this theory to dried fermented products had conflicting results. Two studies by Faith et al., (1998) resulted in a greater reduction of *E. coli* O157: H7 in high fat (32%) content samples compared to low fat (15%) content samples. The first study examined dried pepperoni inoculated with *E. coli* O157:H7, although the pathogen was mixed in the batter, samples were drawn from the pepperoni after it had been sliced and cooked in the oven with temperatures ranging from 135°C to 246°C for 0 to 20 min. The heat treatment included would affect the inhibition of *E. coli* O157:H7 producing variable results. By heating the product, the fat content would play a role in the temperature distribution of the product. It is possible for the high fat pepperoni slices to have been heated thoroughly in comparison to the low fat counterparts resulting in a greater inhibition of *E. coli* O157: H7. Another possibility is the difference in moisture between the two
cooked products, with the low fat product having a higher $a_w$ allowing *E. coli* O157: H7 cells to survive over a longer period of time thus allowing for greater growth. With a decrease in moisture over the drying process causing an increase in salt concentrations which can be related to the higher VTEC reduction noted. In their following study Faith et al. (1998) examined the effect of fat content on the inactivation of *E. coli* O157:H7 in beef jerky slices with results similar to that of the pepperoni study. Jerky is typically a very small diameter product with a faster drying time than pepperoni or other DFS products. As the jerky was not a fermented product there is no subsequent change in pH helping to inhibit microbial growth, thus inhibition is solely based on a reduced $a_w$ from the addition of salt and drying. Low fat products typically have higher moisture contents. Because of this, pathogens are able to survive at a higher level in low fat products as the previously mentioned studies have shown.

A study done by Heir et al. (2010), evaluated the inhibition of *E. coli* O157: H7 populations based on high (21%) and low (12%) fat levels in DFS products. Results in this study showed that there was a lower inhibition of *E. coli* O157:H7 in high fat level DFS compared to the low fat DFS.

While in the study by Stoltenberg et al., (2006) there was no significant difference in bacterial growth based on fat levels. This study used snack sticks composed of beef and a venison/beef fat blend. The product was also acidified with citric or lactic acids to a pH ranging from 4.8 to 5.2. Contrary to dried fermented sausages, this product did not contain a starter culture as it was an acidified product and it was also heat-treated. As the product was heated, the reduced $a_w$ was noted in both the high fat (25%) and low fat (10%) products as it dropped from
0.96 and 0.95 to 0.939 and 0.935, respectively. Results showed that heat treatment provided a 2 log reduction, while upon drying a 5 log reduction was noted. The type of acid used (lactic and citric) had no significant difference in the pH of the end product (5.1 and 4.9, respectively). The inhibition of \textit{E. coli} O157: H7 could be affected by all three factors; the low pH of the added lactic or citric acid, heat treatment, and low \(a_w\). Thus, further analysis of whether fat content had an effect would need to be done. Future research to determine the effects of fat levels on dry fermented sausages should be studied as current results vary.

2.5 Effect of Caliber Size on Pathogen Inactivation

The size of casing diameter (caliber) has also shown to alter the inhibition of bacterial growth. As longer processing time are known to increase the lethality of bacteria, larger diameter DFS inherently need a longer drying time in order to achieve the necessary \(a_w\) levels. Heir et al., (2010) demonstrated that reduction in \(a_w\) was strongly correlated with two main factors; casing size and NaCl content. The smaller casing diameter had faster drying times. By lowering the \(a_w\) quicker than the large diameter products, there was a greater inhibition of \textit{E. coli} O157:H7 noted as the pathogen had more time to survive in the larger diameter products. Although contradictory results were found in a study done by Dalzini et al. (2015), where low fat, large diameter Italian salami was inoculated with \textit{E. coli} O157:H7, \textit{S. enterica} and \textit{L. monocytogenes}. After the salami was fermented and dried there was a 2.5, 1.65, and 0.5 log reduction of \textit{E. coli} O157:H7, \textit{S. enterica} and \textit{L. monocytogenes}, respectively. These results are slightly lower than those of typical DFS products but still fall within the common 2 to 3 log reduction that is often noted. Due to these conflicting findings, further research pertaining to optimal drying times based on DFS diameter are needed.
2.6 Summary

Pathogenic *E. coli* O157:H7 and *S. enterica* can contaminate and later survive in fermented meats at levels sufficient to cause serious illness, as evidenced by several outbreaks of *E. coli* O157:H7 and *S. enterica* infections in DFS. A number of studies presented here focused on the kinetics of inactivation of *E. coli* O157:H7 and *S. enterica* and have examined various factors such as temperature, pH, aw, starter cultures, and product formulations for enhanced and effective inactivation of these pathogens. Results from current studies vary in their respective ability to provide a 5 log reduction of the pathogenic bacteria needed, whilst maintaining organoleptic properties in a cost efficient matter. Hence, further research is needed in areas such as processing parameters (e.g., determine optimal fermentation and drying times) based on fat levels, and determining processing times required for different DFS casing diameters.

2.7 References


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Chapter 3  Effects of Caliber Size and Fat Level on the Inactivation of E. coli O157:H7 in Dry Fermented Sausages

3.1  Abstract
Dry fermented sausages (DFS) have been subject to numerous validation studies, as pathogen reduction heavily relies on both ingredients and processing. In this study the effects of product caliber (casing diameter) size (32, 55, 80 mm), and fat level (low, 9.67%; high, 18.46% w/w) on the inactivation of E. coli O157:H7 during DFS production was examined. Sausages containing a five-strain cocktail of E. coli O157:H7 at $10^7$ CFU/g were manufactured and monitored for changes in E. coli O157:H7 counts, pH, $a_w$ and moisture protein ratio (M:Pr) during the 8 week process. Results were subjected to ANOVA for significance and linear models were produced. A significant increase in the inactivation time was observed in larger caliber sizes. Increasing caliber sizes from 32 to 55 and 80 mm increased the time for 5 log reduction from 29 to 38 and 49 days, respectively. Increased fat level significantly increased inactivation times. The difference between low and high fat levels decreased the averaged reduction from 7.2 log CFU/g to 6.6 log CFU/g respectively at the end of the process overall. DFS manufactured with higher fat level and larger caliber size require longer processing time to achieve a 5 log inactivation of E. coli O157:H7.
3.2 Introduction
Dry Fermented Sausages (DFS) are produced by mixing ground meat and fat, spices, curing salts, and starter cultures are then added. The sausages are then fermented to a pH ≤ 5.3 and dried until a water activity (a_w) of ≤0.90 is attained, the drying process usually results in a moisture loss of 20-50%. The final DFS product must have a pH ≤ pH 5.0, a_w of ≤0.90 and a moisture protein ratio (M:Pr) of ≤1.9:1 (CFIA, 2014). As there is no heat treatment in the process, pathogen reduction heavily relies on ingredients (e.g., salt) and processing parameters (e.g., drying rate). During manufacturing, the addition of salt helps to lower the a_w of the sausage; starter cultures lower the pH during fermentation; and the a_w is further lowered by the drying process.

The processing of some DFS products has been linked to outbreaks of *E. coli* O517:H7 (Conedera et al., 2007; CDC, 1995; MacDonald et al., 2004; Sartz et al., 2008; Williams et al., 2000). Regulatory protocols have been put in place by some government agencies (USDA, 1996; Health Canada, 1996) giving producers the choice of using one of five options to control *E. coli* O517:H7 in DFS products. Two of these options include the use of a process that has already been validated to achieve a 5 log reduction, and the implementation of a HACCP system using a process that has been validated to achieve a 2 log reduction of *E. coli* O517:H7.

There have been limited studies researching the effects of fat level and caliber size on the inactivation of *E. coli* O517:H7 in DFS products. Of the studies that have been conducted, there have also been varying results. One study concluded that high fat levels and large caliber individually reduced the inactivation time of *E. coli* O517:H7 (Heir et al., 2010), but the combined effect was not analyzed. Other studies supported these findings, stating that low fat DFS resulted in a faster drop in pH during fermentation and a_w during drying (Olivares, Navarro,
Salvador, & Flores, 2010; Soyer, Ertaş, & Üzümcüoglu, 2005). A low pH and aw has been seen to increase the inactivation of E. coli O517:H7 (Hwang et al., 2009) in Soudjuk style DFS. In contrast, Faith et al. (1998) reported that low fat level DFS products resulted in a higher final aw. The variation in results could be due to differences in processes/starter culture used or type of DFS products examined in these studies. To the best of our knowledge there have been no studies to date that examined the combined effect of fat level and caliber size on changes in pH and aw in the inactivation of E. coli O157:H7 in DFS products when utilizing identical processes and ingredients (e.g. Salt, spices, and nitrates). Thus, the objectives of this study were to evaluate the effect of caliber size and fat level on the changes in pH and aw as well as their effect on inactivation of E. coli O517:H7 during DFS production.

3.3 Materials and Methods

3.3.1 Bacteria and Growth Conditions

A five strain cocktail of E.coli O157:H7 was used; strain information and source of isolates are shown in Table 3.1. Each strain of E. coli O157:H7 was maintained in a mixture of Trypticase Soy Broth (TSB) and glycerol (1:1 vol/vol) and stored at -80°C. Colonies were isolated individually by streaking on Trypticase Soy Agar (TSA) and incubated for 24h at 37°C. Upon incubation a colony of each strain was transferred to three 250 mL centrifuge bottles containing 200 mL of TSB and 1% glucose and incubated for 24h at 37°C at 120 rpm (New Brunswick Scientific Innova 44, Ependorph, Germany). This preparation allowed for acid adaptation of STEC isolates (Buchanan & Edelson, 1996).

<table>
<thead>
<tr>
<th>E.coli O157:H7 ATCC</th>
<th>Toxin</th>
<th>Source of Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli O157:H7 ATCC</td>
<td>VT1, eae, hlyA</td>
<td>Human feces; American Type Culture</td>
</tr>
</tbody>
</table>
The culture was then centrifuged (Sorvall LYNX 4000 Superspeed Centrifuge, ThermoFisher Scientific, Waltham, MA) at 4000 rpm for 10 min. The supernatant was decanted from each bottle and the remaining pellet was suspended in 10 mL of sterile distilled water. The suspension of each strain was then combined and mixed thoroughly by vortexing. Two 125 mL aliquots of the suspension were stored in a centrifuge bottle on ice (approx. 10-15 min) before being inoculated into two 12.5 kg meat batches at a level of approximately $2 \times 10^7$ CFU/g.

### 3.3.2 Meat Batter Preparation

Dry fermented sausages with and without *E. coli* O157:H7 were manufactured in a dedicated biosafety level 2 containment pilot plant at the Guelph Research and Development Centre (GRDC), Agriculture and Agri-Food Canada, Guelph, ON. All equipment used was chilled overnight at 4°C to prevent fat smearing. The following procedures were completed for three independent replicates for each batter formulation (Table 3.2).

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
<th>High Fat</th>
<th>Low Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>18.46% wt/wt</td>
<td>9.67% wt/wt</td>
<td></td>
</tr>
</tbody>
</table>

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Table 3.2 High and low fat formulations used to produce dry fermented sausages
<table>
<thead>
<tr>
<th>Component</th>
<th>Weight (wt/wt)</th>
<th>Weight (wt/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean Pork, Beef</td>
<td>68.57%</td>
<td>77.36%</td>
</tr>
<tr>
<td>Salami cure</td>
<td>3.71%</td>
<td>3.71%</td>
</tr>
<tr>
<td>Salt, Dextrose and corn syrup solids, Sodium ascorbate, Sodium nitrate, Sodium nitrite</td>
<td>0.44%</td>
<td>0.44%</td>
</tr>
<tr>
<td>Starter culture (dissolved in mL deionized water)</td>
<td>0.03% wt/wt (10^7 CFU/g)</td>
<td>0.03% wt/wt (10^7 CFU/g)</td>
</tr>
<tr>
<td>E. coli O157:H7 cocktail</td>
<td>10^7 CFU/g</td>
<td>10^7 CFU/g</td>
</tr>
</tbody>
</table>

The salami batter was prepared by adding the pre-weighed raw meat (12.5 kg) into a meat mixer (LEM 868 Tilt mixer, West Chester, Ohio) and mixed for 30 sec to break down large clumps, before adding 125 mL of *E. coli* O157:H7 inoculum cocktail resulting in an inoculation level of 10^7 CFU/g. Following the addition of inoculum, salt, cure, spices, and starter culture (CSL Pellet Bactoferm®, CHR Hansen, Denmark) were added to the meat batter and mixing continued for an additional 5 min. Fibrous cellulose casings (Nalo Fibrous, Kalle GmbH, Wiesbaden, Germany) were precut to the desired length and clipped shut one end prior to the processing day. The mixed sausage batter was then placed into a decontaminated pre-cooled stuffer (Talsa H20 hydraulic stuffer, Xirvella, Spain) and stuffed into moistened casings (soaked in deionized water for 20 min) with a caliber size of 32, 55, or 80 mm at a length of approximately 100, 150, and 200 mm i.e., to at least twice the diameter of the stuffed casing as is required by the Canadian Food...
Inspection Agency (CFIA) Challenge Study Protocol (CFIA, 2014). The stuffed casings were then clipped shut (Poly Clip EZ 700, Hattersheim am Main, Germany) using metal clips and a string was attached to one end. The sausages were then hung in a fermentation cabinet (Stagionello STG100 MTO, Crotone, Italy) for five days at an initial temperature of 26°C for 24 hrs followed by a subsequent drop in temperature; 2°C every 24 hrs for the following 48 hrs. At that point the 2°C decrease in temperature occurred every 12 hrs until a final temperature of 14°C was achieved. The relative humidity (RH) was set to 88% for 24 hrs and was then dropped to 80% for another 24 hrs, after this point a 2% drop in RH occurred every 24 hrs until an RH of 75% was reached and maintained. The sausages were then moved to a drying cabinet (Caron Enviro-Chamber 6020, Marietta, OH) for the subsequent 48 days of the study with a temperature set to 14°C and a RH of 75%.

3.3.3 Microbiological analysis
Sampling of DFS was done on days 0, 1, 2, 3, 4, 5, 11, 18, 25, 32, 39, 46, and 53 of the process. One sausage of each caliber size was collected by placing an open stomacher bag around the sausage ensuring that no other sausages were touched. The string holding the sausage was then cut allowing the sausage to fall into the bag. This was done to minimize possible contamination during sampling. Each sausage size was sampled once per sampling day and repeated for 3 batches of each formulation. Sausage samples were cut along the cross section into three separate sections using a stainless steel scalpel (Integra Life Sciences, York, PN). From there approximately 8 g were taken from each of the cut sections to retrieve approximately 25 g composite sample. The 25 g of composite sample was placed in a sterile filter bag containing 225 mL of 0.1% sterile peptone water (Difco Peptone Water, Bectin, Dickinson and Company, Sparks, MD) and homogenized by stomaching (Stomacher 400 circulator, Seward Laboratory Systems Inc., FL) for 2 min at 230 rpm. The stomached samples were then serially diluted with
0.1% peptone water and surface plated (100 µL) in duplicate onto Sorbitol MacConkey agar plates (Difco MacConkey Agar), and incubated for 24 hr at 37°C, after which colonies were enumerated and recorded with a detection limit of 25 CFU/g. When no colony growth was noted during the 53 day process, samples were enriched in TSB supplemented with Novobiocin (7694, Neogen Corporation, Lansing, MI) and incubated for 24 hr at 37°C to account for injured cells. The enriched samples were then spread-plated in duplicate onto Sorbitol MacConkey agar plates for detection of *E.coli O157:H7*.

Uninoculated DFS samples were enriched in TSB supplemented with Novobiocin and incubated for 24 hr at 37°C. The enriched samples were then spread plated in duplicate onto Sorbitol MacConkey agar plates for detection of *E.coli O157:H7*.

### 3.3.4 Physicochemical Analysis

Approximately 5 g samples from all three sections of the sausage cross section were taken to create an approximately 15 g composite sample. The composite sample was then placed in a food processor (Blixer 2, Robot Coupe U.S.A., Jackson, MS) and blended until the sample was uniform (approximately 15 to 20 sec) and analyzed for pH, $a_w$, moisture, fat, and protein. The pH was analyzed using a VWR symphony SP7-P pH reader and surface pH probe (B10P Benchtop Meter, VWR, Radnor, PN). The $a_w$ was measured using the Dew point activity meter 4TE (Aqua Lab, Pullman, WA). Moisture and fat content were analyzed with a Meat Trac Fat and Moisture Analyzer; microwave moisture analyzer and LF-NMR, respectively (AOAC method 2008.06CEM, Matthews, NC). Protein content was analyzed by a CEM Sprint Rapid protein analyzer (AOAC Official Method 967.12, 930.33, and 930.29.). The average value and standard deviation of each test was calculated for each sampling point in triplicates.
3.3.5 Statistical Analysis

The *E. coli* O157:H7 counts (CFU/g) were transformed to log CFU/g + 1. The value 1 was added before logarithm transfer in order to accommodate the value of zero CFU/g. Analysis of variance (ANOVA) was carried out to evaluate the difference of pH, *a*<sub>w</sub>, moisture, protein, and fat composition and microbial counts at each sampling point, using R 2.14.2 (R Foundation for Statistical Computing, Vienna, Austria) at a 95% confidence level (P<0.05). Linear modeling was completed using R2.14.2. Slopes and Y-intercepts of the log CFU/g + 1 of each DFS type were obtained and differences were analyzed at a 95% confidence interval (P<0.05). Linear equations were derived using the significantly different slopes and Y-intercept and the log CFU/g + 1 was plotted for the corresponding equation.

3.4 Results and Discussion

No *E. coli* O157:H7 was detected in any of the uninoculated DFS samples tested throughout the 53 days sampling plan. Changes in pH, *a*<sub>w</sub> or M:Pr of the uninoculated DFS were not significantly different from the inoculated samples (P > 0.05; data not shown) indicating that inoculation with very high concentrations of *E. coli* O157:H7 does not affect the physicochemical changes of the DFS.

3.4.1 Effect of Caliber Size on pH, *a*<sub>w</sub>, and M:Pr

Changes in pH, *a*<sub>w</sub> and M:Pr of DFS products of varying caliber size during the fermentation and dry curing process is presented in Figure 3.1. The DFS production process examined in the present study could be split into two main steps; fermentation/curing and the drying process. Over the course of the 5 day fermentation and curing process a significant reduction in pH was observed (P < 0.05). However, no significant change between DFS products of varying caliber size (small, medium, large) were seen in pH, *a*<sub>w</sub>, or M:Pr (P>0.05). The pH dropped from 5.88 ± 0.08 to 5.02 ± 0.10 in 24 hr and to 4.86 ±0.08 after 72 hr of the fermentation cycle. These results
are similar to those observed by Muthukumarasamy and Holley (2006) who examined sensory qualities of DFS. However, examining the full process, caliber size had a significant effect on the differences in both $a_w$ and M:Pr between the small, medium, and large caliber DFS ($P < 0.05$). These results are in agreement with those previously reported by Heir et al. (2010) who examined the reduction of *E. coli* O157:H7 by process and recipe optimisation in DFS. No significant difference in the pH between the different caliber sized DFS was observed during the drying phase of DFS production. However, caliber size had a significant effect on $a_w$ ($P < 0.05$).

A finished DFS product typically has an $a_w$ of < 0.90 and an M:Pr < 1.9 (CFIA, 2014). In the present study, small and medium caliber DFS reached these values on day 18 while the large caliber DFS reached the same value at a significantly longer time on day 39 ($P < 0.05$). In relation to the time of reaching a 5 log CFU/g reduction, the $a_w$ for the small, medium, and large caliber DFS were 0.78, 0.89, and 0.86 on days 32, 39 and 53 respectively (Figure 3.1B). The small caliber DFS was the only sample of the three caliber sizes not within the typical finished DFS $a_w$ range when the 5 log CFU/g reduction was achieved. Therefore, the integrity of the physiochemical properties of the small caliber DFS could be in question due to the low $a_w$ and final moisture content. There was a significant difference in M:Pr between the different caliber sized DFS. The prescribed M:Pr (<1.9) was reached on day 11 for the small and medium caliber sizes, while the large caliber DFS reached this range by day 25. In relation to the time of reaching a 5 log CFU/g reduction of *E. coli* O157:H7, the M:Pr for the small, medium, and large DFS on these days was 0.89, 1.1, and 1.3, respectively. This again shows that the M:Pr of the small caliber size was below the typical range of a finished product, having an M:Pr this low can bring the physiochemical properties of the final product into question. Although the medium caliber size DFS had an $a_w$ within range of commercial products, the M:Pr was slightly low.
Comparable to the small caliber DFS, the physiochemical properties for this product might also be in question when the 5 log reduction is achieved. The M:Pr of the large caliber DFS was within the range of a typical DFS, showing that the full 53 day process is needed to provide a 5 log reduction of *E. coli O157:H7* while maintaining the *a*ₘ and M:Pr required for a shelf-stable product.

![Graph A: Effect of Product Caliber on the pH During the Fermentation of DFS](image1)

![Graph B: Effect of Product Caliber on *a*ₘ During the Manufacture of DFS](image2)
3.4.2 Effect of Caliber Size on Microbial Inactivation

DFS products produced with three differing caliber sizes of 32, 55 and 80 mm (small, medium, and large, respectively) resulted in no significant 

\( P > 0.05 \) changes between sizes in regards to 

\textit{E. coli} O157:H7 reduction during fermentation in both the high and low fat level DFS (Figure 3.1). A reduction of 1.34, 1.30, and 1.36 logs was observed in the small, medium and larger
caliber size high fat DFS, respectively, during the initial fermentation/curing stage. However, examining the full process, caliber size has a significant impact on the reduction of \textit{E. coli} O157:H7. A significant difference in the reduction was seen between the small and large caliber sizes as well as the medium and large caliber sizes ($P < 0.05$) during the drying process. The $5\log$ CFU/g reduction needed for products not undergoing a heat treatment (CFIA, 2014) step was achieved by days 32, 39 and 53 for the small, medium, and large caliber DFS, respectively (Figure 3.1D). Alternatively, the $2\log$ reduction needed for products made using a HACCP certified process (CFIA, 2014), was achieved on days 14, 17, and 26 respectively (Figure 3.1D). As there was a significant difference in both $a_w$ and M:Pr between the small, medium, and large caliber DFS ($P < 0.05$) and no significant difference in the pH between the different caliber DFS (Figure 3.1A), the difference in reduction may be attributed to the respective differences in $a_w$ (Clavero & Beuchat, 1996; Shadbolt, Ross, & McMeekin, 1999). Clavero and Beuchat (1996) studied the survival of \textit{E. coli} O157:H7 in broth as well as salami influenced by varying temperature, pH, and $a_w$, where a larger inactivation was recorded when temperatures were increased and $a_w$ was decreased. Shadbolt, Ross and McMeekin (1998) studied the effects of $a_w$ and pH on non-pathogenic \textit{E. coli} (\textit{E. coli} M23) in broth, showing a larger reduction was observed in a low $a_w$ and high pH broth.

### 3.4.3 Effect of Fat Level on Changes in pH, $a_w$, and M:Pr

There was a significant reduction in pH, fat levels did not significantly affect the reduction in pH nor had a significant effect on $a_w$, and M:Pr during fermentation and curing ($P > 0.05$). Similarly during the full 53 day fermentation, curing, and drying process of the two fat levels there was no significant difference seen between changes in pH, $a_w$, or M:Pr when comparing the high and low fat DFS of all 3 caliber sizes ($P > 0.05$; Figure 3.2). Studies have shown a significant difference in pH drop between high and low fat level salami during the fermentation process (Olivares et
al., 2010; Soyer et al., 2005) however, longer fermentation times were used in those studies. Olivares et al (2010) recorded a faster pH decline when fat was reduced from 30% wt/wt to 10% wt/wt after 9 days of fermentation/curing. In the present study, when the 5 log reduction was achieved (day 39 of the process), the high and low fat DFS had an $a_w$ of 0.821 and 0.830, respectively. This is close to the typical commercial range of $a_w$ but on the lower side of the spectrum, and falls short of the 0.87 $a_w$ observed in another study analyzing the effect of fat level (Olivares et al., 2010). As previously mentioned, the small caliber DFS physiochemical properties may be compromised at this $a_w$ range. In terms of M:Pr, when the 5 log reduction was achieved for both fat levels (day 39) the M:Pr of the high and low fat DFS was 1.23 and 1.07 respectively. Similar to the $a_w$ of the high and low fat DFS, this too is below the usual range of M:Pr, adding to the questioning of the physiochemical properties of both the high and low fat DFS at the time that a 5 log reduction is achieved.
A  

Effect of Fat level on pH During the Fermentation of DFS

- pH High Fat
- pH Low Fat

B  

Effect of Fat Level on a_w During the Manufacture of DFS

- High Fat (25.12% w/w)
- Low Fat (17.55% w/w)
Figure 3.2 Changes in pH (A), \( a_w \) (B), M:Pr (C), and log CFU/g reduction of \textit{E. coli} O157:H7 in dry fermented sausages manufactured with high (25.12 % wt/wt) and low (17.55 % wt/wt) fat levels.

3.4.4 Effect of Fat Levels on Microbial Inactivation
Unlike DFS of different caliber sizes, there was a significant difference in the reduction of \textit{E. coli} O157:H7 seen during the fermentation and curing process between the high and low (18.46% wt/wt; 9.67% wt/wt) fat level DFS. After the 5 day process, there was a 1.32 log reduction in the
high fat DFS compared to a 1.12 log reduction in the low fat DFS (Figure 3.2D). However, when looking at the whole 53 day fermentation, curing, and drying process there was no significant difference seen in log reductions between the two fat levels. Both high and low fat levels of each caliber size reached the required 5 log reduction on similar days of the process (Figure 3.2D). Although there are few studies that have researched the effect of fat levels on *E. coli* O157:H7 inactivation in DFS products, results from studies on the role of fat level on *E. coli* O157:H7 in products such as cooked pepperoni, raw ground beef, and dried beef jerky show conflicting results (Faith, et al., 1998; Faith, et al., 1998; Tamplin, 2002). Dried beef jerky demonstrated a higher inactivation in high fat products, while cooked pepperoni demonstrated a slower inactivation in high fat, and ground beef stored at 10 °C had an increased growth in lower fat samples. Caliber sizes for both the high and low fat level DFS showed similar outcomes, suggesting that fat levels studied here, do not have a significant effect on the reduction of *E. coli* O157:H7 during the whole manufacturing process of DFS.

### 3.4.5 Modeling Inactivation

Two sets of models were developed in order to describe the inactivation. The models were prepared separately for the two stages of the process; fermentation and drying (Figures 3.3, 3.4). This was done because the reduction rate was affected by different variables for each stage. During fermentation, the inactivation of *E. coli* O157:H7 is reliant on the drop in pH which is brought on by the production of lactic acid at a relatively higher temperature and RH, while the inactivation of during the drying period is reliant on the drop in $a_w$ which is lowered because of the reduced RH and temperature over time. Overall, there was a significant difference in the rate of inactivation of *E. coli* O157:H7 during the fermentation and drying stages of the DFS manufacturing process; for example low fat medium caliber size DFS had an inactivation rate of -2.42 log CFU/g/day during fermentation and -1.32 log CFU/g/day during the drying process.
Figure 3.3 Reduction of \textit{E. coli} O157:H7 over time during fermentation of dry - Log CFU/g \textit{E. coli} O157:H7 over time (Days) during the fermentation process of the manufacturing process of DFS. 3.3A-C represent the high fat level formulation DFS and small (32 mm), medium (55 mm) and large (80 mm) caliber sizes respectively. Figures 3.3D-F represent the low fat level formulation DFS and small, medium and large caliber sizes respectively.

Although fat levels had a significant effect on the reduction of \textit{E. coli} O157:H7 numbers during the fermentation stage (Figure. 2A), the inactivation rates of \textit{E. coli} O157:H7 were not significantly different ($P>0.05$). It should be noted that the higher fat level DFS models showed a generally higher inactivation rate during fermentation with an average slope of -0.250±0.026 log CFU/g/day versus the low fat DFS which had an average slope of -0.219± 0.033 log CFU/g/day (Figure 3.3).
Figure 3.4 Reduction of *E. coli* O157:H7 over time during drying of DFS. Figures 2A-C represent the high fat (25.12% wt/wt) level formulation DFS and small (32 mm), medium (55 mm) and large (80 mm) caliber sizes respectively. D-F represent the low (17.55% wt/wt) fat level formulation DFS and small (32 mm), medium (55 mm) and large (80 mm) caliber sizes respectively.

In agreement with the significant differences in the inactivation of *E. coli* O157:H7 of different caliber sized DFS, there were significant differences (P<0.05) in the inactivation rate of *E. coli* O157:H7 between the small caliber (-0.137±0.012 CFU/g/d) and large caliber (-0.094±0.008 CFU/g/d) as well as the medium (-0.127±0.008 CFU/g/d) and large (-0.094±0.008 CFU/g/d).
caliber sizes (P<0.05) during the drying process of both the high and low fat levels (Figure 4). As reported in a previous study (Heir et al., 2010), this can be attributed to the faster rate of $a_w$ drop in different caliber sizes. The high fat, large caliber DFS showed the lowest rate of inactivation (-0.094±0.008 CFU/g/d), while the low fat, small caliber DFS had the highest rate of inactivation (-0.137±0.012 CFU/g/d). As there was no difference in the rate of inactivation in the small and medium DFS, a linear equation of $y = -0.137x + 6.61$ (where $x =$ time in days, and $y = \log_{10}$ CFU/g E. coli O157:H7) could be used to describe the inactivation during the drying process of the smaller diameter DFS (32 and 55mm) of both high and low fat levels. While linear equations of $y = -0.094x + 7.11$ and of $y = -0.132x + 8.24$ could be used to describe the inactivation of E. coli O157:H7 during the drying process of large caliber of the high and low fat level DFS, respectively.

### 3.4.6 Enrichment of Samples

Results from the enrichment of samples showed that E. coli O157:H7 was still present at the end of the 53 day process in all three caliber sizes as well as in both fat levels within the 6 DFS types. This accounts for the presence of injured cells when no viable cells could be enumerated. Future studies could include end-product holding and testing in order to determine whether these injured cells can become viable over time with the potential for temperature abuse under the already low $a_w$ and pH conditions.

### 3.5 Conclusions

Changing caliber sizes showed an effect on the drying time and, therefore, the inactivation of E. coli O157:H7. As caliber size increased from small and medium to large, the drying and inactivation rates of E. coli O517:H7 decreased. A large caliber sausage took longer to reach a 5 log reduction. A change in fat level had an effect on the inactivation of E. coli O517:H7 during fermentation as a larger reduction was observed in high fat DFS. Linear models showed that the
rate of inactivation generally increased as caliber size and fat levels were reduced. It can be concluded that processors using a larger caliber size, and a higher fat level require longer processing times in order to achieve a 5 log reduction of *E. coli* O517:H7.

### 3.6 References


Faith, N. G., Le Coutour, N. S., Alvarenga, M. B., Calicioglu, M., Buege, D. R., & Luchansky, J. B. (1998). Viability of *Escherichia coli* O157:H7 in ground and formed beef jerky prepared at levels of 5 and 20% fat and dried at 52, 57, 63, or 68°C in a home-style dehydrator. *International Journal of Food Microbiology, 41*(3), 213-221. doi: http://dx.doi.org/10.1016/S0168-1605(98)00058-0


Chapter 4  The Inactivation of *Salmonella enterica* serovars and *E. coli* O157:H7 During the Manufacture of Dry Fermented Sausages Produced with Different Caliber Sizes and Fat Levels

4.1  Abstract
This study examined the effect of caliber size and fat level on the inactivation of *S. enterica* serovars and *E. coli* O157:H7 in dry fermented sausage (DFS *S. enterica* serovars and *E. coli* O157:H7 cocktails were prepared and individually inoculated into sausages of two fat levels (low, 9.67%; high, 18.46% w/w) which were then stuffed into casings of three caliber sizes (32, 55, 80 mm). The sausages were fermented for five days, and then dried for an additional fort-eight days where sampling took place every day during fermentation and once a week during
drying. There was a significantly faster reduction of *S. enterica* serovars seen in comparison to *E. coli* O157:H7. There was no significant difference in the reduction of *S. enterica* serovars among the six sausage types, while high fat levels and larger caliber sizes required longer drying times in order for a 5 log reduction of *E. coli* O157:H7 to be achieved. The rates of reduction were significantly higher in the linear models produced for the DFS inoculated with *S. enterica* serovars compared to those of *E. coli* O157:H7.
4.2 Introduction

*E. coli* O157:H7 and *S. enterica* serovars are foodborne pathogens that have been linked to various outbreaks related to the consumption of contaminated foods, including dry fermented sausages (DFS) (Bremer et al., 2004; Conedera et al., 2007; MacDonald et al., 2004; Moore, 2004; Paton et al., 1996; Prevention, 1995; Sartz et al., 2008; Williams et al., 2000). This resulted in a number of revised regulations and also various validation studies done to examine factors associated with the manufacturing process and their effects on pathogen reduction (Clavero & Beuchat, 1996; Heir et al., 2010; Holck et al., 2011; Hwang et al., 2009; Lahti, Johansson, Honkanen-Buzalski, Hill, & Nurmi, 2001; Nightingale, Thippareddi, Phebus, Marsden, & Nutsch, 2006). Due to the broad nature of the product, manufacturing methods, and ever-changing ingredients and processes, further validation studies are required. There have been fewer outbreaks of *E. coli* and *Salmonella* by DFS in recent years, but the risk is still prevalent as some cases continue to emerge. One of the reasons being that DFS do not include a heat treatment step in the process where *E. coli* O157:H7 and *S. enterica* serovars are able to contaminate DFS in several ways throughout the process. This can happen via contaminated ingredients; namely raw meat but cases of contaminated spices have become known, as well as cross contamination during the process whether during manufacture or potentially during slicing and before packaging steps (Levine, Rose, Green, Ransom, & Hill, 2001).

The USDA and Health Canada (2001) have put in place regulations and guidelines for producers to follow in order to manufacture a safe product (CFIA, 2014). These guidelines are based on recommendations from the US Blue Ribbon Task Force, and give producers the option of using one of five mandatory options in order to control *E. coli* O157:H7 and *S. enterica* serovars (Blue Ribbon Task Force, 1996). A heat treatment step is a viable option that producers may use, but studies have shown that there is a negative effect on the sensory attributes of DFS that have
undergone a heat treatment step. A common option is the use of a validated process that provides a 5 log reduction of *E. coli* O157:H7 and *S. enterica* serovars.

The manufacturing of DFS begins with raw lean meat and fat, which is ground and mixed along with spices, and curing salts. Traditional methods included fermentation by natural microflora as well as back slopping processes, but over the past four to five decades, processors include lactic acid bacteria based starter cultures (Ammor & Mayo, 2007). The sausages are then fermented at temperatures as high as 32 °C and a relative humidity (RH) as high as 95%. The fermentation is complete when the reducing sugars have been used up, producing a fermented sausage that is firm, red in colour, and maintains a pH ≤ 5.3. The fermented sausage is then stored under drying conditions, which include a lower temperature, ranging from 9 to 15 °C and a RH of 70 to 85%. The fermented sausage is dried until 20 to 50% of the moisture has been removed depending on the type of product. This manufacturing process must produce a DFS that is shelf stable with a pH ≤ 5.0, a water activity (*a*<sub>w</sub>) ≤ 0.90, and a moisture protein ratio (M:Pr) of ≤ 1.9:1. (CFIA, 2014).

The possible variations of ingredients, formulations, and processing conditions can result in a different final DFS (Calicioglu, Faith, Buege, & Luchansky, 1997; Hwang et al., 2009; Nightingale et al., 2006). Thus numerous validation studies have been completed on specific DFS products and they may only be applicable to those specific types of DFS. To our knowledge, there is a limited amount of published scientific articles that examine the effects of fat levels, and caliber sizes on the inactivation of *E. coli* O157:H7 as well as *S. enterica* serovars. From these studies, specific water activity (*a*<sub>w</sub>), pH and moisture protein ratio (M:Pr) values were obtained, meaning the results may not be comparable to other DFS products (Heir et al., 2010), indicating that there is a lack of knowledge for manufacturers as the validation studies are for specific DFS products.
The objectives of this study were to evaluate the effect of caliber size and fat level on the drying time and inactivation of *E. coli* O157:H7 and *S. enterica* serovars during the manufacturing processes (fermentation and drying) of DFS.

4.3 Materials and Methods

4.3.1 Inoculated Pathogens

Five strains of *S. enterica* serovars and five strains of *E. coli* O157:H7 were used in this study as shown in Table 4.1. Strains were stored individually in Trypticase Soy Broth (TSB) containing glycerol (1:1 vol/vol), and maintained at -80°C. Each strain was streaked on Trypticase Soy Agar (TSA) and incubated for 24 hrs at 37°C. One colony of each strain was then transferred into 200 mL of TSB containing 1% glucose in a 250mL centrifuge bottle and incubated for 24 hrs at 37°C while being shaken at a speed of 120 rpm (New Brunswick Scientific Innova 44, Ependorph, Germany).

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Source of Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> O157:H7 ATCC 43890</td>
<td>Human feces; American Type Culture Collection</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7 ATCC 43895</td>
<td>Raw hamburger meat from a hemorrhagic colitis outbreak; American Type Culture Collection</td>
</tr>
</tbody>
</table>

Table 4.1 Pathogen serotype and source of isolate of the individual five strain *E. coli* O157:H7 and *S. enterica* serovars cocktails used in this study
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> O157:H7 ATCC 43894</td>
<td>Human feces from raw hamburger meat hemorrhagic colitis outbreak; American Type Culture Collection</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7 E318N</td>
<td>Human isolate; Public Health Agency of Canada</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7 Ec20000859 98-3099</td>
<td>Genoa salami associated outbreak; Public Health Agency of Canada</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>enterica</em> serovar Heidelberg ATCC 8326</td>
<td>American Type Culture Collection</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>enterica</em> serovar Enteritidis ATCC 13076</td>
<td>American Type Culture Collection</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>enterica</em> serovar Berta ATCC 8392</td>
<td>American Type Culture Collection</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>enterica</em> serovar Typhimurium ATCC 14028</td>
<td>Pig body fluid/excretion isolate; American Type Culture Collection</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>enterica</em> serovar Newport ATCC 6962</td>
<td>Isolated from food poisoning fatality; American Type Culture Collection</td>
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</tbody>
</table>

This procedure was repeated three times for each strain allowing for a final volume of broth for each strain to reach 600 mL. Upon incubation, the inoculated broth was centrifuged at 4,000 rpm (Sorvall LYNX 4000 Superspeed Centrifuge, ThermoFisher Scientific, Waltham, MA) for 10 min. The supernatant was decanted (from each bottle) and 10 mL of sterile water was added to the remaining pellet. The pellet from each strain was then shaken until visibly suspended, and combined. This was stored on ice before being inoculated into the raw ground meat, creating an approximate inoculation level of $2 \times 10^7$ CFU/g.
4.3.2 Dry Fermented Sausage (DFS) Manufacturing

All ingredients and processing guidelines were supplied by a large commercial industry manufacturer. Three replicates of each formulation were performed individually for both inoculated pathogens; formulations are provided in Table 4.2.

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
<th>High Fat</th>
<th>Low Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>Fat</td>
<td>Lean Pork, Beef</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>--------------</td>
<td>----------------</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>18.46% wt/wt</td>
<td>9.67% wt/wt</td>
<td></td>
</tr>
<tr>
<td>Salami cure</td>
<td>3.71% wt/wt</td>
<td>3.71% wt/wt</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.44% wt/wt</td>
<td>0.44% wt/wt</td>
<td></td>
</tr>
<tr>
<td>Starter culture</td>
<td>0.03% wt/wt (10^7 CFU/g)</td>
<td>0.03% wt/wt (10^7 CFU/g)</td>
<td></td>
</tr>
<tr>
<td>(Dissolved in 5mL deionized water)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathogen cocktail (E. coli O157:H7 or S. enterica serovars)</td>
<td>2x10^7 CFU/g</td>
<td>2x10^7 CFU/g</td>
<td></td>
</tr>
</tbody>
</table>

A meat mixer (LEM 868 Tilt mixer, West Chester, Ohio) and stuffer (Talsa H20 hydraulic stuffer, Xirivella, Spain) were chilled overnight at 4°C before the manufacturing of the DFS. Each batch was made by first mixing the raw meat for 30 s in order to spread apart large pieces of meat. Once mixed, 125 mL of the inoculum suspension were added. After mixing for an additional minute, the salt, sugar, curing salts, and spices were added to the meat batter followed by a starter culture (SP362 TEXEL, Danisco, Denmark). The meat batter was mixed for an additional 5 min before being placed in the stuffer. Fibrous cellulose casings (Nalo Fibrous, Kalle GmbH, Wiesbaden, Germany) with a caliber size of 32, 55, and 80 mm were pre-measured and precut to a length at least twice the size of the diameter required by the Canadian Food Inspection Agency (CFIA) Challenge Study Protocol (CFIA, 2014), and soaked in...
deionized water for 20 min as recommended by the manufacturer. The meat batter was then stuffed into each casing, and clipped shut (Poly Clip EZ 700, Hattersheim am Main, Germany) with metal clips and a string for hanging. The stuffed sausages were hung on rods and spaced approximately a diameter length apart to allow for uniform air flow. The sausages were placed in a fermentation cabinet (Stagionello STG100 MTO, Crotone, Italy) at an initial temperature of 26°C and a relative humidity (RH) of 88%. After 24 hrs the temperature was lowered by 2°C every 24 hrs for an additional 48 hrs, while the RH was lowered to 80% for 24 hrs and a subsequent 2% drop every 24 hrs was set. After the 72 hr period, a 2°C decrease in temperature occurred every 12 hrs until a final temperature of 14°C and RH of 75% was reached and maintained. After this five day period, the sausages were transferred into a drying cabinet (Caron Enviro-Chamber 6020, Marietta, OH) where they were hung under drying conditions of 14°C and a RH of 75% for the remaining 48 days of the study.

4.3.3 Sample Collection
The DFS were sampled once a day during the fermentation period and once a week during the drying period, this accounted for thirteen total sampling points in the study per each of three replicates. All three DFS caliber sizes were sampled at each sampling point by first collecting the DFS from the cabinets by placing an open stomacher bag around each sausage and cutting the string attached, causing the sausage to fall into the sample bag minimizing any handling of the DFS.

4.3.4 Microbial Analysis
Using a sterile stainless steel scalpel (Integra Life Sciences, York, Pennsylvania), cross-sections of the DFS were cut and an approximate 25g composite sample was added to a sterile filter bag along with 225 mL of 0.1% peptone water (Difco Peptone Water, Bectin, Dickinson and Company, Sparks, MD). The sample was then stomached (STOMACHER 400 CIRCULATOR,
Seward Laboratory Systems Inc., FA) for 2 min at 230 rpm. Samples were serially diluted using 0.1% peptone water, and 100 uL was surface plated onto E. coli O157:H7 and S. enterica serovars selective agar plates; Sorbitol MacConkey and Xylene-Lysine-Tergitol 4 (XLT-4) agar plates, (Difco Macconkey Agar, Bectin, Dickinson and Company, Sparks, MD) respectively. The plates were incubated for 24 hr at 37°C, and enumerated with a detection limit of 25 CFU/g. When colony growth was not present during the study, injured cells were detected by enriching DFS samples inoculated with E.coli O157:H7 in TSB supplemented with NovoBiocin (7694, Neogen Corporation, Lansing, MI) and DFS samples inoculated with S. enterica serovars were enriched in a Selenite Cystine broth (Difco Selenite Cystine Broth, Bectin, Dickinson and Company, Sparks, MD). Enriched samples were incubated for 24 hr at 37°C and spread plated on Sorbitol MacConkey agar plates or XLT-4 in duplicate for detection of E.coli O157:H7 and S. enterica serovars, respectively.

4.3.5 Physiochemical Analysis
A 15 g composite sample was made from the cross-section of each DFS sample, the sample was blended (Blixer 2, Robot Coupe U.S.A., Jackson, MS) for approximately 15 to 20 sec in order to create smaller DFS particles. The ground sample was analyzed for pH and a_w. The pH was measured by a VWR symphony SP7-P pH reader and surface pH probe (B10P Benchtop Meter, VWR, Radnor, PN) in triplicates and the a_w was measured using the dew point activity meter 4TE (Aqua Lab, Pullman, WA). Each test was completed in triplicate, the average value and standard deviation were calculated at each of the sampling points.

4.3.6 Statistical Analysis
Microbiological and chemical differences in the recorded data were analyzed by an analysis of variance (ANOVA) using R 2.14.2 (R Foundation for Statistical Computing, Vienna, Austria) at a 95% confidence level (P<0.05). Microbial counts were transformed to log CFU/g +1 to
accommodate the value of zero CFU/g. The reduction rates of each DFS type were calculated by obtaining slopes and Y-intercepts of the log CFU/g + 1 and differences were analyzed at a 95% confidence level (P<0.05).

4.4 The Effect of Fermentation on the Inactivation of *E. coli* O157:H7 and *S. enterica* serovars in Dry Fermented Sausage

Differences were observed between the reductions of *E. coli* O157:H7 and *S. enterica* serovars during the fermentation process. Both high and low fat DFS demonstrated a larger reduction in *S. enterica* serovars than *E. coli* O157:H7 after the five day fermentation period (Figure 4.1). There was no significant difference in reduction of *E. coli* O157:H7 noted between the fat levels as well as caliber size during fermentation.

![Figure 4.1 Log CFU/g Reduction of *E. coli* O157:H7 and *S. enterica* serovars in dry fermented sausages after a 5 day fermentation process](image)

However, a difference in *S. enterica* serovars reduction was noted between high and low fat levels. The fat level increased, there was less inactivation of *S. enterica* serovars compared to the low fat level DFS. The reason for this can be attributed to fat being able to protect *S. enterica* serovars from acidic conditions (Waterman & Small, 1998). After the five day fermentation, a
1.53±0.07 log CFU/g reduction was recorded in high fat DFS, while a larger reduction of 2.30±0.14 log CFU/g was recorded in the low fat DFS, in comparison to the reduction of *E. coli* O157:H7, where a 1.56±0.45 log CFU/g reduction was observed after fermentation of DFS of both fat levels. As previous studies indicated, the pathogen reduction during fermentation is heavily reliant on the drop in pH (Clavero & Beuchat, 1996; Nightingale et al., 2006). The reduction of *S. enterica* serovars and *E. coli* O157:H7 in this study are consistent with the findings of previous studies, where an approximate 2 log cfu/g reduction was seen in *S. enterica* serovars after fermentation, as well as a larger reduction of *S. enterica* serovars than *E. coli* O157:H7 after fermentation (Heir et al., 2010; Holck et al., 2011; Nightingale et al., 2006). Although in those cases the fat level was not being accounted for and fermentation time and conditions varied. There was no significant difference in pH drop between all six sausage types during fermentation. All DFS had a pH between 4.78 and 4.89 after fermentation, indicating that the growth of the starter culture was not affected by the change in fat level or caliber size. The pH after fermentation was similar to previous studies, where DFS had a pH ranging from 4.4 to 5.0 (Heir et al., 2010; Hwang et al., 2009; Lahti et al., 2001).

Table 4.3 shows a significant difference in the rate of reduction between *E. coli* O157:H7 and *S. enterica* serovars in low fat level DFS, as there was a faster reduction of *S. enterica* serovars during fermentation. However, this was not seen in high fat level DFS, where there was no change in the rate of reduction of *E. coli* O157:H7 and *S. enterica* serovars during fermentation.

**Table 4.3 Reduction Rates of dry fermented sausages composed of high (18.56% wt/wt) or low fat (9.67% wt/wt) levels and small (32 mm), medium (55 mm), or large (80) caliber sizes, inoculated with a *S. enterica* serovars or *E. coli* O157:H7 cocktail**

<table>
<thead>
<tr>
<th>Dry Fermented Sausage</th>
<th>Slope</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Fat Small <em>S. enterica</em> serovars</td>
<td>-0.27014</td>
<td>0.05798</td>
</tr>
<tr>
<td>High Fat Medium <em>S. enterica</em> serovars</td>
<td>-0.31541</td>
<td>0.04962</td>
</tr>
<tr>
<td></td>
<td>Reduction (log)</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>High Fat Large S. enterica serovars</td>
<td>-0.30840</td>
<td>0.05065</td>
</tr>
<tr>
<td>High Fat Small E. coli O157:H7</td>
<td>-0.24233</td>
<td>0.02517</td>
</tr>
<tr>
<td>High Fat Medium E. coli O157:H7</td>
<td>-0.24154</td>
<td>0.02928</td>
</tr>
<tr>
<td>High Fat Large E. coli O157:H7</td>
<td>-0.26639</td>
<td>0.02207</td>
</tr>
<tr>
<td>Low Fat Small S. enterica serovars</td>
<td>-0.37653</td>
<td>0.05331</td>
</tr>
<tr>
<td>Low Fat Medium S. enterica serovars</td>
<td>-0.35560</td>
<td>0.06407</td>
</tr>
<tr>
<td>Low Fat Large S. enterica serovars</td>
<td>-0.35095</td>
<td>0.05048</td>
</tr>
<tr>
<td>Low Fat Small E. coli O157:H7</td>
<td>-0.19855</td>
<td>0.03587</td>
</tr>
<tr>
<td>Low Fat Medium E. coli O157:H7</td>
<td>-0.22740</td>
<td>0.03288</td>
</tr>
<tr>
<td>Low Fat Large E. coli O157:H7</td>
<td>-0.23067</td>
<td>0.02965</td>
</tr>
</tbody>
</table>

The difference in reduction rate can be attributed to the difference in $a_w$ of the DFS after fermentation. As S. enterica serovars are known to be more sensitive to $a_w$, the slight difference in $a_w$ between the high and low fat level DFS ranging from 0.90 to 0.94 and 0.93 to 0.95 respectively.

4.4.1 **The Effect of Drying on the Inactivation of E. coli O157:H7 and S. enterica serovars in Dry Fermented Sausages**

The reduction of S. enterica serovars between the three different caliber sizes did not show significant differences in both the high and low fat level DFS. In high fat level DFS, a 5 log reduction was achieved by day 32 of the study for all three caliber sizes (small, medium, and large); seen in Figure 4.2A, and by days 32, 39, and 53 for the small, medium, and large low fat DFS, respectively.
Figure 4.2 Log CFU/g reduction of (A) *S. enterica* serovars and (B) *E. coli* O157:H7 in dry fermented sausages after the 53 day process

The longer time to achieve the 5 log reduction of *S. enterica* serovars may be attributed to the difference in $a_w$ between the high and low fat DFS, as the low fat DFS had a higher $a_w$ throughout the drying process of all three caliber sizes in comparison to the high fat (Figure 4.3). There were no significant differences in the time to achieve a 5 log reduction of *E. coli* O157:H7 between the high and low fat DFS, where a 5 log reduction was achieved for the high fat level treatment by days 32, 46 and 53 for the small, medium, and large caliber DFS respectively.
(Figure 4.2B), and by days 32, 39, and 53 for the small, medium, and large low fat DFS, respectively.

Figure 4.3 Changes in $a_w$ during the drying process of (A) low (9.67% wt/wt) and (B) high fat (18.46% wt/wt) level dry fermented sausage manufacturing.

S. enterica serovars reduction numbers showed no significant difference between sizes or fat levels by the end of the study. There was a faster reduction rate of S. enterica serovars in low fat DFS compared to high fat DFS. This trend was also noted with the reduction of E. coli O157:H7.
but was only significant in the large caliber sized high fat DFS as seen in Table 4.3. The large caliber DFS showed a significantly less reduction of *E. coli* O157:H7 than the small and medium sizes. It should be noted that for both the high and low fat DFS, the final $a_w$ of the large caliber size was significantly lower than that of both the medium and small caliber products.

In terms of caliber size, there was a significant difference noted in the inactivation of *E. coli* O157:H7 between the small and medium caliber sizes compared to the large. This is similar to previous studies which indicated that larger caliber sizes demonstrate a lower inactivation of *E. coli* O157:H7 in DFS (Heir et al., 2010). The reduction of *S. enterica* serovars showed no significant differences between the small, medium, and large DFS in both the high and low fat level formulations. There was no significant difference in the $a_w$ between the small and medium DFS for both low and high fat levels, but there was a significant difference between the small and medium, and large DFS, as the final $a_w$ after the drying period was 0.76±0.01, 0.78±0.02, and 0.84±0.02 for the small, medium, and large DFS, respectively.

Results from the enrichment of samples showed that *E. coli* O157:H7 and *S. enterica* serovars were still present at the end of the 53 day process in all caliber sizes as well as fat levels within the 6 DFS types. This accounts for the presence of injured cells when no viable cells could be enumerated. Future studies could include end product hold and testing in order to determine whether these injured cells can become viable over time with the potential for temperature abuse during distribution of commercial products.

### 4.5 Conclusions

There was no effect of fat level or caliber size on the inactivation of *S. enterica* serovars, although the final $a_w$ was significantly lower as the caliber size decreased. Contrary *S. enterica* serovars, the inactivation of *E. coli* O157:H7 was affected by fat level during fermentation and
by caliber size during drying. A 5 log reduction of \textit{S. enterica} serovars was reached by day 32 for all 6 DFS types, whereas a 5 log reduction of \textit{E. coli} O157:H7 was reached in longer periods especially as the caliber sizes increased. Linear models showed that \textit{S. enterica} serovars had a faster rate of reduction compared to \textit{E. coli} O157:H7 during both the fermentation and drying steps of the process. As \textit{S. enterica} serovars was inactivated more quickly than \textit{E. coli} O157:H7, processors should ensure longer processing times when formulating DFS products with higher fat levels or larger caliber size. While in terms of \textit{S. enterica} serovars, it can be noted that fat levels and caliber size have no effect on the microorganisms reduction during the manufacturing process of DFS.

4.6 References


**Chapter 5  Conclusions**

The objectives of this study were to evaluate the effect of caliber size and fat level on the inactivation of *E. coli* O157:H7 and *S. enterica* serovars in Dry fermented sausages. To our knowledge, this study was the first of its kind to focus on both fat levels and caliber size in relation to pathogen inactivation in DFS.

There was a significant difference in the $a_w$ reduction between the three different caliber sizes during the drying process. The $a_w$ showed no difference in values between DFS made with high
and low fat levels. The pH of the DFS was also comparable between both fat level as well as the three caliber sizes during the fermentation and drying stages.

It was observed that DFS produced with a high fat level resulted in a lower inactivation rate of *E. coli* O157:H7 during fermentation. There was no difference in *E. coli* O157:H7 inactivation between the high and low fat levels. Generally speaking, the rate of inactivation was increased as fat levels were reduced. The change in caliber size had no effect on inactivation during the fermentation period, but during the drying stages there was a significantly larger reduction in the small and medium calibers compared to the large. Linear modelling showed increased inactivation rates as caliber sizes were increased and fat levels were reduced.

It was also observed that *S. enterica* serovars was not affected by the change in caliber size or fat levels. There was no significant difference in the reduction of *S. enterica* serovars observed in each trial. In comparison to *E. coli* O157:H7, *S. enterica* serovars had faster reduction rates, reaching a 5 log reduction by day 32 of the 53 day process, while the time needed for a 5 log reduction of *E. coli* O157:H7 ranged from 32 to 53 days.