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## THE USE OF *LACTOBACILLUS SALIVARIUS* L28 AS A BIOPROTECTIVE CULTURE IN DRY FERMENTED SAUSAGES

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Dr. David Harmon, Director of Graduate Studies

THE USE OF  
*LACTOBACILLUS SALIVARIUS* L28  
AS A BIOPROTECTIVE CULTURE  
IN DRY FERMENTED SAUSAGES

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THESIS

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A thesis submitted in partial fulfillment of the requirements  
for the degree of Master of Science in the College of Agriculture,  
Food and Environment at the University of Kentucky

By

Kathy Flynt Collins

Lexington, Kentucky

Director: Dr. Melissa Morgan, Associate Professor of Animal and Food Sciences

Lexington, Kentucky

2017

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## ABSTRACT OF THESIS

### THE USE OF *LACTOBACILLUS SALIVARIUS* L28 AS A BIOPROTECTIVE CULTURE IN DRY FERMENTED SAUSAGES

A challenge study to validate a 5 log<sub>10</sub> CFU/g reduction of non-O157 Shiga-toxin producing *Escherichia coli* (STEC) in dry fermented sausage (DFS) was performed. A 4.49 ± 0.474 log<sub>10</sub> CFU/g was achieved over two trials. The results indicated that the process was not effective in reducing the pathogen to the level required of most pathogens by the USDA.

*Lactobacillus salivarius* L28 (L28) was screened in vitro for the ability to inhibit STEC utilizing the paper disk diffusion method. This strain is a known bacteriocin producer. The results revealed that L28 would be a good candidate for use as a protective culture as large zones of inhibition were noted against the STEC. No zones of inhibition were noted against the commercial starter culture; therefore, it would not adversely impact the quality of the DFS.

The supplementary L28 strain was added to a commercial starter culture to provide an additional hurdle in the protection against STEC. The sausage trial showed the additional strain did not offer a significant difference in reduction of the pathogen (p > 0.05). Further study will be required before L28 could be considered for use as a bioprotective culture.

**KEYWORDS:** Fermentation, Lactic Acid Bacteria, Dry Fermented Sausages, Bioprotective cultures, Bacteriocin

\_\_\_\_\_  
Kathy Flynt Collins

\_\_\_\_\_  
November 14<sup>th</sup>, 2017

THE USE OF  
*LACTOBACILLUS SALIVARIUS* L28  
AS A BIOPROTECTIVE CULTURE  
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By

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Nov 14th, 2017

To Stefan,

Thank you for your constant love and support.  
You make life an adventure.

To Jack,

I am so proud of the young man you have become.

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## Table of Contents

ACKNOWLEDGEMENTS .....	iii
LIST OF TABLES .....	vi
LIST OF FIGURES .....	vii
CHAPTER 1 .....	1
INTRODUCTION .....	1
CHAPTER 2 .....	3
LITERATURE REVIEW .....	3
2.1 Fermented Sausages.....	3
2.1.1 History.....	3
2.1.2 Classifications of Fermented Sausage .....	4
2.2 STEC.....	7
2.3 Food Safety and Fermented Sausages.....	9
2.4 Factors Used to Control Microbial Growth in Fermented Sausages .....	14
2.4.1 Water Holding Capacity and Water Activity ( $A_w$ ) .....	14
2.4.2 pH.....	16
2.4.3 Salt .....	17
2.4.4 Nitrite .....	19
2.4.5 Fat Content.....	20
2.5 Desirable Characteristics of Fermented Sausage Starter Cultures.....	21
2.5.1 Homofermentive and Heterofermentative properties .....	21
2.5.2 Bacteriocin Production.....	22
2.5.3 Absence of Amino Acid Decarboxylase Activity .....	25
CHAPTER 3 .....	28
PROCESSING STUDY TO DETERMINE REDUCTION OF NON-O157 <i>ESCHERICHIA COLI</i> IN DRY-FERMENTED SAUSAGE .....	28
3.1 Summary.....	28
3.2 Introduction.....	29
3.3 Materials and Methods.....	32
3.3.1 Bacterial Strains and Culture Media .....	32
3.3.2 Preparation and Inoculation of Sausage.....	33
3.3.3. Physiochemical Analysis .....	34
3.3.4 Microbial Analysis.....	34
3.3.5 Calculation of Log Reduction and Statistical Analysis .....	35
3.4 Results and Discussion .....	36
3.5 Conclusions.....	38
CHAPTER 4 .....	45



INHIBITION OF <i>ESCHERICHIA COLI</i> BY <i>LACTOBACILLUS SALIVARIUS</i> ...	45
4.1 Summary .....	45
4.2 Introduction.....	45
4.3 Materials and Methods.....	48
4.3.1 Bacterial Strains and Culture Media .....	48
4.3.2 Susceptibility Testing.....	48
4.3.2 Statistical Analysis .....	49
4.4 Results and Discussion .....	50
4.5 Conclusion .....	50
CHAPTER 5 .....	54
DIRECT APPLICATION OF <i>LACTOBACILLUS SALIVARIUS</i> IN THE PRODUCTION OF DRY FERMENTED SAUSAGE TO CONTROL THE SURVIVAL OF NON-O157 STEC.....	54
5.1 Summary .....	54
5.2 Introduction.....	55
5.3 Materials and Methods.....	56
5.3.1 Bacterial Strains and Culture Media .....	56
5.3.2 Preparation and Inoculation of Sausage.....	57
5.3.3. Physiochemical Analysis .....	59
5.3.4 Microbial Analysis.....	59
5.3.5 Statistical Analysis.....	60
5.4 Results and Discussion .....	60
5.4.1 Effectiveness of <i>Lactobacillus salivarius</i> L28 in reduction of STEC counts	60
CHAPTER 6 .....	68
OVERALL CONCLUSIONS .....	68
REFERENCES .....	71
VITA.....	82

## LIST OF TABLES

<b>Table 2. 1</b> Characteristics of different fermented sausage types .....	6
<b>Table 2. 2</b> Pathogenic strains of <i>Escherichia coli</i> and their associated virulence genes....	9
<b>Table 2. 3</b> Outbreaks of foodborne illness linked to fermented meats.....	13
<b>Table 3. 1</b> Analysis of pH, water activity ( $A_w$ ), and $\text{Log}_{10}$ CFU/g reduction of STEC during the production of chorizo sausage for trial #1 .....	39
<b>Table 3. 2</b> Analysis of pH, water activity ( $A_w$ ), and $\text{Log}_{10}$ CFU/g reduction of STEC during the production of chorizo sausage for trial #2 .....	40
<b>Table 3. 3</b> Overall $\text{Log}_{10}$ reduction of non-O157 <i>Escherichia coli</i> in chorizo production after 42 days of production .....	41
<b>Table 4. 1</b> Inhibition assay <i>Lactobacillus salivarius</i> L28 against pathogenic strains of <i>Escherichia coli</i> measured by zone of inhibition diameter (mm) .....	51
<b>Table 4. 2</b> Inhibition assay <i>Lactobacillus salivarius</i> L28 vs. Bactoferm™ commercial starter culture ( <i>Pediococcus pentosaceus</i> and <i>Staphylococcus xylosus</i> ) measured by zone of inhibition diameter (mm) .....	52
<b>Table 5. 1</b> $\text{Log}_{10}$ CFU/g of STEC, pH, $a_w$ , and moisture measurements during chorizo production for treatment containing Shiga toxin producing <i>Escherichia coli</i> (STEC) and <i>Lactobacillus salivarius</i> L28.....	63
<b>Table 5. 2</b> $\text{Log}_{10}$ CFU/g of STEC, pH, $a_w$ , and moisture measurements during chorizo production for treatment containing Shiga toxin producing <i>Escherichia coli</i> (STEC) as a control .....	64
<b>Table 5. 3</b> Comparison of $\text{Log}_{10}$ reduction CFU/g of non-O157 Shiga toxin producing <i>Escherichia Coli</i> in chorizo production after 17 days .....	64

## LIST OF FIGURES

<b>Figure 3. 1</b> Log <sub>10</sub> CFU/g reduction of STEC in chorizo sausage at the end of production (42 days total) for two independent trials .....	42
<b>Figure 3. 2</b> Change in pH during chorizo sausage over 42 day production period for two independent trials .....	43
<b>Figure 3. 3</b> Change in water activity during chorizo sausage over 42 day production period for two independent trials .....	44
<b>Figure 4. 1</b> Zones of inhibition of pathogenic <i>Escherichia coli</i> by <i>Lactobacillus salivarius</i> L28 .....	53
<b>Figure 5. 1</b> Comparison of Log <sub>10</sub> CFU/g reduction of non-O157 Shiga toxin producing <i>Escherichia coli</i> (STEC) in dry fermented sausage containing <i>Lactobacillus salivarius</i> L28 and control during chorizo sausage production .....	65
<b>Figure 5. 2</b> Comparison of pH between treatments of dry fermented sausage containing <i>Lactobacillus salivarius</i> L28 and control during chorizo sausage production .....	66
<b>Figure 5. 3</b> Comparison of water activity between treatments of dry fermented sausage containing <i>Lactobacillus salivarius</i> L28 and control during chorizo sausage production .....	67

## CHAPTER 1

### INTRODUCTION

From time immemorial meat has been preserved through the addition of salt and the process of drying. Sausage makers utilized the practice of “backslopping” wherein a small portion of the previous batch would be reworked into the new batch. Native bacterial flora would be transferred in the process. This was known to improve the texture and flavor of the sausage, however, the mechanism of action from the fermentation process by the native flora would not have been understood until the end of the 19<sup>th</sup> century. The intrinsic factors of pH, produced by Lactic Acid Bacteria (LAB), and water activity ( $A_w$ ) rendered a product safe from *Escherichia coli* proliferation in most instances, but, recent outbreaks of this foodborne illness have increased awareness of the pathogenic shiga-toxin producing *E. coli*'s (STEC) ability to adapt to low pH and  $A_w$  environments. This knowledge has led to certain STEC serotypes to be declared as an adulterant in non-intact meat (Baker et al., 2016).

The first modern outbreak of STEC was documented during the summer of 1982 (Riley et al., 1983). Dubbed as the “Washington Experience”, most cases were traced to a popular fast-food chain. The source of the illness being due to undercooked hamburger patties (Bell et al., 1994). In total, there were 731 confirmed cases, 170 hospitalizations, 56 cases of hemolytic uremic syndrome (HUS) and 4 deaths. This led authorities to determine that the pathogen had developed virulence factors that enabled it with a lethal

potency at a low infectious dose (Baker et al., 2016). A similar outbreak also in Washington State two years later was traced to the consumption of dry-cured salami (CDC, 1995c). An unrelated occurrence later that year in Australia was attributed to *E. coli* O111 contamination of Mettwurst, another type of fermented sausage (CDC, 1995a). After these outbreaks, the USDA-FSIS required dry cured fermented sausage (DFS) manufacturers to validate if their process would result in a 5 log<sub>10</sub> reduction in STEC cells (Reed, 1995). Several studies have shown that STEC, specifically *E. coli* O157:H7, can survive the preventative controls of fermentation, drying, and storage if the pathogen exists in high numbers at the beginning of processing (Glass et al., 1992; Hinkens et al., 1996; Holck et al., 2011; Glass et al., 2012). Other outbreaks of the non-O157 variety have also been noted in dry-cured fermented sausage, but have not been investigated to the same extent. The objectives of this study are:

1. To evaluate the viability of non-O157 STEC cells by utilizing the standard methods of dry fermented sausage production.
2. To quantify the *in vitro* bacteriocinogenic and antimicrobial ability of *Lactobacillus salivarius* L28 on non-O157 *E. coli* using the paper disc diffusion method to determine if it has bioprotective action against the pathogen.
3. To quantify the bacteriocinogenic and antimicrobial ability of L28 in dry fermented sausage during the fermentation and drying process to determine if it adds an additional protective level to the finished product.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Fermented Sausages

##### 2.1.1 History

Fermented sausages consist of ground meat mixed with salt, curing agents, and spices that are stuffed into casings and subjected to fermentation by microorganisms which provides shelf stability with no thermal processing step (Lücke, 1994). The definitive origin of this process is lost to history, but evidence exists from Iraq and China that predates the Christian era (Sebranek, 2004; Vignolo et al., 2010; Hui and Evranuz, 2012). Homer may have given the first written reference to sausage in his epic *The Odyssey* where he mentions men sitting by the fire consuming “gizzards filled with blood and fat”(Hui and Evranuz, 2012). In Europe, the pagan fertility festivals of Lupercalia and Floralia used the spicy, phallic shaped sausages in their rituals leading to their outlaw by Constantine the Great until 494 AD (Smith, 1987). One legend recounts that the fermented sausages carried into Gaul by Caesar’s army provided them with the strength for victory and further conquests (Breasted, 1938; Leistner, 1985; Zeuthen, 2007). This meat preservation practice quickly spread through Europe where regional differences developed. Northern Europeans with their cooler climates traditionally produced milder, blander sausages that were smoked. Their Southern cousins had a climate more adapted to air-drying. Due to the greater availability of spices attributed to the geographic

location, Southern style sausages are predominately spicy with added ingredients such as paprika and pepper. While many civilizations perfected this process, the science of fermentation was not understood until the age of Louis Pasteur. Jensen and Paddock were pioneers in development of starter cultures which allowed control of the fermentation process by the addition of *Lactobacillus* bacteria (Roca and Incze, 1990). This microbiological knowledge along with advances in industrialization including refrigeration made it possible to produce high quality safe sausages.

#### 2.1.2 Classifications of Fermented Sausage

The literature concerning the ingredients and manufacture of fermented sausages is vast as many regional and cultural varieties exist. The current definition under 9 CFR 319 is under review. As there are many types and styles of dry fermented sausages, this study will attempt to generalize the process and focus mainly on the safety aspects of production and the microbiology of the starter cultures. Ordinarily, fermented sausages fall into two general categories: fast fermented, semidry sausage or slow fermented, dry sausage. Definitions and Characteristics are listed in Table 2.1. As the name suggests, semidry sausages contain a higher moisture content than dry sausages (Incze, 2007). Due to the faster fermentation time, lower pH, and reduced drying interval. Semidry sausages contain a distinct pungent flavor and less firm texture than dry sausages (Ockerman and Basu, 2007). The parameters of pH, water activity ( $A_w$ ) and moisture to protein ratio (MPR) are the distinguishing factors which are utilized to determine shelf life and safety

conditions (Incze, 2004; Sebranek, 2004). Semidry sausages contain a MPR between 2.3-3.7 to 1.0 with a final pH between 4.7 and 5.4 and  $A_w$  range of 0.91 to 0.95 (Campbell-Platt, 1994; Vignolo et al., 2010). Acidification is more rapid, leading to a shorter processing time. These factors will not render shelf stability; therefore, a smoking or additional heat treatment is often applied as a safety hurdle or refrigeration is required.

Dry sausages are considered shelf stable at room temperature without requiring any additional heat treatment or refrigeration due to their low moisture content (MPR < 1.9:1.0,  $A_w$  between 0.85 to 0.91) and drying process where additional bacterial cultures, usually Gram-positive, catalase positive cocci (Heir et al., 2010; Holck et al., 2017), stimulate enzymatic and proteolytic change to produce flavor and odor compounds (Vignolo et al., 2010). The final pH is higher than semidry sausages (pH range of 5.3 to 5.8) rendering them with a less tangy, milder taste.



**Table 2. 1** Characteristics of different fermented sausage types

Adapted From *Handbook Of Fermented Meat And Poultry* (Toldrá et al., 2007)

Sausage Type	Definition
<p>Dry; long ripening, e.g., dry or hard salami, saucisson, pepperoni, chorizo; shelf stable</p>	<p>Chopped and ground meat            Commercial starter culture            Fermentation temp 15-35°C for 1-5 days            Not smoked or lightly smoked            Bacterial action reduces pH to 4.7-5.3            0.5-1.0% lactic acid; total acidity 1.3%, which facilitates drying by denaturing protein resulting in a firm texture; MPR &lt; 1.9:1, moisture loss 25-50% moisture level &lt; 35%            fat 39%, protein 21%, salt 4.2%, A<sub>w</sub> 0.85-0.86            yield 64%            less tangy taste than semi-dry</p>
<p>Semi-dry; sliceable, e.g., summer sausage, Holsteiner, Cervelat, Tuhringer; refrigerated</p>	<p>Chopped or ground meat            Bacterial action reduces pH to 4.7-5.3 (lactic acid 0.5-1.3%, total acidity 1%) processing time 1-4 wks            Dried to remove 8-30% moisture by heat; contains 30-50% moisture, 24% fat, protein 21%, salt 3.5%, A<sub>w</sub> 0.92-0.94, yield 90%            Usually packaged after fermentation/heating            Generally smoked or refrigerated            MPR 2.3 to 3.7:1.0</p>

## 2.2 STEC

In the production of dry fermented sausages, pathogenic *Escherichia coli* contamination is a growing cause of concern. *E. coli* are Gram-negative, rod-shaped, motile, facultative anaerobic, nonsporulating bacteria belonging to the family *Enterobacteriaceae*. These are natural denizens of the gastrointestinal tracts of mammals and birds. Most are non-pathogenic and are commonly used as an indicator organism to measure fecal or other enteric bacterial contamination in food and water, including those that could be pathogenic. Isolates are segregated by serotypes which are differentiated by surface antigens. The O-antigen is found in the outer portion of the lipopolysaccharide (LPS) that makes up the cell wall of the gram-negative bacteria. It consists of repeating saccharide units that are exposed on the bacterial cell's surface and is encoded by the *rfb* gene cluster. As of 2017, 181 O-groups have been designated. The H-antigen consists of flagellin protein subunits that make up the surface of the flagella, a long tubular structure that enables motility. This antigen is encoded by the *fliC* gene and 53 groups have been identified. *E. coli* may also contain capsular K-antigens and fimbrial F-antigens (Koluman and Koluman, 2017). The six major pathotypes of *E. coli* which cause diarrheal disease are known as enteropathogenic (EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), diffusely adhering (DAEC), enteroaggregative (EAEC), and enterohemorrhagic (EHEC) (Brooks et al., 2005; Ray and Bhunia, 2007). These pathotypes have different mechanisms of action in their ability to invade and colonize the host with some producing toxins. All are classified as mesophilic bacteria with a growth range between 45-115° F (7-46°C),  $A_w > 0.95$  and a pH range of 4.4-9.0 although it has

been noted that the bacterium can tolerate extremely stressful environments such as a pH of 2.5 and desiccation for several months (Hiramatsu et al., 2005).

The EHEC pathotype is the major cause of concern among the six pathotypes of *E. coli* as it can swiftly lead to severe illness in otherwise healthy hosts and is resistant to production methods used to control its growth. This species is particularly deadly due to the production of shiga-like toxins (Stx1 and Stx2) that are produced and the low infective dose required for illness (10-100 cells)(Molina et al., 2003). In one case it is estimated that illness was attributed to O111 contamination in Mettwurst sausage at an infective dose of 1 cell per 10g of product (Paton et al., 1996). A subgroup of this pathotype is the Shiga-toxin producing *E. coli* (STEC) which contain one or both of the AB<sub>5</sub> shiga-like toxins (Stx1 and Stx2). These six subunit toxins are composed of a central A subunit surrounded by 5 B non-covalently linked subunits. The toxin gains entry into host cells by binding of the 5 B subunits to glycosphingolipid globotriaosylceramide (Gb3) receptors and delivering the A subunit by endocytosis. This receptor is commonly found in high concentrations on human renal cells and other primates however, it is not common to ruminants. This allows these animals to act as reservoirs for the bacteria. These toxins can lead to hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombocytopenic purpura (TTP)(Baker et al., 2016).

Other virulence factors associated with STEC include the locus of enterocyte attachment and effacement (LEE) a pathogenicity island that contain the gene that encodes for the formation of intimin proteins (*eae*) an attachment and adhesion outer membrane protein. Once surface microvilli are eroded, the bacteria can tightly adhere by

the formation of pedestals. This disrupts the integrity of intestinal epithelium and leads to diarrheal disease and allows for invasion of the host where the Stx toxins are released (Koluman and Koluman, 2017). The “big-six” non-O157 and their associated virulence genes are shown in **Table 2.2**.

**Table 2. 2** Pathogenic strains of *Escherichia coli* and their associated virulence genes

Target Strain (ATCC No.)	Virulence Genes
<i>Escherichia coli</i> O26:H11 (ATCC BAA-2196)	<i>stx1+</i> / <i>stx2+</i> / <i>eae+</i>
<i>Escherichia coli</i> O45:H2 (ATCC BAA-2193)	<i>stx1+</i> / <i>stx2-</i> / <i>eae+</i>
<i>Escherichia coli</i> O103:H11 (ATCC BAA-2215)	<i>stx1+</i> / <i>stx2-</i> / <i>eae+</i>
<i>Escherichia coli</i> O111 (ATCC BAA-2440)	<i>stx1+</i> / <i>stx2+</i> / <i>eae+</i>
<i>Escherichia coli</i> O121:H19 (ATCC BAA-2219)	<i>stx1-</i> / <i>stx2+</i> / <i>eae+</i>
<i>Escherichia coli</i> O145 (ATCC BAA-2192)	<i>stx1+</i> / <i>stx2+</i> / <i>eae+</i>

### 2.3 Food Safety and Fermented Sausages

The burden of foodborne illness in the United States is not only a public health concern, it contributes to heavy economic losses from contaminated products, recalls, and the demise of consumer trust. The Centers for Disease Control and Prevention estimate that foodborne illness results in 128,000 hospitalizations and 3,000 deaths annually (Scallan, 2011). These illnesses come at a price for producers as well. Economic losses to manufacturers are extensive and can result in a cascade of costs through trade restrictions

and diminished brand reputation. New and innovative methods to control foodborne illness are necessary to protect the public health and economic stability of American producers.

Meat provides microorganisms with a hospitable environment to flourish due to a favorable pH, high moisture content (>70%), and its extensive availability of amino acids, fermentable sugars, and minerals (Holck et al., 2017). While subcutaneous muscle meats are normally sterile, the methods of harvest and processing most likely will result in some degree of microbial contamination. Several strategies are used in tandem to maintain control over both pathogenic and spoilage bacteria that could be a potential contaminate in fermented sausages. In the industry, these methods are commonly known as “hurdle technology”. Some of these hurdles used in fermented sausage production include, time and temperature control, direct or indirect acidification by addition of organic acids or production of lactic acid by selective microorganisms (pH), reduction of water activity ( $A_w$ ), addition of nitrates, control of oxidation-reduction potential (Eh), and the addition of spices and preservatives that contain antimicrobial properties. Conventionally, sausages that were fermented to a low pH (< 5.3) and dried to a water activity below 0.91 were considered safe from pathogenic contamination (Leistner, 2000; Holck et al., 2011). Prior to 1994, no rules were in place to control the manufacture of dry fermented sausage. This was reevaluated when an outbreak of *Escherichia coli* O157:H7 from a dry cured sausage product was identified from Washington state (CDC, 1995b; Tilden Jr et al., 1996). Other outbreaks that followed (see **Table 2.3**) led to the recognition that fermented sausage could pose a significant food safety risk. The USDA

Food Safety and Inspection Service (FSIS) created new performance standards for RTE fermented sausages requiring that the production process must provide for a 5 log<sub>10</sub> reduction in *E. coli* O157:H7. This could be exhibited as one of the following:

1. Apply a thermal treatment as defined by 9 CFR 318.17 or 318.23
2. Apply a thermal treatment of equal lethality to 9 CFR 318.17 or 318.23
3. Test product using International Commission on Microbiological Specifications for Foods (ICMSF) lot acceptance criteria
4. Apply a validation study which shows a 5 log<sub>10</sub> reduction or a process that results in < 1.0 CFU of *E. coli* O157:H7 per 100 grams of finished product
5. Development of a HACCP system including sampling of raw ingredients combined with a process that enables a 2 log<sub>10</sub> reduction

The fifth option was established by The Blue-Ribbon Task Force of the National Cattlemen's Association and accepted by the FSIS after several studies noted that established production processes were insufficient in attaining a 5 log<sub>10</sub> reduction of *E. coli* O157:H7 in a validation study due to the acid and heat tolerance of the microorganism. Most validation studies using non-thermal processes have only attained a 2 to 3 log<sub>10</sub> reduction (Reed, 1995; Chacon et al., 2006; Graumann and Holley, 2008; Balamurugan et al., 2017). Thermal treatment and high pressure processing have been used to reach the 5 log<sub>10</sub> reduction but not without causing changes to the typical texture, sliceability, and mouthfeel of the products (Bamforth and Ward, 2014). Irradiation also remains an option, but it is not well received due to labelling requirements and consumer misunderstanding of the process.

Process validation studies and regulations have been designed around *E. coli* O157:H7 without clear guidelines to determine if these processes are also sufficient to control the non-O157 STEC type. One recent study discovered that the serogroups O145, O26, O103 showed significantly higher acid resistance during fermentation of sausage as compared to O157:H7 (Balamurugan et al., 2017). An outbreak of *E. coli* 0103:H25 in Norway from DFS contributed to the illness of 18 people leading to the development of HUS in 10 and 1 death (Nørrung and Buncic, 2008; Pragalaki et al., 2013). It is estimated that non-O157 strains are responsible for 20-50% of foodborne infections leading to 37,000 illnesses annually (Brooks et al., 2005; Hughes et al., 2006; Conrad et al., 2014). The serotypes identified as 026:H11, 045:H2, 0103:H11, 0111, 0121:H19, and 0145 are progressively more identified with HC and HUS. The USDA Food Safety and Inspection Service (USDA-FSIS) considers these “big six” non-O157 to be adulterants in beef (USDA, 2011) but pork is also at risk from these strains. A study from South Africa reported that pigs routinely shed STEC in their fecal material which leads to environmental contamination and persistence at the farm level (Ferens and Hovde, 2011; Rajkhowa and Sarma, 2014). Additional studies collected non-O157 STEC and O157:H7 from porcine herds in England and Japan (Chapman et al., 1997; Nakazawa and Akiba, 1999). These findings and the ever-present possibility of cross-contamination stress the importance of accepting that porcine products, including pork fermented sausage to be a high-risk food for STEC contamination.

**Table 2. 3** Outbreaks of foodborne illness linked to fermented meats

<b>Location</b>	<b>Agent</b>	<b>Food Source</b>	<b>Probable Fault</b>	<b>Reference</b>
USA	O157:H7	Fermented sausage containing beef	Contaminated raw material	(Tilden Jr et al., 1996)
Australia	O111	Mettwurst	Contaminated raw material	(Paton et al., 1996)
Canada	O157:H7	Genoa salami	Contaminated raw material, poor fermentation	(Williams et al., 2000)
Canada	O157:H7	Fermented sausage containing beef trim	Contaminated raw material	(MacDonald et al., 2004)
Denmark	O26:H11	Fermented sausage containing beef	Contaminated raw material	(Ethelberg et al., 2004)
Norway	O103:H25	Fermented sausage containing mutton	Contaminated raw material	(Sekse et al., 2009)
Sweden	O157:H7	Fermented sausage containing beef trim	Fermentation at too high temperature, aging time too short	(Sartz et al., 2008)

Adapted from *The Oxford Handbook of Food Fermentations* (Bamforth and Ward, 2014)



## 2.4 Factors Used to Control Microbial Growth in Fermented Sausages

### 2.4.1 Water Holding Capacity and Water Activity ( $A_w$ )

Meat contains approximately 70% water. In the production of dry cured sausages, control of moisture content ensures that the growth of pathogenic and spoilage bacteria is contained. Moisture in meat exists in three different fractions that have marked differences on their participation in chemical reactions. Free water is found inside and outside of the muscle myofibers and makes up approximately 10% of total water found in meat. As its name suggests, is not strongly hindered by the capillary force attraction due to the organization of thick and thin filaments, therefore it can flow freely from the muscle tissue, especially after rigor occurs. This water is free to participate in chemical reactions by which bacteria utilize enzymes to hydrolyze proteins and gain nutrients for growth.

Entrapped or immobilized water is found in the matrix of the myofibril but will not be bound to protein. This water is trapped by steric forces or attraction to bound water molecules, but it can be removed by physical means such as drying or freezing. This fraction constitutes the major portion of water in meat at in the region of 80% total water and is also readily available for use by microorganisms in the meat (Damodaran et al., 2007).

Bound water is held tightly by the amino acids of myofibrillar proteins namely glutamic acid, lysine, glutamine, and tyrosine which bind through charged side groups or the strong electronegative pull of nitrogen and oxygen making them virtually impossible

to remove unless by extreme conditions which do not include conventional cooking methods (Apple and Yancey, 2013). This constitutes a very small fraction at 0.5% total water and does not participate in chemical reactions and will not act as a solvent. In the production of fermented sausage, the focus is the removal of the entrapped moisture by slow drying and manipulation of the pH.

The water holding capacity of meat is greatly influenced by pH. The isoelectric point of actin and myosin is a pH between 5.1 to 5.4. At this point the proteins in the meat will have a zero-net charge (an equal number of positive and negative charges on the proteins). This leads to less attraction to water molecules by the proteins and the water will be released. The proteins will also be attracted to one another restricting space in the myofibrillar matrix leading to the expulsion of water. After fermentation, a steady pH of approximately 5.4 will ensure the best environment for drying (Acton, 1978). By dropping the fermentation temperature below 10°C (50°F), production of lactic acid by LAB will cease.

Moisture diffuses outward in a stuffed sausage casing. The coarse grind of the meat increases surface area and creates a pathway for moisture to travel to the surface. The finer the meat grind, the faster the moisture loss. It is critical to maintain an equilibrium of moisture removal from the center and the surface. The diameter of the casing will be a factor to consider; a larger diameter will hold water longer and slow the diffusion rate. The diffusion rate should be equal to the evaporation rate (Roca and Incze, 1990). If moisture is removed too quickly from the outside, the surface will become dry and hard while moisture will be trapped inside leading to spoilage. If diffusion is more

rapid than evaporation, the surface will appear slimy. This will also increase the growth of yeasts and molds on the surface which will raise the pH level confounding moisture control further. Producers should consistently monitor water activity to ensure that moisture loss is balanced. Dry cured sausages should have an endpoint moisture content of 35% which constitutes a MPR of approximately 1.9:1 and  $A_w$  of 0.85 (Marianski and Marianski, 2009).

Water activity is a measure of the free water available for biochemical reactions. It is calculated as the ratio of the food vapor pressure over that of pure water ( $A_w$  of 1.00). This ranges from 0.1 to 0.99. Dry fermented sausage is rendered safe when the  $A_w$  is  $\leq 0.91$  and the pH is  $\leq 5.3$ . Water activity can be lowered by the addition of solutes and the control of relative humidity. Microorganisms have both optimum and minimum water activity values for growth and control. Gram-positive bacteria are normally controlled by a water activity of 0.90 with Gram-negative minimum at 0.93. *Staphylococcus aureus*, yeasts, molds and halophilic bacteria are exceptions and can withstand drier environments of 0.85 to 0.60. These values can be adjusted with pH. Water activity and pH have a synergistic relationship whereby a microorganism's minimal  $A_w$  for survival will be raised by a lower pH.

#### 2.4.2 pH

Manipulation of acidity is an important hurdle concept in food safety. Preservation of food through the direct addition of organic acids or by fermentation is an ancient practice. Acidification takes place when protons are released from an acidulant. Common food acidulants include lactic, acetic, citric, malic and tartaric acids. The

strength of the acid is measured on how readily the molecule releases a proton, or how quickly the proton dissociates from the acid molecule (Damodaran et al., 2007). pH is defined as the negative logarithm of the hydrogen ion concentration in the food matrix. Acidic values are those less than 7 and values below 4.6 will inhibit most microbial growth. During fermentation, weak organic acids including lactic acid and acetic acid are produced as a byproduct of microbial metabolism. These weak acids have pKa values of 3.86 and 4.76 respectively, and will remain in the undissociated state at a lower pH. In this undissociated state, the molecules are lipophilic and can permeate the cell membrane of the bacteria. Once entrance is gained, the more neutral pH of the cytoplasm causes the dissociation of the molecule and the hydrogen ion and anion are released. The charged ions cannot diffuse back through the membrane and the internal pH of the cell begins to drop. This leads to the disruption of the proton motive force, denaturation of metabolic enzymes, and increased solute concentration in the cytoplasm which increases osmotic pressure disrupting the plasma membrane (Lambert and Stratford, 1999).

#### 2.4.3 Salt

The addition of salt to meat has many functions in the matrix besides flavor. It will have a profound effect on the texture and shelf stability. Solubility of the proteins are strongly affected by the concentration of sodium chloride. High concentrations will increase myofibrillar and sarcoplasmic solubility and diffusion leading to the formation of a gel texture between meat and fat particles which secures cohesion of the mixture to form a meat matrix (Työppönen et al., 2003; Holck et al., 2011; Hui and Evranuz, 2012). This ensures sliceability and acceptable texture. Insufficient matrix formation stems from

lack of salt, improper mixing, or premature acidification caused by high levels of contamination or improper use of organic acids. This leads to a mushy texture and deficient bind. High concentrations of salt result in excessive protein extraction and extreme matrix formation leading to fat and protein smearing which is not acceptable. Protein smearing leads to a one-way directional orientation of the meat fibers during casing stuffing. This is the common cause of “pepperoni cupping” (Holdren et al., 1999).

When a food contains a high concentration of a solute such as salt, the osmotic pressure of bacterial cells is disrupted. Microbes contain approximately 80% water in their cytoplasm. When this microbe is placed in a heavy saline environment, this water will diffuse through the plasma membrane towards the solute in an attempt to reach equilibrium. This movement of water outside the cell leads to plasmolysis and will inhibit bacterial growth by slowing cellular metabolism.

The strong ionic nature of sodium chloride will cause it to readily bind to water molecules preventing them from participating in biochemical reactions and in turn reducing the water activity. At saturation level (26.5g/100g) a saline aqueous solution will have a  $A_w$  of 0.753 (Ruiz, 2007). The addition of 3% sodium chloride to ground pork will reduce the water activity from 0.99 to 0.96 (Marianski and Marianski, 2009). This is crucial protection in the beginning stages of fermentation when the starter culture is in the lag phase and no protection is offered by production of lactic acid. Research with differing salt concentrations in meat has shown that with no added salt, all bacteria present will flourish. At 1% (w/v) reduction of spoilage bacteria is significantly different than the 0% (w/v). A 3% (w/v) concentration of sodium chloride will have marked

protection from both pathogenic and spoilage bacteria without heavy reduction of halotolerant LAB. However, at higher concentrations > 4%, organoleptic properties are compromised with inhibition of starter culture interrupting fermentation (Smith and Palumbo, 1973).

#### 2.4.4 Nitrite

Nitrate and nitrite have been used for centuries in curing and preserving meats and fish (Cammack et al., 1999). The addition of saltpeter (potassium nitrate) to meat was commonly used although the mechanism of action was not understood. Nitrite has several functions in cured meat products such as color and flavor formation. Nitrite will react with the iron molecule in myoglobin to produce nitrosylhemochrome which gives sausage a distinctive pink color that is prized by consumers. In the application of food safety, nitrate contains an antimicrobial action that reduces pathogenic and spoilage organisms namely, *Clostridium botulinum*. The inhibitory effect is due to the conversion of nitrate and nitrite to intermediate products which target membranes, interfere with enzyme production, and the DNA of competing bacteria. Nitrite will inhibit oxidative phosphorylation in *C. botulinum* leading to a decrease in metabolic function including active transport. This prevents sporulation, inhibits growth, and leads to death of the cell (Honikel, 2008; Hammes, 2012).

Health and safety concerns have led to use of alternative or omittance of nitrate in fermented meats. Some of these alternative methods include vegetable juices that are naturally high in nitrates the most popular being celery juice powders. By adding compounds that are high in nitrates in conjunction with starter cultures that produce

nitrate reductase, manufacturers can cure meats without directly adding sodium nitrite. The antimicrobial activity of these alternatives may be lower than that of conventional nitrites. The USDA requires refrigeration in fermented meats with less than 120 ppm added (FSIS Directive 7620.3). Celery powders are not approved for use as a curing agent under 9 CFR 424.21(c). These products must be labelled as “uncured” under 9 CFR 319.2 and the label must state “no nitrates or nitrites added except for those naturally occurring in celery powder.”

Some manufacturers are omitting nitrate/nitrite in order to produce a “clean label”. Studies have shown that this is often at the expense of sensory and microbiological quality (Pichner et al., 2006). One study confirmed that the addition of nitrite provides similar protections comparable to pH and  $A_w$  against the growth of *Salmonella typhimurium* in dry fermented sausages. The omission of nitrite to the meat batches led to an increase of 2 - 2.5 log CFU/g in the *Salmonella* population in the end products (Hospital et al., 2014). The maximum limit for nitrate in a comminuted product is 156 ppm and 625 ppm for a dry cured product (Honikel, 2008).

#### 2.4.5 Fat Content

Fat content of the sausage will have an effect on the drying process as it contains little water (approx. 10-15%) and will reduce the initial water activity. The ground meat should contain fat with a high melting point at levels between 20 to 30% to allow for proper drying and formation of the meat matrix to ensure good sliceability. Grinding at too high a temperature (above 54°F) or using a fat source with high levels of polyunsaturated fats with lower melting points (such as pork fat) can lead to fat smearing.

Smearred fat will create an oily layer around the meat particles and inhibit moisture removal (Ruiz, 2007). High levels of PUFAs are prone to oxidative rancidity which can lead to an inferior product with a warmed-over flavor.

## 2.5 Desirable Characteristics of Fermented Sausage Starter Cultures

### 2.5.1 Homofermentive and Heterofermentative properties

Fermentation is the method used by bacteria to chemically produce energy by the production of adenosine triphosphate (ATP) through substrate level phosphorylation without oxygen. *Lactobacillus* utilizes glucose through the Embden-Meyerhof (glycolysis) pathway where the major end-product is lactic acid (90%). Meat does not contain a large source of carbohydrate and must be added to the meat matrix for the fermentation process to start. Some mixed cultures contain facultative heterofermentative organisms that ferment pentoses as well as hexoses through the 6-phosphogluconate phosphoketolase pathway producing lactic and acetic acid. Heterofermentative cultures are often discouraged as they can form gas which can lead to defects in the sausage or acids that promote unpalatable flavors. Catalase positive, nitrate reducing strains are often added for the promotion of color and flavor. Other metabolic products include diacetyl, CO<sub>2</sub>, H<sub>2</sub>O<sub>2</sub>, bacteriocins, and antimicrobials. (Kandler, 1983; Puolanne and Petäjä-Kanninen, 2014). During sausage production, fermentation begins after the meat batter has been ground, mixed with salts and spices. Starter cultures including lactic acid bacteria are added to the mix which is then stuffed into casings. This process should



occur at a temperature of less than 4°C to prevent the outgrowth of spoilage and pathogenic bacteria. Once the sausages are formed they will be placed into an environmental chamber where temperature and relative humidity are tightly controlled. Initial fermentation temperatures will be high to encourage the growth of the lactic acid bacteria. The fermentation rate will be dependent on the optimal temperature requirements of the starter culture. It is imperative to monitor the time the product is held above 60°F (15.6°C); the optimal temperature for *Staphylococcus aureus* growth and toxin production. The FSIS requires less than 1200 degree-hours for products fermented at temperatures between 90°F (32°C) and 60°F (15.6°C). Degrees are measured as the excess of 60°F multiplied by the number of hours held above this temperature until the pH reaches 5.3 (Chacon et al., 2006; Toldrá et al., 2007). When this pH is achieved, the fermentation process is completed and the temperature must be adjusted below 60°F (15.6°C) where lactic acid bacteria will stop converting dextrose to lactic acid and allow for the drying process to begin.

### 2.5.2 Bacteriocin Production

Bacteriocins are antimicrobial peptides that are ribosomally synthesized by bacteria and inhibit growth of other bacterial strains in a shared system (Dobson et al., 2012). It has been determined that most bacterial strains in general produce at least one bacteriocin (Klaenhammer, 1988), with some being very prolific. According to (Gálvez et al., 2007), bacteriocins have eight applications in the preservation of food:

1. Decrease the risk of food poisoning
2. Decrease cross-contamination in the food chain
3. Improve shelf-life
4. Give protection from temperature abuse
5. Decrease economic loss from spoilage bacteria
6. Reduce levels of chemical preservatives
7. Reduce physical treatments
8. Provide alternative preservation barriers for novel foods

Several studies inoculated a food matrix with the parent strain in order for the bacteriocin to be produced in situ. This is an important concept as currently only one bacteriocin, Nisin, has been approved for direct application to products in the United States. *Lactobacillus sakei* C2 research verified that the bacteria could impede harmful microorganisms without interfering with the starter culture during the production of fermented sausages. The sausage quality was not diminished by the treatment (Gao et al., 2014). *Lactobacillus sakei* C2 was pre-applied to food production surfaces to determine if the strain could prevent biofilm formation by interfering with adhesion (Pérez-Ibarreche et al., 2016). Lactiguard, an antimicrobial compound comprised of three lactic acid producing bacterial strains, was evaluated to determine the preventative impact on *Escherichia coli* 0157:H7 in fresh spinach (Calix-Lara et al., 2014). *E. coli* 0157:H7 was applied allowed to dry for 60 minutes then followed by Lactiguard treatment. Growth of the pathogen was inhibited by a significant level. In a similar study, a LAB isolated from fermented pao cai (Chinese pickled vegetables) was found to have antagonistic activity

against *Salmonella enteritidis* on fresh-cut apple (Luo et al., 2015). The use of bacteriocinogenic strains in fermented sausage would provide an extra hurdle against pathogenic bacteria. According to (Vignolo et al., 2010) these strains should meet the following criteria to be a good candidate for selection:

1. The producer strain should have GRAS status (generally recognized as safe)
2. It should be adaptive to the meat environment
3. It should not produce gas
4. It should not inhibit the Gram-positive, catalase positive cocci in the mixed starter culture
5. It should not be impeded by the recipe (NaCl, NaNO<sub>2</sub>, spices)
6. It should have a broad spectrum of inhibition
7. The bacteriocin should be heat stable to withstand processing
8. It should provide no associated health risks (biogenic amines)
9. The bacteriocin activity must only provide beneficial effects (improved safety, quality, flavor, improved health)

In addressing these concerns, it is noted that LAB is ubiquitous in the environment and has been determined by scientific procedure that its use is generally recognized as safe (GRAS) as a competitive inhibitor of pathogenic bacteria in meat and poultry products (21 CFR 170.36). Bacteriocins are considered non-toxic to eukaryotic cells (Cotter et al., 2005). *L. salivarius* has been cultured from fermented and dry cured pork products and ground beef (Luo et al., 2013; Ayala et al., 2017) which shows an adaptability to the meat environment. In vitro studies have also shown that *L. salivarius*

can thrive in acidic conditions similar to a fermented sausage environment with a pH range of 2 to 9. The bacterial strain has been described by Rogosa as homofermentative (Rogosa et al., 1953) with the main by-product of glucose fermentation being lactic acid with no gas production. Bacteriocins produced by *L. salivarius* have been found to contain broad spectrum activity against both Gram positive and Gram negative bacteria (Stern et al., 2006; O'Shea et al., 2011; Messaoudi et al., 2013; Ayala et al., 2017). One bacteriocin, ABP-118, produced by *L. salivarius* is capable of inhibiting several food-borne pathogens including *Bacillus*, *Listeria*, *Enterococcus*, and *Staphylococcus* without antagonizing related *Lactobacillus* (Flynn et al., 2002; Riboulet-Bisson et al., 2012; Messaoudi et al., 2013). Agar inhibition studies with this bacterium by Danisco show strong inhibition of *S. typhimurium*, *S. aureus*, pathogenic *E. coli*, and *L. monocytogenes*. Several bacteriocins have been isolated from *L. salivarius* strains from human, porcine, and avian gastrointestinal tracts. Most belong to the class II non-lanthionine containing bacteriocins. These are low molecular weight peptides (< 10 kDa) that are heat stable and not subject to post-translational modification. The mechanism of action focuses on the disruption of the membrane, pore formation, and leakage of ions leading to cell death (Corr et al., 2007; Dobson et al., 2012).

### 2.5.3 Absence of Amino Acid Decarboxylase Activity

Fermented meats by their nature are high in amino acids, allochthonous and autochthonous cultures with amino acid decarboxylase activity. Biogenic amines (BAs)

are molecular structures that are formed from the enzymatic decarboxylation of free amino acids by microbial organisms. Although they are the product of normal metabolic activity, high concentrations have shown to be toxic to humans (Alvarez and Moreno-Arribas, 2014). Histamine (HIS) and Tyramine (TYR) formed by the decarboxylation of histidine and tyrosine, are the most problematic BAs. Patients that are effected can present with symptoms including flushing, headaches, nausea, heart palpitations, hypertension or hypotension. This reaction is commonly known as “histamine poisoning” or “the cheese reaction”. In extreme cases, high BA consumption has been linked to depression, schizophrenia, and other neurological maladies. TYR induces the release of noradrenaline from the sympathetic nervous system with has an effect on heart rate and blood pressure (Bardócz, 1995). Alcohol may enhance the toxicity of BAs by promoting absorption through the intestinal wall and increasing levels in the bloodstream by inhibition of amino oxidase activity (Maintz and Novak, 2007). Some individuals are more at risk to these effects, especially those that are currently taking monoamine oxidase inhibitors (MAOIs), commonly prescribed antidepressants. These medications prevent the activity of monoamine oxidase enzymes that are used to remove and degrade BAs from synapses leading to an increased concentration of the compounds which act as neurotransmitters in the brain (Suzzi and Gardini, 2003). Persons usually prescribed these medications are advised to avoid foods considered high in BAs including fermented sausage. In healthy individuals, concentrations of 400 mg/kg for HIS and 125 mg/kg for TYR are considered the threshold for toxicity. In susceptible patients, this level is much

lower at 75 mg/kg for HIS and 6 mg/kg for TYR (McCabe, 1986; Taylor and Eitenmiller, 1986).

Not only will these compound have an effect on human health, but they can degrade the organoleptic properties of the product and are commonly used as indicators of quality (Ruiz-Capillas and Jiménez-Colmenero, 2005). Fermented foods in general contain a higher concentration of these compounds due to the nature of the production process which promotes proteolytic activity for color and flavor. *Enterobacteriaceae*, *Pseudomonadaceae*, *Micrococcaceae*, and LAB have all been attributed to high BA concentrations. Good manufacturing procedures can alleviate most of the contamination by pathogen and spoilage bacteria, but the choice of starter cultures that do not contain decarboxylase activity could be another hurdle to enhance food safety (Roig-Sagués and Eerola, 1997) It has been determined that some cultures have the capacity to degrade these compounds and render them nontoxic in a susceptible food source (Leuschner et al., 1998). Several studies have shown that *Lactobacillus salivarius* does not form biogenic amines (Bover-Cid and Holzapfel, 1999; Holzapfel, 2002; Martín et al., 2006), therefore it may enhance the food safety of fermented sausage without negative health effects when used as a starter culture.

## CHAPTER 3

### PROCESSING STUDY TO DETERMINE REDUCTION OF NON-O157 *ESCHERICHIA COLI* IN DRY-FERMENTED SAUSAGE

#### 3.1 Summary

The purpose of this study was to determine if the processing parameters used in the standard manufacture of dry-fermented sausage (DFS) could render a 5 log<sub>10</sub> reduction of pathogenic non-O157 shiga-toxin producing *E. coli* (STEC) as required by the USDA (Reed, 1995). This process relies on the factors of salt content, nitrate reduction, production of lactic acid by starter microorganisms, decrease in water activity (*A<sub>w</sub>*), decrease of redox potential, and outgrowth of competitive starter cultures during manufacture. A challenge study was performed using a 6-strain cocktail of non-O157 *E. coli* known as the “big six”: O26:H11, O45:H2, O103:H11, O111, O121:H19, and O145. Two treatments (uninoculated vs. inoculated) were established in triplicate with two trials (n=2). Environmental conditions and ingredients were held constant with the inoculum being the only variation between treatments. The process exhibited a  $4.49 \pm 0.474$  log<sub>10</sub> CFU/g reduction of non-O157 STEC, falling short of the required 5 log<sub>10</sub> reduction required. Pathogenic cells could still be recovered with enrichment at the end of the drying period with very low water activity. Since the infectious dose of STEC can be as low as 1 to 10 cells per gram, it is paramount to ensure the inactivation of the microorganism by the manufacturing process. Due to limited published data concerning

the survival of non-O157 in DFS, additional studies are required to extend the scope of knowledge and assist producers in adopting practices that ensure a safe product.

### 3.2 Introduction

Traditional dry-fermented sausages do not utilize a thermal processing step. This product's safety is reliant on a successful fermentation process where the critical control points or "hurdles" of  $\text{pH} \leq 5.3$  at the end of fermentation and water activity ( $A_w$ )  $\leq 0.91$  are achieved. This correct application is paramount to control the outgrowth of pathogenic bacteria. "Hurdle technology" focuses on the control of microorganisms through the disruption of their homeostasis, by creating a stressful environment which leads to metabolic exhaustion. Multiple hurdles have a synergistic effect rather than an additive effect (Leistner, 2000). In fermented sausage, the sequence of hurdles is an important concept throughout the production process.

The first hurdle is the addition of salt to the sausage batter. In high salt concentrations, life is energetically expensive. At a concentration of 346 g/L or 34.6 %, most microbial processes cease (Oren, 2011). This concentration, however, would have a disastrous impact on organoleptic properties of a sausage matrix. Therefore, salt must be used in combination with other factors to impede microbial growth. Salt content is normally added at 2.5 to 3.0% of the initial meat weight. As the sausage loses moisture, this percentage increases. The initial water activity of the sausage batter will decrease immediately with the addition of sodium chloride. This immobilizes free water in the batter and prevents its use by spoilage or pathogenic bacteria for metabolic processes.



This action will delay the growth of existing bacteria in the meat before the starter bacteria reaches logarithmic growth. This give the LAB a competitive advantage. Salt will also influence the osmotic pressure on the bacterial cell wall. As solute concentration increases in the interstitial area, cytoplasmic water will be drawn out of the cell causing plasmolysis; a hypotonic environment where the cell dehydrates and shrinks disrupting metabolic processes. Bacteria differ with their ability to cope with osmotic pressure. LAB are salt tolerant and thrive under normal fermented sausage salinity concentrations. Some halophilic bacteria can withstand relatively high concentrations. *Staphylococcus aureus* can grow in media containing as much as 8.0% salt (Ventosa et al., 1998). This is why is it important to have other factors in place that can control for these outliers.

Nitrite, at the beginning of the process, is another hurdle providing protection before the added starter cultures reach logarithmic growth and begin to produce lactic acid. Curing salts can contain nitrite alone or be in combination with nitrate to provide an immediate source of nitrite for protection against *Clostridium botulinum*. The additional nitrate acts as a storage source for color and flavor development as fermentation progresses. Nitrate will be reduced by the enzymatic action of nitrate reductase produced by added Gram positive cocci starter co-cultures such as *Staphylococcus xylosum*. Nitrite can diffuse through the bacterial membrane in its undissociated state ( $\text{HNO}_2$ ) and disrupt the function of bacterial enzymes which in turn interferes with metabolism and growth (Erkkilä, 2001). As the pH drops from the production of lactic acid by LAB, the reduction of nitrate to nitrite will be enhanced (Puolanne and Petäjä-Kanninen, 2014)

giving an example of the synergistic effect of hurdles. This added nitrate/nitrite combination must be under 625 ppm by federal guidelines 9 CFR 172.15.

In the process of fabricating the meat matrix, the surface area is increased by chopping and excessive oxygen is introduced into the system. After the meat is stuffed into casings, the redox potential ( $E_h$ ) will be decreased due to the use of oxygen by the normal flora bacteria found in the meat. This creates an environment hostile to spoilage bacteria but advantageous for the facultative anaerobic LAB giving them a competitive advantage (Leistner, 1995; Holck et al., 2011).

As the time passes, the LAB reach logarithmic growth and begin to produce lactic acid which disrupts the homeostasis of pathogenic and spoilage bacterial cytoplasmic pH. During the fermentation process, LAB ferment the added dextrose in the sausage batter to produce lactic acid. Lactic acid in its undissociated state is lipophilic which allows diffusion through the bacterial membrane where it will decrease the cytoplasmic pH. In order to maintain homeostasis, the protons must be pumped out at a significant energy cost to the bacteria. This leads to metabolic exhaustion when combined with other factors such as competition for nutrients or osmotic stress from added salts and desiccation. Bacteriocins could also be produced at this time which disrupt the membrane stability of competing bacteria.

Lastly, during the drying process the control of the relative humidity and air flow will result in the loss of water available for chemical reactions which will halt the metabolism, growth, and survival of pathogenic bacterial.

This sequence of result should result in a safe and stable product, but recent outbreaks and studies have shown that when present in high numbers, the process will not inactivate STEC completely. This leaves the consumer at risk.

The objective of this study was to validate a 5 log<sub>10</sub> reduction of STEC cells by utilizing the standard methods of DFS production.

### 3.3 Materials and Methods

#### 3.3.1 Bacterial Strains and Culture Media

Six strains of non-O157 *E. coli* were received from the American Type Culture Collection (Manassas, Virginia, USA) known as the Big-Six *E. coli* panel (O26:H11 ATCC® BAA-2196™ strain designation 2003-3014, O45:H2 ATCC® BAA-2193™ strain designation 2000-3039, O111 ATCC® BAA-2440™ strain designation O111, O121:H19 ATCC® BAA-2219™ strain designation 2002-3211, O145 ATCC® BAA-2192™ strain designation 99-3311, O103:H11 ATCC® BAA-2215™ strain designation 2006-3008). The cultures were stored on Brain Heart Infusion agar slants (BHI, Difco™ Laboratories, Sparks, MD, USA) at 4°C and incubated in Brain Heart Infusion broth (BHI, Bacto™ Laboratories, Mt. Pritchard, NSW, Australia) at 37°C for 24h. Bacteria cultures were transferred 3 times before the study to ensure pathogenicity. CHROMagar STEC base (CHROMagar™ Microbiology, Paris, France) was prepared in plastic petri dishes to enumerate bacterial counts. This media contains the antimicrobial compounds

cefixime and tellurite to inhibit the growth of fecal coliforms and other bacteria and differentiates STEC by utilizing a chromogenic agent.

### 3.3.2 Preparation and Inoculation of Sausage

Raw ground pork was obtained from the University of Kentucky Meat Laboratory (Lexington, KY, USA) and ground using a 3/8 plate. Sausage batter was prepared by mixing 2.5 kg of raw ground pork, 3% NaCl (Morton International, Inc. Chicago, IL), 0.3% Curing salt #2 (Nitrate 4%/Nitrite 6.25%) (Anthony's Goods, St. Louis, MO), 0.3% Paprika, 0.3% Black pepper, 0.3% Garlic powder (McCormick® Spice Company, Sparks, MD) and 0.3% dextrose (Difco™ Laboratories, Inc, Detroit, MI). A commercial starter culture containing the LAB organisms *Pediococcus pentosaceus* and *Staphylococcus xylosus* was prepared following the manufacturer's instruction and added to the batter (Bactoform™ TSPX, Chr Hansen, Graasten, Denmark). The initial fat content of the pork was approximately 18% and the moisture content was approximately 65%.

The batter was mixed by hand in a 1 gallon commercially sterile Ziploc® bag (SC Johnson Company, Racine, WI) and divided into two samples of approximately 1250 g. A cocktail of the 6 strain STEC was prepared by centrifuging the individual strains together in a sterile 50 ml polypropylene conical tube. One batter sample was inoculated with the multi-strain mixture of non-O157 STEC to achieve a cell concentration of approximately  $6 \log_{10}$  CFU/g of batter. The batter was stuffed into natural sheep casings

(Quality Casings, Hebron, KY) using a manual stuffer and formed into 10 cm links by twisting. Each sausage link was approximately 50 g. The links were then separated and placed in sterile test tube racks and positioned in digital dry curing cabinet (Lunaire, New Columbia, PA, USA). The sausages were sampled for microbial counts, pH,  $A_w$ , at day 0,1,2,7,14, 28, and 42. Two trials were conducted (n=2) with three sausages of each treatment being sampled in duplicate.

### 3.3.3. Physiochemical Analysis

The temperature setting for the initial fermentation step was at 20 °C with relative humidity of 90% until the internal pH of the sausage reached  $\leq 5.3$ . pH measurements were recorded with a portable meat pH meter (Hanna Instruments, Ann Arbor MI, USA) from three samples with the reported being the mean of the samples. Water activity was measured by an Aqualab Pawkit water activity meter (Meter Group, Pullman, WA, USA) with means of three samples reported. The sausages were then dried at 16°C with a relative humidity of ~80% for 42 days. Relative humidity and temperature were monitored using the Thermadata™ temperature and humidity logger (ThermoWorks Inc., USA).

### 3.3.4 Microbial Analysis

Microbial analysis was performed on three randomly selected sausages of each treatment. A sample was extracted from the center portion of each sausage which

represented approximately 10% of the total weight. The sample was transferred to a stomacher bag and diluted with sterile peptone water (Difco™ Laboratories, Becton, Dickinson and Company, USA) for a 1:10 dilution. On sample days 3-42, the samples were diluted with lactose enrichment broth (Difco™ Laboratories, Becton, Dickinson and Company, USA) to enumerate injured cells. Samples were agitated for 1 minute at 230 rpm in a Stomacher Lab-blender 400 (Worthing, West Sussex, UK). Aliquots were extracted from the stomacher bag and serial diluted to desired concentration using phosphate buffer diluent. The dilutions were plated on CHROMAgar using the EddyJet™ Spiral Plater (IUL, Farmingdale, NY, USA). Agar plates were incubated aerobically at 37°C for 24h. STEC colonies were confirmed by the mauve color. The plate counts were enumerated into Log<sub>10</sub> CFU/ml using a Flash and Go™ computerized plate reader (IUL, Farmingdale, NY, USA).

### 3.3.5 Calculation of Log Reduction and Statistical Analysis

The reduction of STEC examined during the processing of dry fermented sausage was represented as a log reduction using the following formula:

$$\text{Log reduction} = \text{Log} (N_t/N_0)$$

Where  $N_t$  represents the plate count for specific sample day and  $N_0$  represents the initial plate count. Statistical Analysis System software 9.4 (SAS Institute, Cary, NC) was used to determine the means and standard deviation of the viable counts of non-O157 STEC

over the two trials. Graphs were produced using SigmaPlot 12.3 (SysTat, Chicago, IL USA).

### 3.4 Results and Discussion

STEC was not detected at any time in the control samples or the initial raw materials. During the 42-day experiment a  $4.49 \pm 0.474 \log_{10}$  CFU/g reduction was seen in the inoculated sausages (See **Table 3.3**). This falls short of the 5  $\log_{10}$  reduction required by the USDA. Although no colonies grew on the CHROMagar medium during the last sample day, the cells could be recovered with enrichment (lactose broth) and subsequent plating. This confirms that approximately 1 to 9 cells are surviving per 10 g of sausage. When the enrichment broth was plated on Rainbow Agar to determine which strain of STEC survived, it was noted that all strains of bacteria were still viable in the enrichment broth. From this experiment, the data establishes that the manufacturing process was not effective in controlling any of the non-O157 pathogenic bacteria to the extend required by the USDA. A rapid drop in pH during the first three days was observed in all samples and therefore, it was established that the starter culture was actively metabolizing the added dextrose to produce lactic acid. The subsequent and consistent decrease in the water activity was observed throughout the experiment with a final  $A_w$  recorded at  $0.66 \pm 0.014$  (See **Table 3.3**). This is extremely low for a dry-fermented sausage. This could be caused by the very small diameter of the sausage due to the use of sheep casings. The quality of the links was very poor at the end of the

experiment and it was difficult to cut and stomach the samples. It can be questioned if the viable counts were accurately recorded or if the counts were higher but unable to be retrieved fully from the sample due to the condition of the specimen. The sausages were not organoleptically acceptable at the endpoint and were not representative of a commercial product. The pH and  $A_w$  reached well below the established critical control points of  $\leq 5.3$  at the end of fermentation and  $\leq 0.91$ , yet with these factors being met, the 5  $\log_{10}$  reduction was not reached. Individually, each hurdle is non-lethal; only by their collective use do they have the capability to inhibit and prevent pathogenic growth. Unintentionally, this type of food production could result in sub-lethally stressed microorganisms that become resistant. Acid resistance mechanisms have been noted in non-O157 STEC which are induced by sub-lethal exposure to moderately low pH. A study by Kim et al, (2016) showed significantly enhanced survival of O111 when compared to the control after being exposed to fruit juices at low temperature. McKellar and Knight (1999) demonstrated that EHEC strains isolated from outbreaks could become acid tolerant and continue to survive and grow in a pH environment of 4.25.

Studies have shown that STEC is adaptive to dry conditions as well. A major outbreak of O104:H4 in Germany was attributed to dry fenugreek seeds (Knödler et al., 2016). It was determined that the contamination had taken place more than a year before the seeds had been used for sprout production. The outbreak resulted in a high number of cases (3816) with 845 cases of HUS and 54 fatalities. The extremely virulent strain sickened not only the immunocompromised but also healthy adults (Knödler et al., 2016). It has been observed that STEC strains can adapt to stressful environments and may



exploit stress response mechanisms to survive in low  $A_w$  environments. Several studies have seen growth of STEC serotypes during the initial stages of fermentation (Nissen and Holck, 1998; Lindqvist and Lindblad, 2009, 2011). Results from inactivation studies of EHEC in fermented sausage show that the current practices are unsatisfactory in reducing the pathogens to an acceptable level (Nissen and Holck, 1998; Lindqvist and Lindblad, 2009). If the initial level of contamination in the raw materials is high, the process will not suffice to ensure a safe product.

### 3.5 Conclusions

From the observations of the experiment, it is reported that the fermentation and subsequent drying process was not sufficient to achieve a 5 log<sub>10</sub> reduction of non-O157 STEC in dry-fermented sausages. FSIS guidelines recognize the difficulty in establishing a validation study which demonstrates a 5 log<sub>10</sub> reduction of pathogens in dry fermented sausages. Other measures to ensure safety may need to be investigated such as the addition of antimicrobial spices, oils or a bacteriocin producing starter culture. The Blue-Ribbon Task Force on *E. coli* O157:H7 at the National Cattlemen's Beef Association agreed that research is needed to further analyze the safety parameters of this product. Additional research needs were identified which included studying the influence that bioprotective starter cultures may have on the survival and destruction of EHEC in a dry-fermented sausage product (Reed, 1995).

**Table 3. 1** Analysis of pH, water activity ( $A_w$ ), and  $\text{Log}_{10}$  CFU/g reduction of STEC during the production of chorizo sausage for trial #1

Trial 1			
Procedure	$\text{Log}_{10}$ CFU/g STEC	pH	$A_w$
STEC Cocktail	9.45	ND	ND
Day 0	5.68	5.45	0.95
Day 1	5.53	5.52	0.92
Day 2	5.15	5.11	0.92
Day 3	5.38	4.99	0.90
Day 7	5.15	5.19	0.82
Day 14	3.81	5.30	0.74
Day 28	2.61	5.60	0.73
Day 42	1.53	5.64	0.65

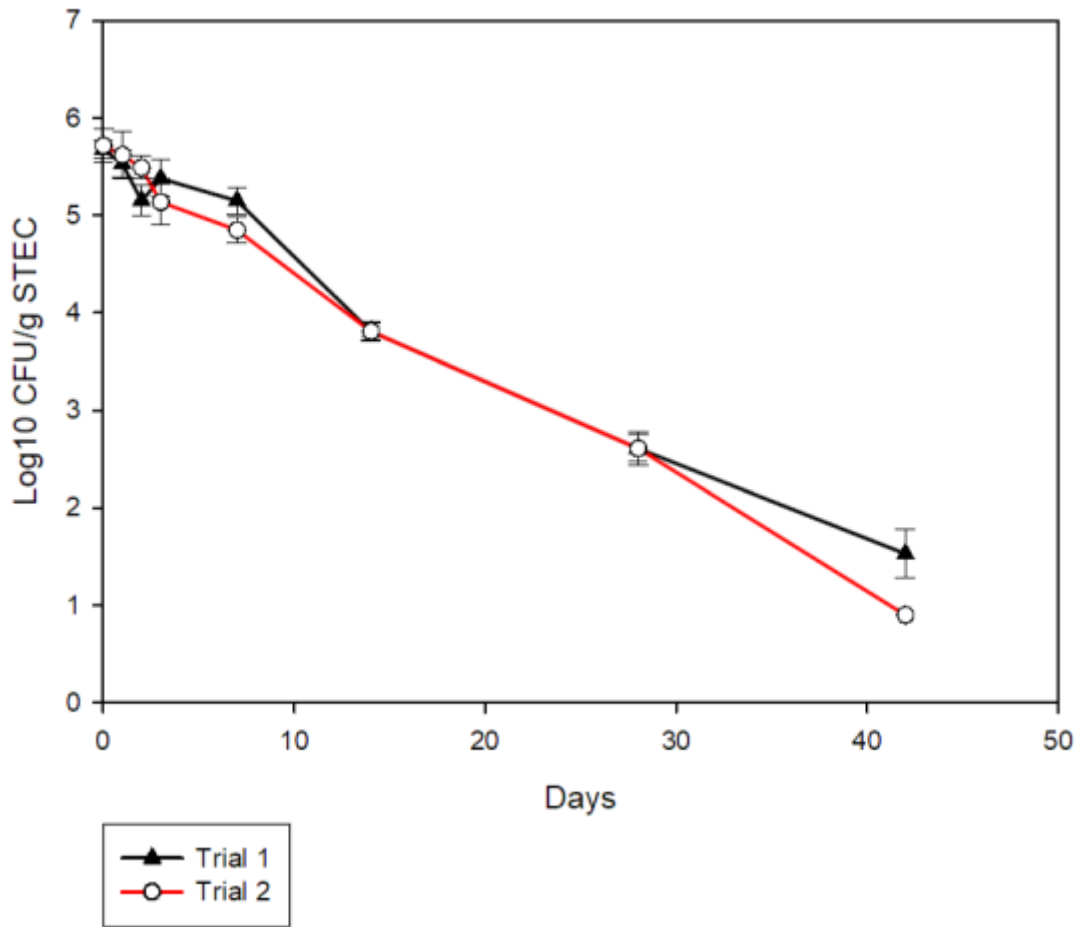
**Table 3. 2** Analysis of pH, water activity ( $A_w$ ), and  $\text{Log}_{10}$  CFU/g reduction of STEC during the production of chorizo sausage for trial #2

Trial 2			
Procedure	Log10 CFU/g STEC	pH	$A_w$
STEC Cocktail	9.29	ND	ND
Day 0	5.72	5.27	0.96
Day 1	5.62	5.28	0.92
Day 2	5.49	5.18	0.89
Day 3	5.14	5.19	0.83
Day 7	4.85	5.11	0.81
Day 14	3.81	5.37	0.79
Day 28	2.61	5.57	0.73
Day 42	0.90	5.58	0.67

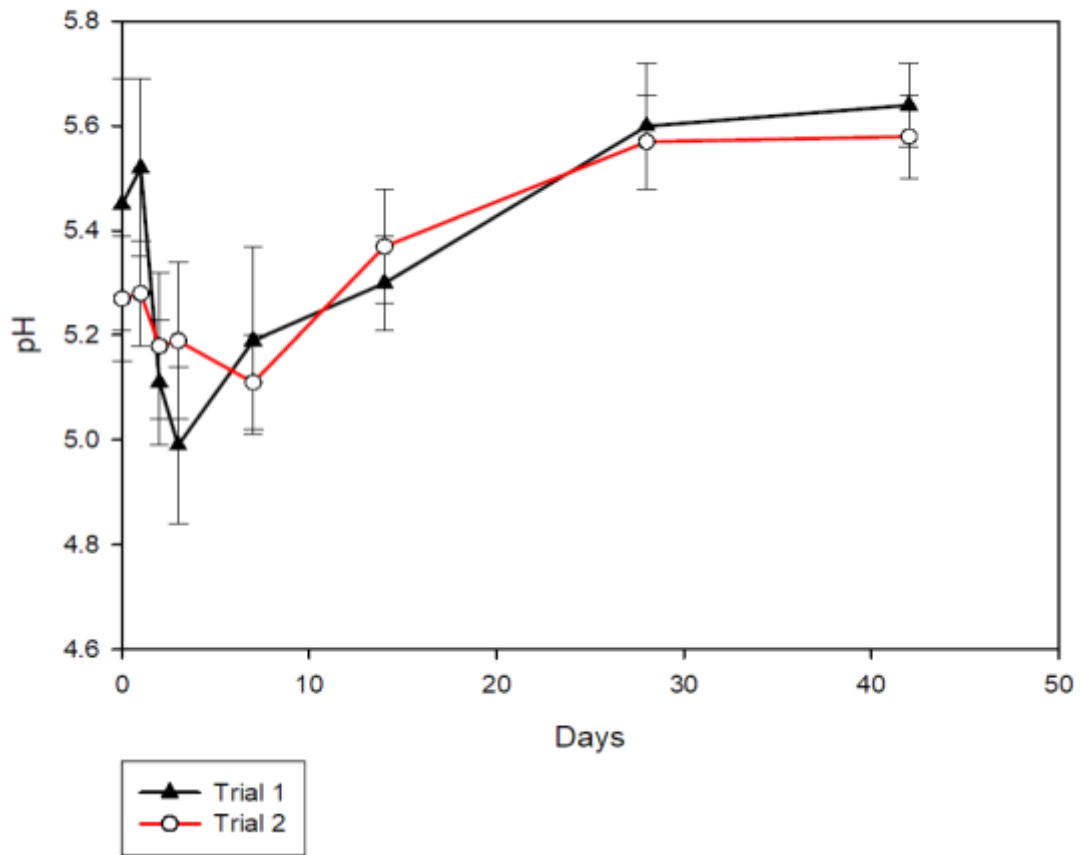
**Table 3. 3** Overall Log<sub>10</sub> reduction of non-O157 *Escherichia coli* in chorizo production after 42 days of production

Trial	Log <sub>10</sub> CFU/g STEC Reduction at endpoint	pH	A <sub>w</sub>
1	4.15	5.64	0.65
2	4.82	5.58	0.67
Mean ± SD	4.49 ± 0.474	5.61 ± 0.042	0.66 ± 0.014

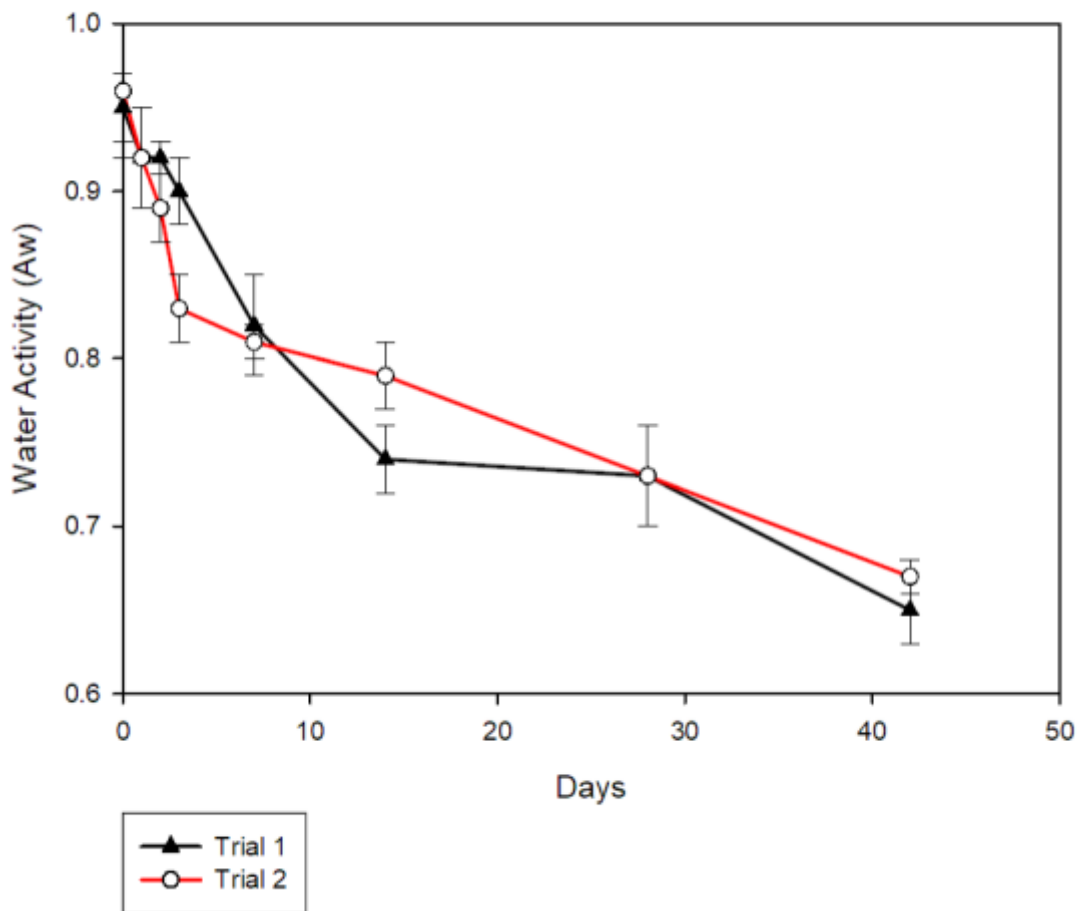
**Figure 3. 1** Log<sub>10</sub> CFU/g reduction of STEC in chorizo sausage at the end of production (42 days total) for two independent trials



**Figure 3. 2** Change in pH during chorizo sausage over 42 day production period for two independent trial



**Figure 3. 3** Change in water activity during chorizo sausage over 42 day production period for two independent trials



## CHAPTER 4

### INHIBITION OF ESCHERICHIA COLI BY LACTOBACILLUS SALIVARIUS

#### 4.1 Summary

The purpose of this experiment was to determine the inhibitory activity of *Lactobacillus salivarius* L28 against the “big six” non-O157 STEC in vitro utilizing the disk diffusion assay or “spot on the lawn” technique. 1 ml aliquots of individual 24 hr cultures of six non-O157 STEC pathogens consisting of O26:H11, O45:H2, O103:H11, O111, O121:H19, and O145 were transferred to Mueller-Hinton agar using a sterile pipette tip. A lawn was made using a sterile swab. One sterile paper disk was placed in the center of the agar plate and inoculated with 50 µl of a 24 hr culture of *Lactobacillus salivarius*. The plates were incubated at 37°C for 24 hrs. The results were compared to a control of a sterile paper disk only upon the *E. coli* lawn. Three trials were performed. Inhibition zones ranged from 23 to 38 mm. All pathogenic strains showed inhibition by the *Lactobacillus* strain. Results of the experiment demonstrated that *Lactobacillus salivarius* L28 does contain an inhibitory effect against non-O157 STEC and should be explored further as a bioprotective culture in fermented sausages.

#### 4.2 Introduction

The trend in protein snack products has been gaining influence over the last decade with fermented meat snacks and charcuterie style sausages growing by 51% between 2011 and 2016 (Mintel Group, 2017). Consumers see these traditionally styled



products as more “authentic” with the perception of being more “natural” and containing higher quality. But with increased production, the probability of outbreak risk is heightened if not handled cautiously.

Ready to eat (RTE) dry fermented sausages are growing in popularity with the rise of the millennial generation (Mintel Group, 2017). Not only are they convenient due to their shelf stability without refrigeration, but as no thermal treatment is required before consumption, many see this as a “traditional” food that evokes a superior measure of quality. This poses a problem as outbreaks of serious foodborne illness and death have been associated with these and similar dried meat products. At the same time, consumers are demanding safe meat products that are more naturally preserved yet remain organoleptically superior, therefore research is currently focusing on innovative alternative technologies that convey good flavor, odor and texture but are effective against pathogenic and spoilage bacteria (Aymerich et al., 2008). Biopreservation by LAB is one alternative to chemical or thermal preservation that can impart an additional safety hurdle to extend the shelf life without detrimental effects on quality.

Lactic Acid Bacteria can have an antagonistic effect on pathogenic bacteria as a result of direct competition for nutrients or by the production antimicrobial products as a result of fermentation. These substances include short chain fatty acids, carbon dioxide, hydrogen peroxide, diacetyl, ethanol, and bacteriocins. It is widely known that LAB have inhibitory activity against common Gram positive food spoilage bacteria and pathogens, especially *Listeria monocytogenes* (De Vuyst and Leroy, 2007; de Souza Barbosa et al., 2015). Research has indicated that some strains of LAB also exert inhibitory activity

against Gram negative bacteria, including EHEC (Gálvez et al., 2007). *Lactobacillus salivarius* L28 has shown to reduce EHEC in vitro by 4.5 log<sub>10</sub> CFU/g (Ayala et al., 2017).

The concept of a “bioprotective” culture has gained increased interest over the last decade as consumers are more concerned with the potentially toxic or carcinogenic ingredients they consume. Traditional “chemical” based preservatives such as sodium benzoate or potassium sorbate are slowly being removed from products as the recent trend focuses on a more natural approach as an alternative to synthetic compounds. Consumers want “clean labels” without scientific sounding ingredient names (Negi, 2012).

This demand from consumers has expanded research into bioprotective cultures. In order to qualify as a protective culture, the bacteria must be determined to have an inhibitory effect against the pathogen of interest. The paper disk diffusion method has often been utilized as a starting point for determining the susceptibility of bacteria against antibiotics or other inhibitors.

It must also be shown in the study that the added bioprotective culture does not interfere with the standard starter culture containing *Pediococcus pentosaceus* and *Staphylococcus xylosus*. As *Lactobacillus salivarius* L28 does not produce nitrate reductase or catalase, it is not suitable for used as an individual starter culture.

The objective of this study was to quantify the *in vitro* bacteriocinogenic and antimicrobial ability of *Lactobacillus salivarius* L28 on non-O157 *E. coli* using the paper disc diffusion method to determine if it has bioprotective action against the pathogen.

## 4.3 Materials and Methods

### 4.3.1 Bacterial Strains and Culture Media

Six strains of non-O157 *E. coli* were received from the American Type Culture Collection (Manassas, Virginia, USA) known as the Big-Six *E. coli* panel (O26:H11 ATCC® BAA-2196™ strain designation 2003-3014, O45:H2 ATCC® BAA-2193™ strain designation 2000-3039, O111 ATCC® BAA-2440™ strain designation O111, O121:H19 ATCC® BAA-2219™ strain designation 2002-3211, O145 ATCC® BAA-2192™ strain designation 99-3311, O103:H11 ATCC® BAA-2215™ strain designation 2006-3008). The cultures were stored on Brain Heart Infusion agar slants (BHI, Difco™ Laboratories, Sparks, MD, USA) at 4°C and incubated in Brain Heart Infusion broth (BHI, Bacto™ Laboratories, Mt. Pritchard, NSW, Australia) at 37°C for 24hr. A commercial starter culture containing the LAB organisms *Pediococcus pentosaceus* and *Staphylococcus xylosus* was prepared following the manufacturer's instruction (Bactoform™ TSPX, Chr Hansen, Graasten, Denmark). Frozen *Lactobacillus salivarius* L28 was received from the Animal Science department of Texas Tech University and kept at 0°C until use. The strain was cultivated in Mann Rogosa Sharpe broth and incubated under CO<sub>2</sub> (BD Difco™ GasPak™ Sparks, MD, USA) at 37°C for 24h.

### 4.3.2 Susceptibility Testing

For the disk diffusion method, 1 ml aliquots of individual 24 hr cultures of six non-O157 STEC pathogens consisting of O26:H11, O45:H2, O103:H11, O111, O121:H19, and O145 were transferred to Mueller-Hinton agar (Difco™ Laboratories, Sparks, MD, USA) using a sterile pipette tip. The same procedure was repeated using the commercial starter culture. A sterile paper disk was applied with sterile tweezers to the center of the Mueller-Hinton plate and 50 ul of *Lactobacillus salivarius* L28 was aliquoted from the cultivation tube directly onto the paper disk by sterile pipette. The control for the experiment consisted of the same 1 ml of pathogen and starter culture to the agar plate with paper disk but no inoculation of LAB. The experiment had three trials (n=3) each trial repeated in duplicate. The plates were incubated at 37°C for 24 hrs. Zones of inhibition were recorded the following day with plates of zones > 10 mm considered to be susceptible.

#### 4.3.2 Statistical Analysis

The results were analyzed using the GLM procedure. Dunnett's range test was used to determine differences in means ( $p < 0.05$ ) between the control and *E. coli* strains. All statistics were performed using SAS 9.4 (SAS Institute, Cary NC). Graphs were produced using SigmaPlot 12.3 (SysTat, Chicago, IL, USA).

#### 4.4 Results and Discussion

The controls presented no zones of inhibition while each strain of non-O157 STEC contained large zones of inhibition >20 mm which were statistically significant against the control ( $p < 0.001$ ). Alternatively, *Lactobacillus salivarius* L28 did not show any antagonistic activity against the commercial starter culture as no zones of inhibition were formed on the agar plate with either culture (L28 vs. TSPX, TSPX vs. L28) as the antagonist.

#### 4.5 Conclusion

From the results of this study, it is concluded that *Lactobacillus salivarius* L28 would be a good candidate as a bioprotective culture against STEC in fermented sausage as it has definitive inhibitory activity against the pathogen of interest but concomitantly does not inhibit or compete with the commercial starter culture which will be used as a co-culture during dry fermented sausage production.

**Table 4. 1** Inhibition assay *Lactobacillus salivarius* L28 against pathogenic strains of *Escherichia coli* measured by zone of inhibition diameter (mm)

<b>Strain</b>	<b>Control</b>	<b>L28</b>
O145	-	31.4 ± 1.1 <sup>b</sup>
O45:H2	-	28.6 ± 2.3 <sup>b</sup>
O26:H11	-	36.3 ± 2.6 <sup>b</sup>
O103:H11	-	34.1 ± 2.2 <sup>b</sup>
O121:H19	-	28.8 ± 1.1 <sup>b</sup>
O111	-	26.6 ± 1.2 <sup>b</sup>

-: no zone of inhibition noted

a: nonsignificant at the 0.05 probability level

b: significant at the 0.05 probability level ( $p < 0.001$ )

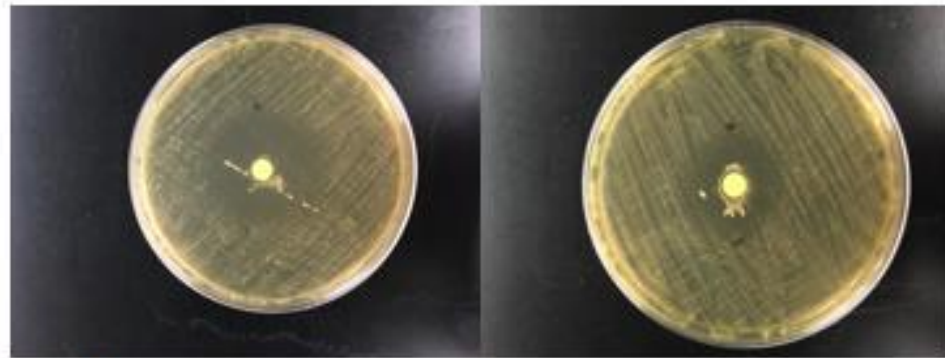
results are the mean of three measurements

**Table 4. 2** Inhibition assay *Lactobacillus salivarius* L28 vs. Bactoform™ commercial starter culture (*Pediococcus pentosaceus* and *Staphylococcus xylosus*) measured by zone of inhibition diameter (mm)

<b>Strain</b>	<b>Control</b>	<b>L28</b>
Bactoform™ TSPX	-	-
<b>Strain</b>	<b>Control</b>	<b>TSPX</b>
L28	-	-

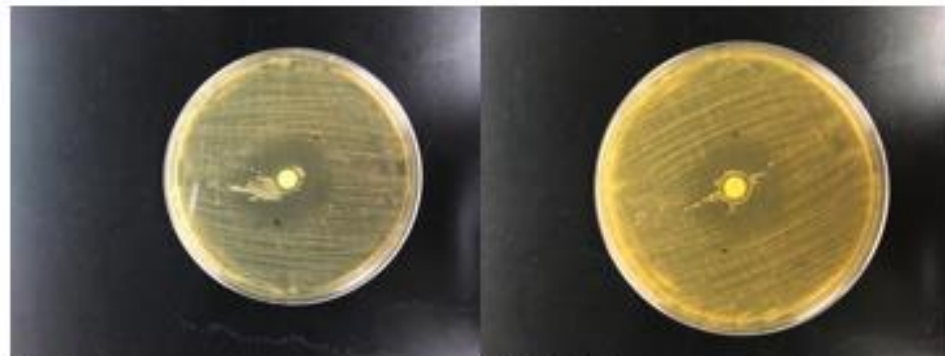
-: no zone of inhibition noted, results are the mean of three measurements

**Figure 4. 1** Zones of inhibition of pathogenic *Escherichia coli* by *Lactobacillus salivarius* L28



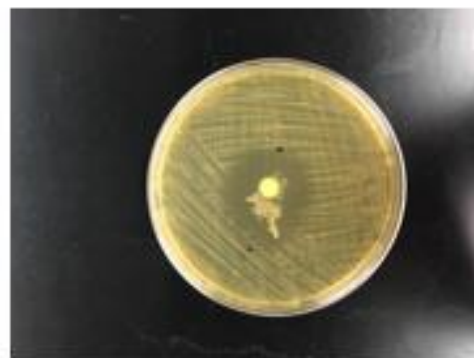
O26:H11

O45:H2



O145

O103:H11



O111



## CHAPTER 5

### DIRECT APPLICATION OF *LACTOBACILLUS SALIVARIUS* IN THE PRODUCTION OF DRY FERMENTED SAUSAGE TO CONTROL THE SURVIVAL OF NON-O157 STEC

#### 5.1 Summary

The purpose of this experiment was to determine the *in carnis* bacteriocinogenic and antimicrobial ability of *Lactobacillus salivarius* L28 against non-O157 STEC during dry fermented sausage production. Dry fermented sausage was produced by traditional methods with four treatments being applied:

- Negative control sausage containing commercial starter culture, no inoculation with pathogen
- Positive control sausage containing commercial starter culture, *Lactobacillus salivarius* L28, no inoculation with pathogen
- Sausage containing commercial starter culture, non-O157 STEC inoculation
- Sausage containing commercial starter culture, *Lactobacillus salivarius* L28, and non-O157 STEC inoculation

All treatments were subjected to the same fermentation and drying procedure to control for variables. Microbial and physicochemical analysis was performed in duplicate on sample days of 0,1,2,7,10, and 17 with three separate trials (n=3). In conclusion, this experiment demonstrated that the addition of *Lactobacillus salivarius* L28 as a co-culture

was not statistically different than the control ( $p > 0.05$ ) and therefore did not provide any additional antimicrobial activity.

## 5.2 Introduction

Meat products are a nutrient dense food and are an intricate part of the human diet. Due to the method of harvest and production, these products are susceptible to high levels of contamination by enterohemorrhagic *Escherichia coli*. In the production of dry fermented sausage, the lack of a thermal processing application makes consumers more vulnerable to foodborne illness. The use of bacteriocins and their producer strains as a non-thermal antimicrobial treatment may contribute to food safety when combined with other hurdle technology.

*Lactobacillus salivarius* L28 (L28) has been shown in preliminary experiments to have antimicrobial activity against pathogenic *E. coli*. In chapter four, L28 was shown to be inhibitory to six individual strains of non-O157 shiga-toxin producing *E. coli* and therefore, may be a good candidate as an additional bioprotective starter culture in the production of dry fermented sausage. In a recent study, compared to controls without L28, STEC was reduced by a 4.5 log<sub>10</sub> CFU/g in a food matrix. In addition, recent genome sequencing of the bacteria has revealed genetic markers for two pre-peptides involved in bacteriocins synthesis (Ayala et al., 2017). L28 is known to produce small heat stable bacteriocins containing broad-spectrum action against both Gram negative and Gram positive bacteria without antagonistic activity towards related LAB commonly used

as starter cultures (Flynn et al., 2002; Busarcevic and Dalgarrondo, 2012; Messaoudi et al., 2012; Messaoudi et al., 2013). The mode of action of the Class IId bacteriocin is effective by altering the permeability of the cell membrane to disintegrate the proton motive force or by interfering with the precursor of peptidoglycan, lipid II during cell wall synthesis. The bacteriocin can form a complex with the lipid II and insert itself directly into the cell membrane forming a pore. This inevitably results in leakage of the cytoplasm and cellular death (Cotter et al., 2005; Woraprayote et al., 2016).

L28 is an autochthonous microorganism of ground beef and ground pork (Corr et al., 2007; Ayala et al., 2017). This intrinsic characteristic makes the strain a good candidate for use as a bioprotective culture in the dry fermented sausage matrix as it can survive well in the product.

## 5.3 Materials and Methods

### 5.3.1 Bacterial Strains and Culture Media

Six strains of non-O157 *E. coli* were received from the American Type Culture Collection (Manassas, Virginia, USA) known as the Big-Six *E. coli* panel (O26:H11 ATCC® BAA-2196™ strain designation 2003-3014, O45:H2 ATCC® BAA-2193™ strain designation 2000-3039, O111 ATCC® BAA-2440™ strain designation O111, O121:H19 ATCC® BAA-2219™ strain designation 2002-3211, O145 ATCC® BAA-2192™ strain designation 99-3311, O103:H11 ATCC® BAA-2215™ strain designation 2006-3008). The cultures were stored on Brain Heart Infusion agar slants (BHI, Difco™

Laboratories, Sparks, MD, USA) at 4°C and incubated in Brain Heart Infusion broth (BHI, Bacto™ Laboratories, Mt. Pritchard, NSW, Australia) at 37°C for 24hr. Bacteria cultures were transferred 3 times before the study to ensure pathogenicity. CHROMagar STEC base (CHROMagar™ Microbiology, Paris, France) was prepared in plastic petri dishes to enumerate bacterial counts. Frozen *Lactobacillus salivarius* L28 was received from the Animal Science department of Texas Tech University (Lubbock, TX, USA) and kept at 0°C until use. The strain was cultivated in Mann Rogosa Sharpe broth and incubated under CO<sub>2</sub> (BD Difco™ GasPak™ Sparks, MD, USA) at 37°C for 24h.

### 5.3.2 Preparation and Inoculation of Sausage

Frozen Pork shoulder and beef trim were obtained (Clem's Refrigerated Foods, Lexington, KY, USA) and ground using a 3/8 plate. Sausage batter was prepared by mixing 5 kg of ground raw meat, 3% NaCl (Morton International, Inc. Chicago, IL), 0.3% Curing Salt #2 (Anthony's Goods, St. Louis, MO), 0.3% Paprika, 0.3% Black pepper, 0.3% Garlic powder (McCormick® Spice Company, Sparks, MD) and 0.3% dextrose (Difco™ Laboratories, Inc, Detroit, MI). A commercial starter culture containing the LAB organisms *Pediococcus pentosaceus* and *Staphylococcus xylosus* was prepared following the manufacturer's instruction and added to the batter (Bactoferm™ TSPX, Chr Hansen, Graasten, Denmark). The initial fat content of the pork was approximately 20% and the moisture content was approximately 60%.

The batter was mixed by hand in a 1 gallon commercially sterile Ziploc® bag (SC Johnson Company, Racine, WI) and divided into four samples of approximately 1250 g. A cocktail of the 6 strain STEC was prepared by centrifuging the individual strains together in a sterile 50 ml polypropylene conical tube. Four treatments were applied:

- A. Negative control sausage containing commercial starter culture, no inoculation with pathogen
- B. Positive control sausage containing commercial starter culture, *Lactobacillus salivarius* L28, no inoculation with pathogen
- C. Sausage containing commercial starter culture, *Lactobacillus salivarius* L28, and non-O157 STEC inoculation
- D. Sausage containing commercial starter culture and non-O157 STEC inoculation

Treatments C and D were inoculated with the multi-strain mixture of non-O157 STEC to achieve a cell concentration of approximately  $7 \log_{10}$  CFU/g of batter. The batter was stuffed into natural hog casings (Quality Casings, Hebron, KY) using a manual stuffer and formed into 30 cm links by twisting then securing with zip-ties. Each sausage link was approximately 150 g. The links were then positioned in digital dry curing cabinet (Sausage Maker, Buffalo, NY, USA). The sausages were sampled for microbial counts, fat, moisture, pH, and  $A_w$ , at day 0,1,2,7,10, and 17. Three trials were conducted (n=3) with two sausages of each treatment being sampled in duplicate.

### 5.3.3. Physiochemical Analysis

The temperature setting for the initial fermentation step was at 20 °C (78°F) with relative humidity of 90% until the internal pH of the sausage reached  $\leq 5.3$ . pH measurements were recorded with a portable meat pH meter (Hanna Instruments, Ann Arbor MI, USA) from three separate readings with the reported being the mean of the readings. Water activity was measured by an Aqualab Pawkit water activity meter (Meter Group, Pullman, WA, USA) with means of three readings reported. The sausages were then dried at 16°C with a relative humidity of 80% for 17 days. Relative humidity and temperature were monitored using the Thermadata™ temperature and humidity logger (ThermoWorks Inc., USA).

### 5.3.4 Microbial Analysis

Microbial analysis was performed on two randomly selected sausages of each treatment. A sample was extracted from the center portion of each sausage which represented approximately 10% of the total weight. The sample was transferred to a stomacher bag and diluted with sterile peptone water (Difco™ Laboratories, Becton, Dickinson and Company, USA) for a 1:10 dilution. Samples were agitated for 1 minute at 230 rpm in a Stomacher Lab-blender 400 (Worthing, West Sussex, UK). Aliquots were extracted from the stomacher bag and serial diluted to desired concentration using phosphate buffer diluent. The dilutions were plated on CHROMAgar and Mann Rogosa Sharp Agar (MRS) using the EddyJet™ Spiral Plater (IUL, Farmingdale, NY, USA).

Agar plates were incubated aerobically at 37°C for 24h for the STEC and under CO<sub>2</sub> at 37°C for 24h for L28. STEC colonies were confirmed by the mauve color. The plate counts were enumerated into Log<sub>10</sub> CFU/ml using a Flash and Go™ computerized plate reader (IUL, Farmingdale, NY, USA).

### 5.3.5 Statistical Analysis

The results were analyzed using a student's t-test, with the significance level set *a priori* at 95%. The reduction of STEC examined during the processing of dry fermented sausage was represented as a log reduction using the following formula:

$$\text{Log reduction} = \text{Log} (N_t/N_0)$$

Where N<sub>t</sub> represents the plate count for specific sample day and N<sub>0</sub> represents the initial plate count. All statistics were performed using SAS 9.4 (SAS Institute, Cary NC). Graphs were produced in SigmaPlot 12.3 (Systat, Chicago, IL, USA).

## 5.4 Results and Discussion

### 5.4.1 Effectiveness of Lactobacillus salivarius L28 in reduction of STEC counts

Preliminary analysis of the raw meat batter before pathogen inoculation with pathogen and commercial starter culture show that the overall quality of the pork shoulder and beef trim was acceptable (< 100 CFU/g total aerobic plate count). Samples were also plated on 3M™ Petrifilm™ *E. coli*/coliform (Sigma-Aldrich, St. Louis, MO,

USA) to ensure the samples were free of pathogenic *E. coli* contamination. The noninoculated sausages (treatments A and B) remained negative for STEC throughout the assay.

The pH of the chorizo decreased from  $5.74 \pm 0.028$  at the beginning of the assay then dropped to  $5.18 \pm 0.012$  at the end of fermentation. The pH rose during storage as an effect of the proteolytic *Staphylococcus xylosus* in the commercial starter culture and a final pH was noted as  $5.63 \pm 0.011$  on day 17. The  $A_w$  initially was recorded as  $0.96 \pm 0.01$  then decreased throughout drying to a final  $A_w$  of  $0.79 \pm 0.01$ .

Results of the statistical analysis show that there was not a significant difference in the means of the log reduction counts ( $p = 0.379$ ) indicating that the additional culture had no significant effect on the  $\log_{10}$  reduction of STEC. As noted in **Table 5.1**, a 5  $\log_{10}$  reduction was not achieved for neither control nor treatment even after the process achieved a pH of  $\leq 5.3$  and water activity of  $\leq 0.91$ . The treatment with the added L28 saw a log reduction of 2.13 CFU/g while the commercial starter culture had a mean reduction of 1.55 CFU/g. The moisture content was reduced to  $< 35\%$  signifying the process was complete with a MPR of  $< 1.9$ .

A greater reduction of STEC cells may have been achieved with a fermentation pH  $< 4.3$ , yet this would have rendered a sausage that had poor organoleptic properties, poor binding and would have been too sour for most consumers. The sub-lethal pH at fermentation temperature may enhance survival of the STEC (Hinkens et al., 1996) by the production of an acid tolerance response and adjustment of the bacterial metabolism. Other factors that may be influencing the viability of the pathogen in the sausage may be



explained by the failure of the *Lactobacillus salivarius* L28 to compete well with the starter culture. Although an experiment was performed to determine if one culture would be inhibitory to the other in chapter four (see **Table 4.2**) with negative results in vitro, the competitive exclusion may be enhanced in the meat matrix. One study of O104:H4 in dry fermented sausage saw the pathogenic bacteria produce a bacteriocin that may have been antagonistic to the starter culture used (Böhnlein et al., 2016)

Overall the sausage retained good organoleptic properties of color and odor. No visible mold or spoilage was noted. The product was representative of a commercial product at the endpoint.

**Table 5. 1** Log<sub>10</sub> CFU/g of STEC, pH, a<sub>w</sub>, and moisture measurements during chorizo production for treatment containing Shiga toxin producing Escherichia coli (STEC) and *Lactobacillus salivarius* L28

Procedure	Log <sub>10</sub> CFU/g STEC	pH	A <sub>w</sub>	Moisture
Day 0	6.36 <sup>a</sup>	5.72	0.96	61.26 %
Day 1	6.13 <sup>a</sup>	5.28	0.92	61.89 %
Day 2	5.49 <sup>a</sup>	5.18	0.90	64.95 %
Day 7	4.68 <sup>a</sup>	5.58	0.86	55.74 %
Day 10	4.58 <sup>a</sup>	5.61	0.81	42.93 %
Day 17	4.23 <sup>a</sup>	5.63	0.79	34.28 %

a: nonsignificant at the 0.05 probability level (p = 0.379)

**Table 5. 2** Log<sub>10</sub> CFU/g of STEC, pH, a<sub>w</sub>, and moisture measurements during chorizo production for treatment containing Shiga toxin producing *Escherichia coli* (STEC) as a control

Procedure	Log <sub>10</sub> CFU/g STEC	pH	A <sub>w</sub>	Moisture
Day 0	5.74 <sup>a</sup>	5.76	0.95	61.74 %
Day 1	5.34 <sup>a</sup>	5.21	0.93	60.72 %
Day 2	4.98 <sup>a</sup>	5.18	0.92	62.21 %
Day 7	4.43 <sup>a</sup>	5.62	0.87	56.67 %
Day 10	4.37 <sup>a</sup>	5.78	0.81	43.33 %
Day 17	4.19 <sup>a</sup>	5.63	0.78	33.82 %

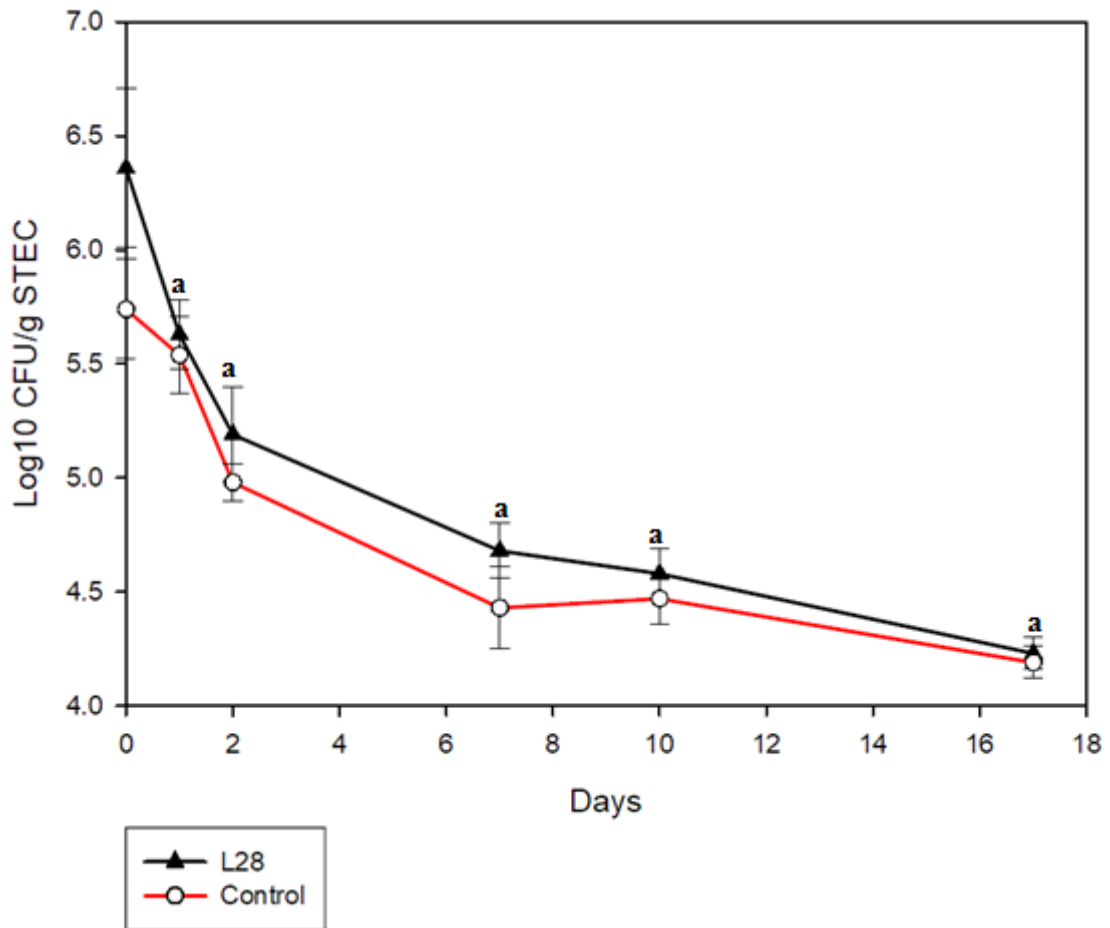
a: nonsignificant at the 0.05 probability level (p = 0.379)

**Table 5. 3** Comparison of Log<sub>10</sub> reduction CFU/g of non-O157 Shiga toxin producing *Escherichia Coli* in chorizo production after 17 days

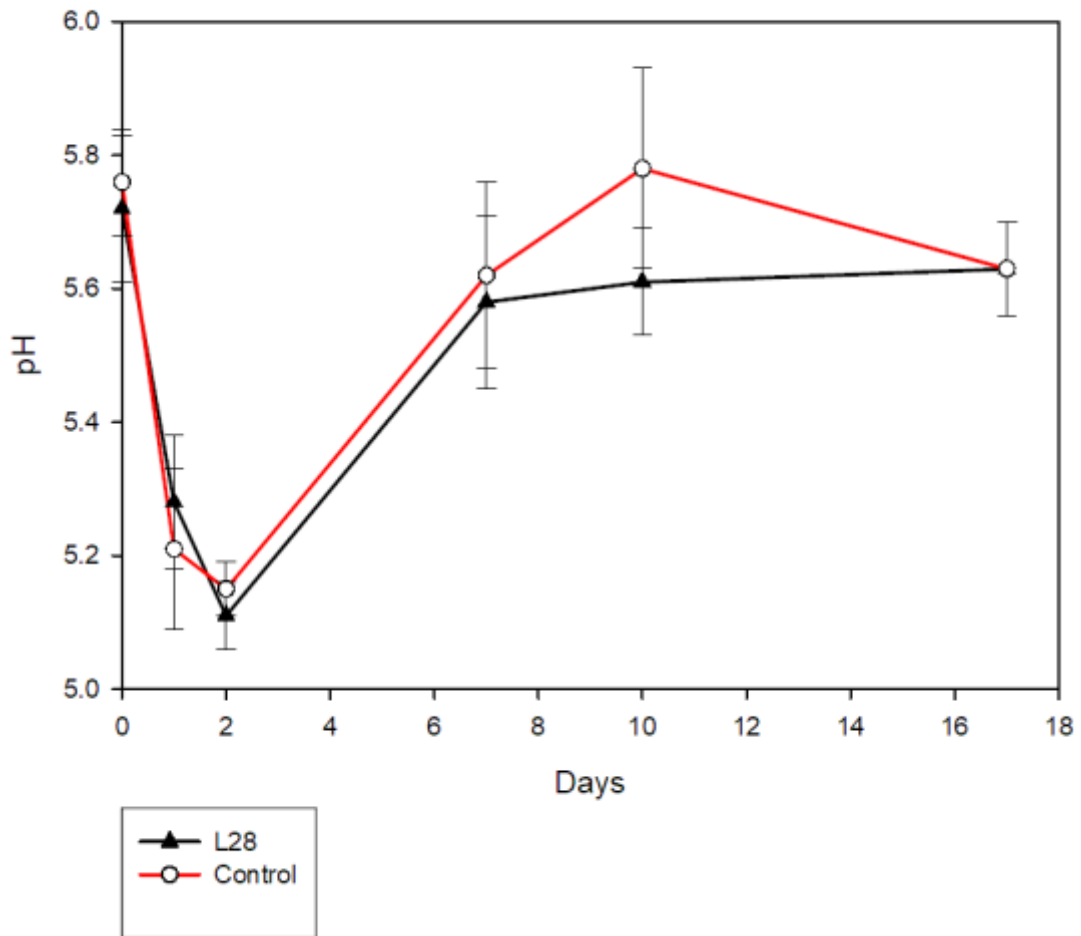
Treatment	Log <sub>10</sub> CFU/g STEC Reduction at endpoint	pH	A <sub>w</sub>
L28 and STEC	2.13 ± 0.881 <sup>a</sup>	5.63	0.79
STEC Only	1.55 ± 0.615 <sup>a</sup>	5.63	0.78

a: nonsignificant at the 0.05 probability level (p = 0.379)

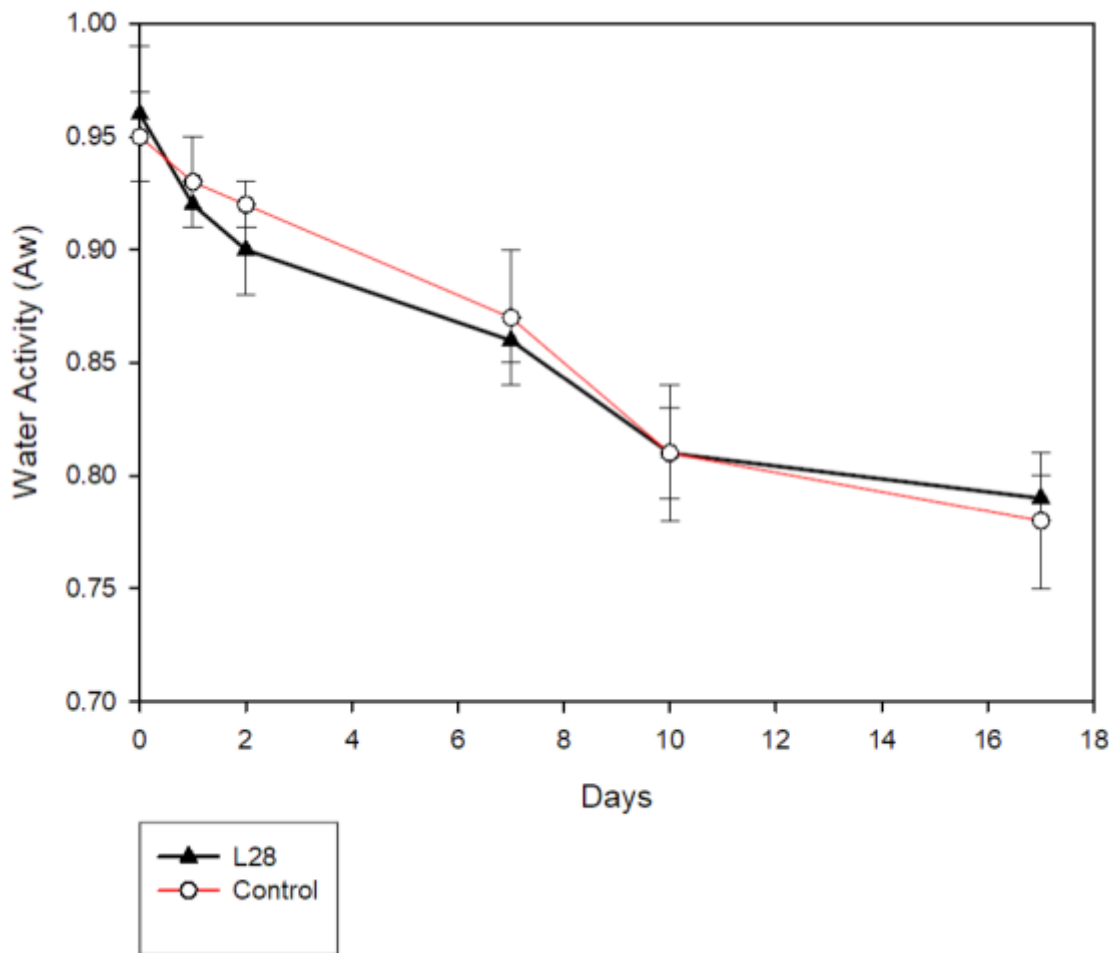
**Figure 5. 1** Comparison of Log<sub>10</sub> CFU/g reduction of non-O157 Shiga toxin producing *Escherichia coli* (STEC) in dry fermented sausage containing *Lactobacillus salivarius* L28 and control during chorizo sausage production



**Figure 5. 2** Comparison of pH between treatments of dry fermented sausage containing *Lactobacillus salivarius* L28 and control during chorizo sausage production



**Figure 5. 3** Comparison of water activity between treatments of dry fermented sausage containing *Lactobacillus salivarius* L28 and control during chorizo sausage production



## CHAPTER 6

### OVERALL CONCLUSIONS

In conclusion, it was observed that the traditional small production method of dry-fermented sausage manufacture will not deem the product safe if it is contaminated intentionally or by chance with high levels of pathogenic *E. coli*. **Table 3.3** shows that a 5 log<sub>10</sub> CFU/g reduction was not achieved ( $4.49 \pm 0.474$  log<sub>10</sub> CFU/g) during the first validation study in chapter three, although a pH of  $\leq 5.2$  was reached after fermentation and a water activity of  $\leq 0.85$  was established and maintained during several weeks of drying. This calls into question the dependence on the combination of water activity and pH for safety measures when no thermal cook step is employed in the production method. Organoleptic deterioration was evident at the endpoint of the experiment and the sausage was unpalatable after day 28. This was similar to a study by Balamuragan (2017) where the researchers did achieve a 5 log<sub>10</sub> reduction of both *E. coli* O157 and non-O157 after 39 days of drying, but the eating quality of the sausage was lost after day 11 when only a 2.5 log<sub>10</sub> CFU/g reduction of STEC was attained.

*Lactobacillus salivarius* L28 (L28) did seem to be a promising candidate for use as a bioprotective culture in the sausage matrix. Previous applications in animal studies show that the bacterial strain produces a bacteriocin that has the potential to impede the growth of Gram negative bacteria (Zhang et al., 2011; Chaves et al., 2017). This broad spectrum action is unusual as most LAB bacteriocins are antagonistic against Gram

positive bacteria only due to their inability to permeate the outer membrane (Cotter et al., 2005; Gillor et al., 2008). From **Table 4.1**, it was established that L28 does contain inhibitory metabolites that interfered with the growth of each of the six strains of STEC, evident from the large zones of inhibition created during the paper disk diffusion assay in chapter four. Unfortunately, this inhibition was not observed during the sausage trial in chapter five. No statistical difference ( $p = 0.379$ ) was noted between the sausage control and the L28 treatment. Neither control nor treatment rendered a  $5 \log_{10}$  CFU/g reduction throughout all three trials of the experiment in chapter five. The L28 treatment attained a  $2.13 \log_{10}$  reduction while the control group had a reduction of  $1.55 \log_{10}$ . No spoilage or contamination could be detected in the product by sensory means and the product was representative of a commercial product at the endpoint. These findings are comparable to other studies of STEC survival in dry fermented sausage (Faith et al., 1998; Erkkila et al., 2000; Hwang et al., 2009). These studies yielded an approximate 2-3  $\log_{10}$  reduction of STEC while continuing to maintain the eating quality.

This research is indicative of the dangers of artisanally produced dry fermented sausages. While there is always a risk from pathogens in dry fermented sausages, large commercial producers are heavily regulated, have strict sanitation procedures, and follow good manufacturing practices (GMPs). Small producers such as local restaurants and delicatessens do not always understand the hazards or follow strict hygienic measures that would be found in industry. This research shows that the process itself does not suffice to control a contaminated product. Further research will be required to identify other methods of bioprotection that can be safely employed for achieving a  $5 \log_{10}$



reduction as recommended by the USDA; thus producing safe artisanal products for consumption.

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