PROCESS VALIDATION FOR THE REDUCTION OF SURROGATE *ESCHERICHIA COLI* DURING THE PRODUCTION OF BILTONG

by

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(Under the Direction of Alex Stelzleni)

ABSTRACT

Biltong, a South African staple that is gaining in popularity in the United States typically comes in medium or dry forms related to total product water activity. Ready-to-eat beef products should achieve a five-log reduction for *Escherichia coli* ***(E. coli)*** O157:H7 and other non-O157 Shiga-toxin strains (**STEC**). Under current guidelines, the recommended method to achieve a five-log reduction is via thermal processing. In contrast, biltong producers prefer non-thermal processing to maintain traditional practices. The objective of this research was to validate a process for *E. coli* reduction during biltong manufacturing, while adhering to traditional, non-thermal, processing techniques. Experiments 1 and 3 showed that it was possible to achieve a 5-log reduction for surrogate *E. coli* in whole muscle dried products without thermal processing. This work should be followed by studies utilizing pathogenic *E. coli* O157:H7 and non-O157 STEC for further validation.

INDEX WORDS: biltong, *E. coli*, reduction, water activity, *shiga-toxin producing E. coli*

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DEDICATION

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CHAPTER 1

INTRODUCTION

For centuries, producers utilized curing as the most common meat product preservation method to protect against bacterial and microbial growth (Leistner, 1987). In response to increasing public health concerns associated with processed meat consumption, the meat industry has begun introducing minimal processing to improve this food category's nutritional and health properties (Toldra and Reig, 2011). In developing countries, consumers eat dried meat products as valuable protein sources, while in industrial countries, these products are consumed as healthy, high-protein snack foods. McLynn et al. (2015) reported demand for meat snacks among American young adults increased by 18% between 2010 and 2015. Biltong, an air dried ready-to-eat (**RTE**) meat product, is becoming a popular U.S. snack food. Biltong typically comes in two forms related to the total water activity ($\mathbf{A_w}$) of the product, medium and dry. Medium and dry biltong are considered shelf stable ready-to-eat products (Leistner, 1987). In the United States, producers manufacture biltong with whole-muscle beef slices seasoned with salt and spices which are allowed to dry to the desired A_w (Jones et al., 2017). With this type of further processed meat product gaining popularity, there is a risk of producers improperly processing products due to not understanding repercussions associated with pathogens such as *Escherichia coli (**E. coli**)*.

Morbidity and mortality associated with several large outbreaks of gastrointestinal disease caused by Shiga toxin-producing *Escherichia coli* (**STEC**) within beef products highlighted threats these organisms can pose to public health (Paton et al., 1998). *Escherichia coli* serotypes found in foods cause approximately 169,600 US foodborne illness cases each year

(CDC, 2021). It is recommended that ready-to-eat beef products, such as biltong, achieve a five-log reduction for *E. coli O157:H7* and other non-O157 Shiga-toxin strains for a process to be considered as validated (USDA, 2014). In 1995, the Blue Ribbon Task Force was created to find ways to decrease the chance of *E. coli* 0157:H7 potential outbreaks, of which they outlined five options. Of the five options, option four states that processors may utilize thermal processing, among other hurdles, to achieve standards for *E. coli* O157:H7 lethality (5D process; decimal reduction dose), while option two recommends processors to test raw product as well as apply a hurdle to achieve at least a 2-log reduction (Nickelson II et al., 1996). Out of the available recommendations, biltong producers prefer to not use thermal processing to stay true to traditional practices which makes it difficult to ensure a safe product. Currently, there are no validated non-thermal processing applications to achieve a 5-log reduction of *E. coli* O157:H7 or STEC on biltong as public knowledge or in the literature.

In the absence of heat, the greatest opponent to microbial growth is low A_w. Typically, bacteria are unable to reproduce below an 0.85 A_w. More specifically, *E. coli* is unable to reproduce below 0.95, *Salmonella* is unable to reproduce below 0.94, and *Staphylococcus aureus* cannot reproduce below 0.86 (Leistner, 1987). However, the bacteria discussed can still survive in a vegetative state on the product for extended periods of time at a low A_w. To reduce the bacterial population as much as possible, other hurdles should be employed. Common hurdles to biltong production include an antimicrobial vinegar-water marinade (acetic acid) as well as a lactic acid dip (Fontana, 2007). Creating a lower A_w, and lower pH product creates an inhospitable environment for the pathogens of concern, which can lead to longer shelf life and a safer product for consumers (Fontana, 2007). The purpose of this research was to validate a process for a 5-log reduction of *E. coli* during the manufacture of whole muscle biltong while

adhering to traditional, non-thermal, processing techniques. The objectives were to validate a standard processing procedure for biltong production, as well as to evaluate antimicrobial effects of the hurdles used in the process.

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CHAPTER 2

THE REVIEW OF LITERATURE

Overview

Consumption of red meat and meat products plays an important role in the human diet. Fresh meats are highly perishable commodities that have elevated water activity ($\mathbf{A_w}$; 0.99) and contain sufficient protein which provides an optimal environment for growth of an abundance of pathogenic bacteria and other organisms. For centuries, salting and drying has been the most common method to preserve meat products protecting against bacterial and microbial growth. In response to increasing public health concerns associated with processed meat consumption, the meat industry has begun introducing innovations, including minimal processing, to improve the nutritional and health properties of this food category (Toldra and Reig, 2011).

With these advancements, biltong, an air dried ready-to-eat meat product, is becoming a popular United States snack food. In developing countries dried meat products provide a valuable protein source. In industrial countries dried meat products are consumed as a healthy, high-protein snack food. McLynn et al. (2015) reported demand for meat snacks among young American adults increased by 18% between 2010 and 2015. With this type of further processed meat product gaining popularity, there /s a risk of producers not understanding the repercussions of pathogens such as *Escherichia coli* (***E. coli***) and improperly processing products or not processing sufficiently to remove adulterants. Morbidity and mortality associated with several large outbreaks of gastrointestinal disease caused by Shiga toxin-producing *Escherichia coli* **(STEC)** highlighted threats these organisms pose to public health (Paton et al., 1998). Except for reports of prevalence of pathogens such as *E. coli* O157:H7 (Abong'o and Momba, 2008), *Listeria monocytogenes, Shigella dysenteriae* (Naidoo and Lindsay, 2010) and *Staphylococcus*

aureus (Mhlambi, 2008) in South African biltong, there is limited data in the literature detailing validated pathogen reduction strategies during the non-thermal manufacturing process of biltong. The purpose of this report is to provide a review of the literature in the following areas:

Characteristics of biltong and its ingredients

Characteristics of other similar dried meat products

Identification of *E. coli* as well as *E. coli* surrogates and their significance

Identification of microbiological hazards and control factors

Biltong Characteristics

Dry-curing products is the oldest known method to preserve meat against spoilage and pathogens (Leistner, 1987). Preserving meat through dry curing is achieved through extraction of water, which causes a reduction of water activity. Biltong, a South African ready to eat (**RTE**) meat product, can be made from raw meat through salting, curing, and drying. Biltong variations are produced with different types of meat, seasonings, and preservatives. Biltong is an intermediate-moisture food for shelf-life stability classification as most falls within a range of 20 to 50% moisture content and a A_w of 0.60 to 0.90 (Leistner, 1987). Unlike some other dried meats, it is not re-hydrated or cooked prior to consumption; therefore, manufacturing and storage conditions could be a source of potential food-borne pathogens.

Manufacturers usually make biltong from beef or game meat (Leistner, 1987), utilizing various muscles. Lean meat is recommended because increased fat levels can affect salt uptake and cause off-flavor development due to oxidation. The whole muscle is cut into strips following or parallel to the muscle fiber orientation with slices varying in length and thickness (up to 400-mm long and 50-mm thick, respectively). Salt, black pepper, coriander, brown sugar, and vinegar

are often used as ingredients with a variety of other seasonings included according to personal and consumer preference. Traditional biltong production involves marination followed by coating product in seasonings and low temperature air drying, but other approaches have been suggested and tested. Petit et al. (2014) distinguished two groups of biltong: those with a moisture content between 21.5 and 25.3% and A_w between 0.65 and 0.68, which they classified as dry, and those with a moisture content between 35.1 - 42.8% and A_w between 0.85 and 0.89, which they classified as 'moist'. However, due to the wide variety of ways to produce biltong, there are no set standards or classifications for A_w, moisture content, salt, or seasonings.

Biltong Processing

Use of Vinegar in Marination

Biltong raw meat is marinated in salt and organic acid mixes and dried to achieve a reduced A_w. The A_w of a food is the ratio between food vapor pressure, when in a completely undisturbed balance with surrounding air media, and vapor pressure of distilled water under identical conditions (FDA, 2018). Final salt concentration of the product increases due to reduction in moisture. The presence of organic acids, salt, and a reduced A_w are important factors for the potential destruction of pathogens and preventing microbial growth. Naidoo and Lindsay (2010) demonstrated salt levels greater than 20% were required to prevent growth of *Staphylococcus aureus* and *Listeria monocytogenes*. Additionally, *Staphylococcus aureus*, which has an optimum pH range of 7-7.5, was not prevented in presence of traditional organic acids like apple cider vinegar and brown spirit vinegar. Salt, spices, and presence of organic acids as individual ingredients were not sufficient to prevent growth of *Staphylococcus aureus* and *Listeria monocytogenes* (Naidoo and Lindsay, 2010).

Cutter and Siragusa (1994) examined efficacy of organic acid concentration to control *E. coli* O157:H7. After spraying either water, 1, 3, or 5% acetic, lactic, or citric acids respectively at 24°C, tissues were incubated for 24 h at 4°C and bacterial populations enumerated. Data indicated there were no acid type differences; however, acid concentration, tissue type, and bacterial strain significantly influenced the reduction of bacterial populations on lean and adipose tissue. Of the concentrations tested on lean tissue, 5% spray treatments were most effective for reducing *E. coli* O157:H7 populations. There were differences observed in resistance of *E. coli* O157:H7 strains to acid washing. The magnitude of bacterial population reductions was consistently greater on adipose versus lean tissue for all bacterial strains, which could be attributed to the ability of weak acids to inhibit microbial growth being related to their lipid permeability (Cutter and Siragusa, 1994). Generally, treatments with acetic, lactic, or citric acids at varying concentrations resulted in population reductions ranging from 1- to 4-log CFU/cm^2 on meat surfaces. While this study indicated organic acids alone may not be sufficient to be considered a single microbiological safety hurdle, they were beneficial as part of a hazard analysis critical control point (**HACCP**) approach to enhance microbiological safety of further processed meat products.

Seasonings

i. Salt, black pepper, coriander, brown sugar, and vinegar are often used as ingredients in biltong production with a variety of other seasonings included according to personal preference. Nitrate and/or nitrite can also be used as a source of color enhancement and to prevent *Clostridium botulinum*. Vinegar and roasted coriander are featured in many biltong recipes as key ingredients, while a vast range of other seasonings have been included depending on preference, including those which help differentiate product flavor, such as chili, garlic, or

Worcestershire sauce, to give the consumer a variety of choice. Coriander (*Coriandrum sativum L.*) is a well known herb widely used as a spice and in pharmacy and food industries. Coriander seed oil is the world's second most common essential oil, exhibiting antimicrobial activity against Gram-positive and Gram-negative bacteria. The direct addition of coriander oil (0.02%) to ground beef caused only a slight reduction in *Enterobacteriaceae* counts (Michalczyk et al., 2012). This indicates that applying essential oils at the highest sensorially acceptable dose (0.02%) was of limited efficacy. Overall, the research on coriander oil application as a food preservative is still scarce and shows very different outcomes in terms of effectiveness. Additives and preservatives also include boric acid, pimaricin, and potassium sorbate (Naidoo and Lindsay, 2010).

ii. Salt level

Salt added to biltong at 2.5 to 4% (Van der Riet, 1976), serves as a method of preservation and causes changes in muscle proteins and texture by modifying myofibrillar protein solubility and muscle fiber swelling. These changes are related increased water-binding and water-holding capacities associated with salt concentration and pH manipulation (Offer & Trinick, 1983). Swelling occurs because of the chloride ions that bind to the protein filaments, which increases the electrostatic repulsive force between them, causing the protein structure to unfold and swell (Cheng & Sun, 2008). Salt preservation also decreases A_w; therefore, less available moisture for microbial growth will increase shelf-life (Cheng & Sun, 2008).

iii. Lactic acid level

Organic acids have been used for decades as an additive and preservative, typically being used for increasing shelf life and decreasing microbial populations (Ricke, 2003). Organic acids target the cell wall, cytoplasmic membrane, and specific metabolic functions within the bacterial pathogen. Lactic acid (PKa: 3.79) is a natural antimicrobial that can inhibit the growth of gram-

negative species in the Enterobacteriaceae family. Lactic acid has been accepted by consumers as natural and healthy and is generally regarded as safe at levels up to 5% (Montville et al., 2001). Lactic acid is able to reduce populations of pathogens due to its ability in the undissociated form to penetrate cytoplasmic membranes, in turn reducing intracellular pH (Alakomi et al., 2000). Currently, lactic acid is used in the beef, poultry, and pork industry as an organic acid spray for carcass surfaces to reduce microbial loads. Hardin et al., (1995) found that a 2% lactic acid spray on beef carcass surface areas (inside round, outside round, clod, brisket) was able to reduce *E. coli* O157:H7 bacterial populations significantly more than acetic acid, the active ingredient in vinegar. In a study conducted by Anderson and others (1991), individual solutions of acetic and lactic acids were evaluated on lean beef surfaces inoculated with *Salmonella typhimurium.* Samples sprayed with 3.0% lactic acid were able to achieve a 1.25 log reduction of *Salmonella typhimurium* while samples sprayed with 3.0% acetic acid were only able to achieve a 0.9 log reduction.

Biltong Drying

Most foods have a water activity above 0.95, which provide sufficient moisture to support growth of bacteria, yeasts, and mold. Amount of available moisture can be reduced to a point which inhibits growth of organisms (FDA, 2021). Water activity, which is related to the product moisture and salt content, is an important parameter in achieving a safe and wholesome product. The water activity of raw meat is approximately 1.0 which is ideal for the growth of many microorganisms. Few pathogenic bacteria can survive or reproduce below a water activity of 0.90 and few microorganisms grow below a water activity of 0.75. Yeasts and molds do not grow below a water activity of 0.60 (FDA, 2021). Van der Viet (1976) found no microbial growth or spore germination detected on biltong samples with a water activity of 0.7 or less.

Allowing for an arbitrary safety margin, a water activity of 0.68 was regarded as the critical moisture content at which biltong could be kept for long periods when pH is not considered. Consequently, achieving a low water activity in a short time is a main goal to create a safe product when dry curing (Van der Riet, 1976).

Effective drying relies on drying time and three inter-related factors: air temperature, relative humidity, and air exchange (Fulton et al., 1987). The traditional drying of biltong is accomplished by hanging the meat strips outside for one to two weeks, depending on ambient conditions (Van der Riet, 1976). Traditionally, biltong is dried outside in the shade during the winter months or in a drying box not achieving temperatures over 22°C (Leistner 1987). Dryers frequently used in industry are only temperature controlled, but some can control relative humidity. Large biltong producers like to use drying chambers in which temperature and relative humidity are controlled. Biltong is usually dried to a 50% weight loss or more to accommodate other consumer preferences (Strydom & Zondagh, 2014). A wide range of commercial units are available for drying foods, but a batch dryer using forced convection is most appropriate for drying biltong or jerky (Strydom & Zondagh, 2014). Most producers are likely to use tray dryers in which meat strips are placed on mesh trays or hung on supports placed in the dryer with the latter being more common. Drying rooms or "tunnels", with product on racks, are suitable for large-scale operations. Small butchers/processors are likely to use ovens which provide little humidity control other than door opening and speed of the fan which may be unreliable, while others rely on separate rooms with ambient temperature and humidity based on environment. Specialized dryers, with dehumidifiers, are available and provide excellent humidity control, but are unlikely to be used by small producers due to cost (Strydom & Zondagh, 2014).

Storage of Biltong

Storage of product by the producer and consumer is important as biltong may reabsorb moisture if stored incorrectly in warm, moist conditions. Van der Riet (1976) suggested biltong with a moisture content less than 24%, or A_w less than 0.68, was microbiologically stable with rancidity limiting shelf life, however, product could reabsorb moisture if stored incorrectly. Large scale manufactured products are packed with nitrogen flushing or vacuum-packed to maintain the shelf life (Attwell, 2003). Although no definitive shelf life for biltong has been determined, “several months”, “very long”, and “indefinite” were noted (Van der Riet,1976; Attwell, 2003). Ingham et al. (2006) found that although factors other than A_w may have some effect on pathogen survival, drying beef products to an A_w of 0.87 or below will ensure that bacterial pathogens cannot grow on vacuum-packaged product stored at room temperature.

Microbiological Issues Related to Biltong

Several studies assessed levels of naturally occurring organisms in a range of different types of biltong (chicken, venison, and beef) from a range of outlets including street vendors, small butchers producing biltong on-site, supermarkets, convenience stores, and medium scale commercial producers (Wolter et al., 2000; Mhlambi, Naidoo and Lindsay, 2010; Naidoo and Lindsay, 2010). The surveys indicated elevated levels of microorganisms are commonly observed in biltong with levels ranging from 6 to 7 log CFU/g. *Enterobacteriaceae* were observed at 3 to 4 log CFU /g, yeasts were present at levels ranging from 2 to 7 log CFU /g and mold up to 5 log CFU /g were found. Lactic acid bacteria were found to be present at levels as high as 8 log CFU/g, and *Staphylococci* counts ranged from 4 to 8.5 log CFU /g. Not only were high levels of potential spoilage organisms observed, some surveys found pathogens were also

occasionally detected. *Salmonella* was found in about 3% of samples tested by Van den Heever (1970). *Escherichia coli* O157:H7 was found 2% of samples tested by Abongo and Momba (2009). *Listeria monocytogenes* was found to be present in 1.3%, and toxin producing *Staphylococci* were present in 2% of samples examined (Naidoo and Lindsay, 2010). Naidoo and Lindsay (2010) also noted mycotoxin producing molds can be present in biltong samples. Van der Riet (1976) found 55% of mold populations present on 20 samples of biltong belonged to *Aspergillus* with 16 of 26 strains identified as having capabilities to produce mycotoxins. In summary, these surveys showed commercial biltong may contain fairly elevated levels (4 log CFU/g or greater) of many spoilage organisms and occasionally samples may contain organisms capable of causing food poisoning including *Salmonella, E. coli*, toxin producing *Staphylococci*, and molds. As raw meat used in biltong production may well contain pathogens, it is important products are manufactured in a way to prevent growth and increase the destruction of these organisms.

As described earlier, raw meat for biltong is marinated in mixes containing salt, organic acids, and other ingredients, then dried to achieve a reduced A_w as quickly as possible. The salt concentration increases due to reduction in moisture, and acetic acid within vinegar can cause sublethal injuries to *E. coli* as well (Hirshfield et al., 2003). Weak acids such as acetic acid can be either charged or uncharged, depending on pKa of the acidic group and pH of the environment surrounding it. The uncharged form of the acid is lipid permeable and can freely diffuse into the microbial cell cytoplasm. Organic acid diffusion reaches equilibrium when internal and external concentrations of the uncharged form of the acid are equal. Since most neutralophilic microorganisms maintain a pH gradient across their cytoplasmic membranes, with

inside being more alkaline than outside, accumulation of high levels of charged-weak acid in the cytoplasm occurs, causing the microbial cell to weaken (Hirshfield et al, 2003).

The presence of organic acids, salt, and a reduced A_w achieved by drying are all controlling factors in the potential destruction of pathogens and play a critical role preventing microbial growth. However, Naidoo and Lindsay (2010) demonstrated salt, spices, and the presence of organic acids as individual factors were not sufficient to prevent growth of *S. aureus* or *L. monocytogenes*. Several studies have assessed reductions of *Salmonella, L. monocytogenes, S. aureus,* and *E. coli* O157 achieved during processing. The reductions achieved for *Salmonella* ranged from 2 to 3 log CFU/g, *E. coli* from 2 to 3 log CFU/g, *L. monocytogenes* from 2 to 4.5 log CFU/g, and *S. aureus* from 1 to 6 log CFU/g. The reductions varied depending upon the method used as Naidoo and Lindsay (2010) found that a greater reduction of *L. monocytogenes* was achieved more quickly when a traditional method was used (vinegar dip then spiced) compared with a modern method (vinegar and spice mixed), but the opposite was observed for *S. aureus*. The reduction in pathogen level will also vary throughout the drying processes with greater reductions achieved as the A_w lowers (Burnham et al, 2008).

In 2020, Karolenko, et al. conducted a study to achieve a 5-log reduction of *Salmonella* on biltong. Beef pieces were inoculated with a 5-strain cocktail of *Salmonella*, dipped in various antimicrobials (lactic acid, acidified calcium sulfate, and sodium acid sulfate) or water, then marinaded while vacuum tumbled. After marination, samples were placed into a curing cabinet set at varying temperatures (22.2 °C, 23.9 °C, or 25 °C) depending on process. The study also examined salt and vinegar concentration levels. Nearly all approaches achieved a 5-log reduction of *Salmonella*, which can be comparable to *E. coli* reductions (Karolenko et al., 2020). However, samples were far smaller than average slices that would be used in commercial productions,

causing a substantial loss in moisture in a short amount of time that may have led to the reduction in counts reported. Some samples also underwent vacuum tumbling according to treatment, causing the product to no longer be intact (Federal Register, 1999). Therefore, the *Salmonella* used for the study may have been inadvertently introduced into the interior of the product, causing the need to sample the whole product, not just the surface of the sample. While the study conducted by Karolenko and others (2020) focused on *Salmonella* reductions, USDA (2021) recommends that establishments use Salmonella as an indicator of lethality because the thermal destruction of Salmonella in cooked products would indicate the destruction of most other pathogens (64 FR 732). However, when evaluating non-thermal processed meat products that rely on A_w, *E. coli* levels should be used as indicators for lethality.

Jerky Previous Research

Jerky is a dried meat product common to the United States that is similar to biltong. Jerky is typically sliced thinner than biltong and therefore dries quicker. Commercial manufacturers of jerky dry the product at an elevated temperature or cook it before drying with high humidity to ensure a safe and wholesome product for consumers (Nummer et al, 2004). Jerky can be made from a variety of sources including beef, venison, poultry, wild game, and other animals. Meat thickness, seasonings and marination's vary considerably. Jerky may also be made from chopped or ground meats formed into strips or stuffed into narrow casings. Jerky must have a moisture-to-protein ratio of 0.75:1 and a A_w of 0.85 or less (USDA, 2014). A A_w of 0.70 is recommended to prevent mold growth (Nummer et al, 2004) and guidelines from the USDA (2014) require a A_w no greater than 0.85 as part of its legal identity which should control pathogens of concern such as *Staphylococcus aureus* and *Clostridium botulinum*, which can survive at A_w levels as low as 0.86 (Nummer et al, 2004). Water activity alone does not consider possible problems associated

with drying rate including growth of *Staphylococcus aureus* and toxin production if the meat is not dried fast enough, and sublethal injury or adaptation of *E. coli*, *Salmonella*, or *L. monocytogenes*. Current guidelines for the commercial production of jerky recommend several processes that can be used to ensure pathogen lethality by combination of high heat with high relative humidity followed by drying (USDA 2021). When processed correctly, commercially packaged jerky can be kept at room temperature for over 12 months (USDA, 2017). Although both are dried products, biltong is not considered jerky and does not have to adhere to the jerky identity standards. However, as a dried RTE meat product it must still meet the requirements for pathogen control and stability (USDA 9 CFR 417.2). Following the FSIS Compliance Guideline for Meat and Poultry Jerky Produced by Small and Very Small Establishments (USDA, 2014) would help ensure biltong could meet pathogen lethality and stability requirements, but traditional biltong is not subjected to temperatures (or temperature plus sufficient humidity) high enough to ensure lethality prior to drying and may be dried to A_w levels greater than those required for jerky products (A_w greater than 0.85 for medium and wet biltong). Therefore, alternative total process methods must be validated to ensure pathogen lethality and product stability for biltong.

Historically, drying meats to produce jerky was considered a safe preservation process, convenience, nutritional aspects, and its flavor made it a popular food product for home food preservers. Recent foodborne illness outbreaks related to both home-dried and commercially manufactured jerky have raised concerns about the product's safety. Some traditional home recipes and drying processes were shown to be inadequate to destroy *E. coli* O157:H7, *Salmonella*, *S, aureus*, and *L. monocytogenes* in both whole-muscle and ground-meat jerky. Several research studies identified processes such as precooking meats before drying, using

acidic marinades, cooking meats after drying, or some combination of treatments that can destroy concerning pathogens to produce microbiologically safe and palatable meat jerky at home (Nummer et al., 2004). Due to similarities in product processing, concerns exist that non-thermally treated biltong processes may not be sufficient to eliminate pathogens of concern, Multiple processing techniques have been employed to produce jerky, with marination as the most common method, high temperature drying, and high temperature heating as a lethality step for pathogen reduction (Nummer et al. 2004).

Many consumers demonstrate an aversion to the cooked flavor of jerky products when the meat was first cooked to 71.2°C and then dried as recommended (Albright et al, 2003). To bridge the gap between safety and quality, studies were conducted to examine effects of predrying treatments on pathogen destruction during the jerky production process. Albright et al. (2003) evaluated survival of inoculated *E. coli* O157:H7 populations on whole-strip beef jerky prepared using four multihurdle, predrying treatments: (i) boiling water immersion, then marinating; (ii) seasoning followed by hot pickling brine immersion (iii) vinegar-water solution (1:1) immersion, then marination; and (iv) marinating, then vinegar-water solution (1:1) immersion. All samples were dried in home-style dehydrators at 62°C and stored for up to 90 days at ambient temperature (21°C) in a room relative humidity of 19–24%. Treatment 2, immersing product into a pickle brine heated to 78°C for 90 seconds, resulted in the greatest bacterial population reduction (5.7 to 5.8 log CFU/cm^2) during drying. The remaining three methods resulted in bacterial population reductions of 4.3 to 5.2 log CFU/cm^2. Bacterial populations continued to decline during storage and were not detectable by direct plating after 30 days in storage. A consumer panel rated all four jerky products quality as acceptable. The authors concluded the pre-drying treatments evaluated significantly destroyed *E. coli* O157:H7 produced in home-dried

whole-muscle beef jerky processing. The work by Albright et al. (2003) demonstrates the possibility of achieving lethality without a full lethality heat treatment (treatments 3 and 4) using similar base ingredients (salt and vinegar) as used in biltong processing.

More recently, Porto-Fett et al. (2008) validated lethality of three time and temperature regimens for whole muscle beef jerky commercial processing. A total of 8.9 log CFU/g per strip of *E. coli* O157:H7, *Salmonella typhimurium*, or *L. monocytogenes* multiple-strain cocktails were separately applied onto the beef strip surfaces and treatments were applied for both non-marinated and marinated samples. Strips were then cooked and dried for 1.5, 2.5, or 3.5 h at 82.2°C with constant smoking, but without humidity. Regardless of how the strips were treated, drying for 1.5, 2.5, or 3.5 h to 82.2°C with constant smoke resulted in a 7.3 log CFU/g decrease per strip for each pathogen cocktail. Marinated strips cooked and dried for 2.5 and 3.5 h or non-marinated strips cooked or dried for 3.5 h also satisfied the U.S. Food Safety and Inspection Service standard of identity (moisture-to-protein ratio 0.75:1) and/or shelf-stability (A_w 0.80) jerky requirements.

Escherichia Coli

Escherichia coli is a gram-negative, rod-shaped bacterium from the *Enterobacteriaceae* family. It is typically found in human and animal digestive systems and can survive for an extended period under a wide variety of environments including high acidity and high heat (Armstrong et al. 1996; Tuttle et al. 1999). Some pathogenic strains were described as verotoxigenic *E. coli* or STEC. Most recently, designation was simplified to STEC in recognition of similarities of toxins produced by *E. coli* and Shigella dysentery (Murray et al., 2007; Fischer-Walker et al., 2012).

In addition to *E. coli* O157:H7 the United States Food and Drug Administration (**FDA**) identified six serogroups that are also considered adulterants on beef and ready-to-eat products, known as the "big six": *E. coli* O26, O45, O103, O111, O121, and O145 (US 76 FR 58157). Serogroups are a designation scientists use to group different *E. coli* serovars, or strains. The "O" refers to the O (somatic) antigen, a surface structure that differs among *E. coli* serovars (Murray et al., 2007). These serovars are the most identified types of non-O157 *E. coli* serotypes found in foods and cause approximately 169,600 US foodborne illness cases each year (CDC, 2021b).

There are many known *E. coli* strains, with *E. coli* O157:H7 being most widely recognized due to severity of foodborne illness causes (CDC, 2021b). Potent toxins can cause severe damage to intestinal lining, even in healthy individuals. *Escherichia coli* produced toxins are additionally responsible for symptoms such as hemorrhagic colitis. Hemorrhagic colitis is associated with bloody diarrhea and hemolytic uremic syndrome (**HUS**), which is typically seen in the very young, immunocompromised, and elderly generations, and causes renal failure and hemolytic anemia. In the very young, in addition to renal failure and hemolytic anemia, this disorder can cause permanent loss of kidney function (Tarr, 1995; Fischer-Walker et al., 2012). Symptoms can appear within hours or up to several days after ingestion of bacteria and illness usually lasts 5 to10 days (Tarr, 1995; Fischer-Walker et al., 2012). *Escherichia coli* O157:H7 was identified as the causative agent in 85 to 95% of cases of HUS in North America. Non-O157 Shiga- toxin producing *E. coli* were responsible for another 5 to15% (Armstrong et al., 1996).

Generally, *E. coli* can survive at temperatures ranging from 7°C to 50°C. It can also survive under acidic conditions (pH of approximately 4.4), enabling it to survive in mildly acidic food products like dry-fermented sausage (Jordan et al., 1999). Additionally, *E. coli* O157:H7 and non-O157 have a low infective dose and can be transmitted from person to person through fecal-

oral route. An asymptomatic carrier state was reported, where individuals showed no clinical signs of disease, but were capable of infecting others (CDC, 2021a). Duration of excretion of STEC is about 1 week or less in adults but can be longer in children. Visiting farms and other venues where public might come into direct contact with farm animals has also been identified as an important STEC infection risk factor (CDC, 2021a).

The first *E coli* O157:H7 outbreaks occurred in Oregon and Michigan in 1982, isolated from individuals who developed bloody diarrhea and severe abdominal cramps after eating hamburgers in a restaurant chain (USDA, 2017). In 1993, United States Department of Agriculture Food Safety and Inspection Service **(USDA-FSIS)** mandated the zero-tolerance standard regarding complete removal of fecal material, ingesta, and udder fluids from beef carcasses. In 1994, USDA-FSIS declared *E. coli* O157:H7 as an adulterant in ground beef (USDA, 2002, US 67 FR 62325). The USDA states that an adulterant shall apply to any carcass, part thereof, meat or meat food product under one or more circumstances (if it contains poisonous substances, pesticides, or chemicals; or if it has been prepared under unsanitary conditions). In 1994, the adulterated definition was extended to include raw ground beef that is positive for *E. coli* O157: H7. The USDA set requirements for meat slaughter establishments to develop and implement HACCP programs (US 61 FR 38868) to ensure control of slaughter processes and further processing of meat with the goal of effectively reducing or eliminating pathogens from beef products.

Surrogates

Core principles of HACCP include hazard analysis, critical control point identification, establishing critical limits, monitoring procedures, corrective actions, verification procedures,

and record-keeping and documentation (US 9 CFR 417; 61 FR 38868). Under such systems, if a deviation occurs indicating that control has been lost, the deviation is detected and appropriate steps are taken to reestablish control in a timely manner to assure that potentially hazardous products do not reach consumers. As foodborne pathogen detection technologies improve, validation and verification methods have become more preventive in nature (FDA, 2020). Using non-pathogenic bacteria as surrogates for pathogens has provided an opportunity to validate various processing parameters. Nonpathogenic surrogate bacteria are currently used in a variety of food challenge studies in place of foodborne pathogens such as *L. monocytogenes*, *Salmonella*, *E. coli* O157:H7, non-O157 STEC, and *Clostridium botulinum* because of safety and sanitary concerns. A validated surrogate responds like the targeted pathogen when tested for inactivation levels, growth parameters, or survivability under given conditions (FDA, 2020) and ideally harbors greater resistance to reduction hurdles than the actual pathogen to ensure a margin of safety. The U.S. Food and Drug Administration defines surrogates as "a non-pathogenic species and strain responding to a particular treatment in a manner equivalent to a pathogenic species and strain." When evaluating the efficacy of a given processing treatment in food systems, indicator or surrogate microorganisms are commonly used in place of foodborne pathogens in challenge tests conducted in either laboratories or food manufacturing plants. Nonpathogenic surrogate microorganisms are specially selected strains prepared in a laboratory and inoculated into food products, or they may be a naturally occurring inoculum that qualifies as a surrogate and has been confirmed to exist at adequate levels in the specific food product (FDA, 2017).

Although use of the target pathogens in challenge studies would be ideal, mishandling a pathogen can have devastating effects for researcher safety, food safety, and safety of the

processing environment. Many companies and academic institutions do not have access to a biosafety level 2 testing laboratory and the introduction of pathogens in a food processing facility is highly discouraged. Surrogate microorganisms function by standing in place of equivalent pathogenic bacteria for determination of inactivation levels of a given intervention treatment without introducing pathogens into a laboratory or a food processing area. Therefore, surrogate microorganism use is a valid approach when an appropriate biosafety laboratory is not available and because it is not acceptable to introduce pathogens into a food processing facility. Desired characteristics of an appropriate surrogate include nonpathogenic behavior, inactivation characteristics, and kinetics that can be used to predict those of the target microorganism when exposed to similar or specific environmental conditions, genetically stable, and susceptibility to injury like that of the target pathogen (Busta et al., 2003).

Borowski et al. (2009) conducted a comparison between a 10-strain cocktail of serovars plus five *E. coli* O157:H7 isolates and three commercial lactic acid bacteria (**LAB**): (i) BioSource (described by the manufacturer as *Pediococcus acidilactici*), (ii) Saga 200 (described as *Pediococcus* spp.), and (iii) Saga 75 (strain information unknown). The LAB strains Saga 200 and BioSource were consistently more thermotolerant than the 10-strain cocktail of *E. coli* O157:H7 and *Salmonella* serovars under both conditions, indicating that these LAB strains could serve as effective surrogates for *Salmonella* and *E. coli* O157:H7 for evaluating the safety of processes used in the manufacture of ground-and-formed beef jerky.

Marshall et al. (2005) conducted a study using prerigor lean and adipose beef carcass tissue that was artificially contaminated with cultures of five *E. coli* beef cattle isolates or a cocktail of five *E. coli* O157:H7 strains in a fecal inoculum. Samples were processed with various microbial interventions that included lactic acid, chlorine, hot water, and trisodium phosphate, or a

combination. For all microbial intervention methods applied regardless of tissue type, the average log reductions of at least two *E. coli* isolates were not significantly different from the average log reduction of the *E. coli* O157:H7 cocktail. Thus, the use of these surrogate isolates, identified as P1, P3, P8, P14, and P68, were able to accurately predict the effectiveness of microbial intervention methods on the reduction of *E. coli* O157: H7 in beef carcass tissue.

Continuing with the work of Marshall et al. (2005), Niebuhr et al. (2008) tested the five nonpathogenic *E. coli* strains identified above (now identified as BAA-1427 through 1431) on pre-rigor beef lean and adipose tissue in summer sausage batter, before and after fermentation. The study found that the strains may be used individually or collectively for specific process validation indicators for Salmonella. In general, when using surrogate organisms and as highlighted by the work of Marshall et al. (2005) and Niebuhr et al. (2008) it is best if a cocktail of validated surrogates are used as a single surrogate rarely accounts for all sources of variation when compared to the pathogen(s) of concern growth and death curves.

Keeling et al. (2009) conducted a study where the aforementioned *E. coli* isolates (noted as *E. coli* biotype 1) were compared with *E. coli* O157:H7 under four common meat processing conditions. The processes that were evaluated included freezing, refrigeration, fermentation, and thermal inactivation. For each study, at least one surrogate organism was not statistically different when compared with *E. coli* O157:H7 (Keeling et al., 2009). However, the four studies did not consistently show the same isolate as having this agreement. The three studies that involved temperature as a method of controlling or reducing the *E. coli* population all had at least one possible surrogate in common. In the fermentation study, only one isolate (BAA-1429) showed no statistical difference when compared with *E. coli* O157:H7. However, the population reductions that were observed indicated the isolates BAA-1427 and BAA-1431 would

overestimate surviving *E. coli* O157:H7 populations in a fermented summer sausage. When all the data from all the surrogates was examined, it was found that isolates BAA-1427, BAA-1429, and BAA-1430 were acceptable surrogates for all four of the processes that were examined in this study. There was no statistical difference noted between these three isolates and *E. coli* O157:H7 in the refrigeration study. These isolates resulted in decreased population reductions than *E. coli* O157:H7 in the frozen, fermentation, and thermal inactivation studies. This indicated that these isolates would overpredict the *E. coli* O157:H7 population in these three instances resulting in an additional margin of safety when using *E. coli* biotype 1 as a surrogate (Keeling et al., 2009).

Diaz et al. (2009) conducted a study where four nonpathogenic *E. coli* biotype I strains, including BAA-1427, 1428, and 1430, isolated from cattle hides (fluorescent protein-marked mutants) were compared with four *E. coli* O157:H7 strains isolated from cattle fecal samples and five *Salmonella* serovars isolated from cattle. No differences were observed in maximum population density and lag time between fluorescent *E. coli* mutant strains and target pathogens. Log reductions of fluorescent mutants were not different from or showed decreased log reductions compared to pathogenic *E. coli* O157:H7 and *Salmonella* strains after exposure to pH 2.5, 3.0, and 3.5 for 0.5, 1.0, 1.5, and 2.0 hours. Mean populations for the fluorescent protein-marked strains were not different or were higher than those observed for *E. coli* O157:H7 and *Salmonella* strains after hot water interventions at 55, 60, and 65°C.

As the five biotypes are considered comparable to both *Salmonella* and *E. coli* O157:H7, they are now able to be used in plant facilities as well as research facilities that do not have Biosafety Level 2 areas. These surrogates can be purchased for educational use from ATCC (Manassas, Virginia) as ATCC *Escherichia coli* (Migula) BAA- 1427,1428,1429,1430, and

1431(ATCC, 2021). Creating safe dried meats relies on achieving the correct balance of several parameters during processing and storage. Using this concept, each hurdle can be applied to produce products that are safe and stable (Betts and Everis, 2008).

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CHAPTER 3

PROCESS VALIDATION FOR THE REDUCTION OF SURROGATE *ESCHERICHIA COLI* DURING THE PRODUCTION OF BILTONG[1]

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ABSTRACT

Biltong is gaining in popularity in the U.S. and is primarily made with whole muscle beef slices seasoned with salt and spices and allowed to dry to the desired water activity (**A_w**). Ready to eat beef products should achieve a 5-log reduction for *Escherichia coli (**E. coli**)* O157:H7 and other non-O157 Shiga-toxin strains (**STEC**). The USDA recommends thermal processing to achieve a five-log reduction. The research objective was to validate a process for *E. coli* reduction during biltong processing while adhering to traditional non-thermal techniques. Beef eye of round slices were inoculated with surrogates for *E. coli* O157:H7. Samples were subjected to varying levels of salt, vinegar, lactic acid (**LA**), and nitrite based on experiment. Samples in Experiment 1 were subjected to 3.75% salt, 21.3% vinegar, 156 ppm $NaNO_2$ and ingredients traditional to biltong. Experiment 2 samples were assigned to 1 of 9 treatments consisting of salt at 1.7, 2.2, or 2.7% and vinegar at 2, 4, or 6% meat weight. Experiment 3 samples were assigned to one of four treatments: T1 (2% NaCl, 2.5% vinegar, no LA), T2 (2% NaCl, 2.5% vinegar, 3% LA), T3 (3.5% NaCl, 5% vinegar, no LA), and T4 (3.5% NaCl, 5% vinegar, 3% LA). Samples were dried at 15.5°C and 65% relative humidity. Samples were removed for microbial analysis at A_w levels of 0.85, 0.80, and 0.75. Experiments 1 and 3 achieved a 5-log reduction for surrogate *E. coli* without thermal processing after reaching A_w 0.85 or lower where Log CFU *E. coli* and A_w decreased with time ($P < 0.01$). Experiment 2 was able to achieve a 2-log reduction after reaching A_w 0.75 for all treatments and a 5-log reduction for 4 of the 9 treatments. This work should be followed by studies utilizing pathogenic *E. coli* O157:H7 and STEC for further validation.

Keywords: biltong, *Escherichia coli*, reduction, water activity

INTRODUCTION

For centuries, producers utilized curing as the most common meat product preservation method to protect against bacterial and microbial growth (Leistner, 1987). In response to increasing public health concerns associated with processed meat consumption, the meat industry has begun introducing minimal processing to improve this food category's nutritional and health properties (Toldra and Reig, 2011). In developing countries, consumers eat dried meat products as valuable protein sources, while in industrial countries, these products are consumed as healthy, high-protein snack foods. McLynn et al. (2015) reported demand for meat snacks among American young adults increased by 18% between 2010 and 2015. Biltong, an air dried ready-to-eat (**RTE**) meat product, is becoming a popular U.S. snack food. Biltong typically comes in two forms related to the total water activity ($\mathbf{A_w}$) of the product, medium and dry. Medium and dry biltong are considered shelf stable RTE products (Leistner, 1987). In the U.S., producers manufacture biltong with whole-muscle beef slices seasoned with salt and spices which allowed to dry to desired A_w (Jones et al., 2017). With this type of further processed meat product gaining popularity, there is a risk of producers improperly processing products due to not understanding repercussions associated with pathogens such as *Escherichia coli (**E. coli**)*.

Morbidity and mortality associated with several large outbreaks of gastrointestinal disease caused by Shiga toxin-producing *Escherichia coli* (**STEC**) within beef products highlighted threats these organisms can pose to public health (Paton et al., 1998). *Escherichia coli* serotypes found in foods cause approximately 169,600 US foodborne illness cases each year (CDC, 2021). Ready-to-eat beef products must achieve a five-log reduction for *E. coli* O157:H7

and other non-O157 Shiga-toxin strains for a process to be considered as validated (USDA, 2014). Under current USDA guidelines, processors may utilize thermal processing, among other hurdles, to achieve standards for *E. coli* O157:H7 and STEC lethality (5D process; decimal reduction dose). Biltong producers prefer to not use thermal processing to stay true to traditional practices which makes it difficult to ensure a safe product. Currently, there is little information in the literature or public domain with a validated process to reduce *E. coli* by 5-log on biltong without using thermal processing.

In the absence of heat, one of the greatest hurdles to microbial growth is low water activity. Typically, bacteria are unable to reproduce below 0.85 $A_{w.}$ An antimicrobial vinegar-water marinade as well as a lactic acid dip adds additional hurdles for bacterial reduction (Fontana, 2007). The purpose of this research was to validate a process for a 5-log reduction of *E. coli* during the manufacture of biltong while adhering to traditional, non-thermal, processing techniques. The objectives were to validate a standard processing procedure for biltong production, as well as to evaluate antimicrobial effects of various hurdles throughout biltong processing.

MATERIALS AND METHODS

Preparation of bacterial cultures

Nonpathogenic *E. coli* surrogates were obtained from ATCC (Manassas, VA), transferred to the University of Georgia Department of Animal and Dairy Science (Athens, GA), and stored at -80°C. Nonpathogenic *E. coli* serovars ATCC BAA-1427, 1428, 1429, 1430, 1431, used as surrogates for *E. coli* O157:H7 (Marshall et al., 2005) and STEC (USDA 2021b), were activated by placing one bead of each surrogate into 9 mL of tryptic soy broth (TSB; Beckton, Dickinson

and Company, Sparks, MD) and incubated at 37°C for 24 h. Two subsequent loop transfers in TSB were conducted and then surrogates were transferred to tryptic soy agar (TSA; Beckton, Dickinson and Company, Sparks, MD) plates and stored at 4°C. Fresh cultures were prepared for all experiments and replicates within an experiment. To prepare the inoculum, a 10 μL loopful of each serotype was deposited in 9-mL tubes of TSB with 2.5% glucose and 100 ppm rifampicin (TSB+R; Fisher Scientific, Waltham, MA) and incubated at 37°C for 24 h to induce acid tolerance and antibiotic resistance. Overnight cultures were each transferred to two 50-mL conical tubes with TSB+R and incubated at 37°C for 24 h. Cultures were centrifuged at 2,000 × *g* for 20 min at 20°C, excess supernatant was removed, the remaining pellet was washed with 50-mL 0.1% buffered peptone (Fisher Scientific, Waltham, MA), and centrifuge and wash steps were repeated once more. The supernatant was removed from all tubes and the residual pellets were combined with 0.1% buffered peptone to a final volume of 500 mL. Prior to inoculation, the inoculum cocktail was serially diluted (1:10) in 0.1% buffered peptone with 100 ppm rifampicin and plated in duplicates onto aerobic plate count (APC) Petrifilm (3M, Saint Paul, MN) then incubated at 37°C for 48 h for enumeration. The methodology to prepare surrogates was followed for all experiments.

Beef preparation, inoculation, and processing

Experiment 1

Five frozen beef eye-of-rounds (Institutional Meat Purchase Specifications #171C; USDA 2014b) were obtained from a national supplier and transferred (155 km) to the University of Georgia Meat Science and Technology Center (Athens, GA) via refrigerated truck and frozen (-20°C) until needed. Frozen beef eye-of-rounds were tempered at 4°C, excess connective tissue and fat cover were removed prior to processing, and 2.5-cm thick slices were cut with a deli

slicer (Hobart EDGE Manual Meat Slicer, Troy, OH) parallel to fiber orientation. Fifteen slices of similar length and width were randomly assigned to 1 of 3 replications equaling 5 slices for each replication. A sterile stainless-steel template 25-cm^2 was used to make impressions on the surface of each slice and food grade carcass ink (Koch Supplies, Kansas City, MO) was used to outline each impression (Figure 1). The ink was allowed to dry (~30 min), and each slice was inoculated with a foam brush containing the five-strain cocktail (ATCC BAA 1427 to 1431) of non-pathogenic surrogate *E. coli* (ca. 9.34 log CFU/mL) across the entire surface and allowed to rest for 30 min (4ºC) for surrogate attachment. Slices were seasoned according to individual weights with 3.75% salt (accounting for $NaNO_2$), 2.0% ground coriander, 1.0% brown sugar, 0.25% finely ground pepper, 0.25% $NaNO_2$, 0.125% ground garlic, and 0.0625% Worcestershire in poly bags (Wilheit Packaging, Gainesville, GA). Bags were tied shut and stored at 2 ± 2°C for 48 h with a manual massage at 24 h. After 48 h samples were aseptically moved to a clean bag and 1.5 times v/w DI-H_2O was added to the clean bag. Samples were massaged to rinse remaining cure from exterior. The DI-H_2O was drained from the bag and the sample was rinsed a second time. Post-rinse, samples were allowed to drip for 30 min on metal racks placed in poly-sausage trays to capture excess moisture and keep the slices out of the drip. Slices were then placed in clean poly bags with brine solution at 1.5 times v/w (pH 3.12) and tied shut. Brine solutions containined 21.3% naturally fermented 5% cider vinegar (Kroger, Cincinnati, Ohio), 10.6% Worcestershire (French's, Springfield MO), and 68.1% DI- H_2O and slices brined for 2.5 h at 4°C with bags being rotated every 15 min to ensure total surface exposure. Slices were aseptically removed from bags and allowed to drip for 30 min as described after rinsing.

After inoculation, slices were randomly assigned to sampling times where slice 1 (n = 3) for each replication was used to obtain 25 cm^2 samples post-inoculation (INOC), post-rinse (PR),

and post-brine (PB) for microbial analysis. Slices 2 and 3 were sampled on d 14 and 27 (n = 6), and slices 4 and 5 were sampled on d 21 and 280 (n = 6). Post-brining, slices 2 - 5 for each replication were placed in a Stagionello curing cabinet with Climatouch controls (Twin 100 + 100 kg, Crotone, Italy) set to 15.5°C, 50% relative humidity, and low ventilation (approximately 0.5 m/s) with a 30 min rest period every 2 h. During the first 7 d, slices were sprayed with 5% acidity apple cider vinegar (pH 3.17) each day to allow for 3 g of solution on each surface. Slices were sampled at their respective times for microbial analysis. Samples were also collected for surface and whole pH and A_w at each microbial sampling point, except for d 280. After 27 d of drying and collection of the d 27 sample the remaining samples from slices 4 and 5 were individually vacuum packaged, boxed, and stored at 23 ± 3°C until d 280 when the final sample was collected.

Microbial sampling

Each 25-cm² sample was aseptically removed to a depth of approximately 4 mm and placed in a sterile filter stomacher bag (VWR, Radnor, PA) with 90 mL 0.1% buffered peptone with rifampicin (100 ppm) and stomached (Seward, Worthing, United Kingdom) for 2 min at 230 rpm for microbial sampling. At all sampling points the originally marked 25 cm^2 area was excised to ensure a greater sampling area was not remove as samples dried and shrank in size. Samples were serially diluted (1:10) in 0.1% buffered peptone with rifampicin (100 ppm) and plated on both APC Petrifilm and TSA with rifampicin (100 ppm; **TSA+R**) via spread plating. All plates were incubated 48 h at 37°C for enumeration based on 25 cm^2. Counts were log transformed for statistical analysis and reporting.

Experiment 2

Slices from 50 beef eye of round subprimals were prepared as described in Experiment 1 to yield 175 slices. From the pool of slices, 108 slices that were the most uniform in length and width were selected and randomly assigned to 1 of 9 treatments across 2 replicates, allotting for a total of 6 slices•treatment•replicate^{-1} in a 3x3 factorial design. Factor one consisted of the amount of salt within cure at 1.7, 2.2, or 2.7% and factor two was amount of vinegar within brine at 2, 4, or 6% based on slice weight. Within treatment and replicate, slices were randomly paired (3 pairs) and within pair, slices were randomly assigned slice 1 and slice 2 designation. Slice 1 was reserved for sampling after inoculation, post-salt (PS), PR, and PB and slice 2 was reserved for sampling when A_w was estimated to be 0.80 and 0.75. As in Experiment 1 a 25-cm^2 stainless steel template was used to make impressions on the slices, the impressions were marked with food grade carcass ink (slice 1 = 4 samples; slice 2 = 2 samples) to show sample locations, and the ink was allowed to dry for ~30 min. Slices were inoculated (ca. 8.3 log CFU/mL using a foam brush with the five-strain cocktail (ATCC BAA 1427 - 1431) of non-pathogenic surrogate *E. coli* and allowed to rest for 30 min (4ºC) for surrogate attachment. An additional 2 slices•treatment•replicate^{-1} were randomly assigned but were not inoculated or marked. The two non-inoculated slices were used to estimate when each treatment combination achieved the target A_w. Slices were individually seasoned with 0.25% $NaNO_2$ and their assigned NaCl level, accounting for the salt in the $NaNO_2$ based on individual slice weight in poly bags and the bags were tied shut and stored at 2 ± 2°C to cure for 48 h with manual massage after 24 h. Subsequent to curing, samples were aseptically moved to a clean bag and rinsed with DI-H_2O following the procedures as Experiment 1. Samples were allowed to dry for 30 min and placed in clean poly bags with 1.5 times v/w of brine solution (pH 3.3). Brine solutions contained vinegar at either 2,

4, or 6% based on meat weight and DI- H_2O. Bags were tied shut and slices were brined for 1 h at 4°C, rotating every 15 min to ensure total surface exposure. After 1 h, slices were aseptically removed from bags and allowed to dry for 30 min. Twenty-five cm² samples were collected from slice 1 after inoculation, PS, and PB for analysis. Slice 2 and A_w test slices were placed in the Stagionello curing cabinet set to 15.5°C, 50% relative humidity, and low ventilation (approximately 0.5 m/s) with a 30 min rest period every 2 h until reaching the targeted water activities of 0.80 and 0.75 at which time they were sampled. When samples were collected for microbial analysis samples were also taken from each slice for surface pH and A_w, whole slice pH and A_w, and moisture to protein.

Microbial sampling

Each 25 cm² sample was aseptically removed to a depth of approximately 4 mm and placed in a sterile stomacher bag (VWR) with 90 mL 0.1% buffered peptone and stomached (Seward) for 2 min at 230 rpm. At all sampling points the originally marked 25 cm^2 area was excised to ensure a greater sampling area was not remove as samples dried and shrank in size. Samples were serially diluted (1:10) in 0.1% buffered peptone and 0.1 mL was plated on TSA+R via spread plating and plates were incubated 48 h at 37°C for enumeration based on the 25 cm^2 of the original mark. All counts were log transformed for statistical analysis and reporting.

Experiment 3

Fifty beef eye-of-round subprimals were prepared as previously described. A total of 144 uniform slices were randomly assigned to replication and to 1 of 4 treatments within replication, equaling 6 slices•treatment•$replicate^{-1}$ for each of 6 replications. Treatments were based on salt and brine vinegar percentages, as well as whether treatments received a lactic acid (LA) (Corbion, Lenexa, KS) dip (3.0%) for 30 s. The treatments included: T1 (2% NaCl, 2.5%

vinegar, no LA), T2 (2% NaCl, 2.5% vinegar, LA), T3 (3.5% NaCl, 5% vinegar, no LA), and T4 (3.5% NaCl, 5% vinegar, LA). Slices within a treatment and replication were then randomly numbered 1-6 and a 25-cm^2 stainless steel template was used to make impressions on the slices (except for slice 6), the impressions were marked with food grade carcass ink to show sample locations, and the ink was allowed to dry for ~30 min. Slices were assigned to sampling at the following time points: Slice 1 – INOC and post LA dip (PLAS), Slice 2 – PS and PB, Slice 3 – A_w 0.85, Slice 4 – A_w 0.80, Slice 5 – A_w 0.75, and Slice 6 – A_w testing slice. All slices, except slice 6, were inoculated (ca. 8.55 log CFU/mL) using a foam sponge brush with the 5-strain cocktail of non-pathogenic surrogate *E. coli* (ATCC BAA 1427-1431) and the slices were allowed to rest for 30 min (4ºC) to allow surrogate attachment. After inoculation and rest, the 25 cm^2 area from slice 1 was aseptically removed for microbial analysis of inoculation level. Slices from T2 and T4 were then subjected to the 30 s 3% lactic acid dip. Lactic acid was prepared from 88% concentrate solution by dilution in DI-H_2O according to manufacture recommendations. The LA solution was poured into plastic lugs with lug liners approximately 7.6 cm deep to ensure complete slice coverage. All 6 slices from one treatment by replication combination were submerged in LA together in a single layer. Lug liners and LA solutions were changed for each treatment by replication combination to prevent cross-contamination. Post LA dip, slices were removed and placed on wire racks in trays to capture excess drip and allowed to rest for 15 min. Slices assigned to T1 and T3 were not exposed to LA and were left in ambient temperature (4ºC) during the LA treatment and rest period of T2 and T4. After LA treatment, 25-cm^2 samples were obtained from slice 1 for all treatments for further microbial analysis. Remaining slices 2 through 6 were individually seasoned based on assigned NaCl levels (2.5% or 3%), and 1% brown sugar in poly bags. Experiment 3 excluded the use of additional

seasonings included in Experiment 1 including $NaNO_2$. Bags were tied closed and stored at 2 ± 2°C to cure for 48 h with a manual massage after 24 h. Following curing, samples were aseptically moved to new bags and brine solutions consisting of 2.5% or 5% naturally fermented cider vinegar (pH: 3.62 and 3.32, respectively) based on slice weight and DI-H_2O for a total volume of 0.25 v/w of each slice. Slices in Experiment 3 were not rinsed with DI-H_2O as done in Experiments 1 and 2 between salting and brining. Bags were again tied closed and brined for 1 h at 4ºC, rotating every 15 min to ensure total surface exposure. Samples were aseptically removed from bags and allowed to drip for 30 min. Twenty-five cm^2 samples were collected from slice 2 for each sample PS and PB, respectively for microbial analysis. Slices 3 through 6 were hung in the Stagionello curing cabinet set to 15.5℃, 50% relative humidity, and low ventilation (approximately 0.5 m/s) with a 30 min rest period every 2 h. During the first 5 d, slices were sprayed with 5% acidity (pH 3.17) cider vinegar daily to allow for 3 g of solution on each surface. Upon reaching A_w approximating 0.85, 0.80 and 0.75 the respective slices were taken out of the Stagionello cabinet and the pre-marked 25-cm^2 area was removed for microbial analysis. At each sampling time point for surrogate *E. coli* additional samples were excised from each slice for determination of whole slice pH, whole slice A_w, and moisture to protein.

Microbial sampling

Each 25 cm^2 sample was aseptically removed to a depth of approximately 4 mm and placed in a sterile filter stomacher (VWR) bag with 90 mL 0.1% buffered peptone with rifampicin (100 ppm) and stomached (Seward) for 2 min at 230 rpm for microbial sampling. At all sampling points the originally marked 25 cm^2 area was excised to ensure a greater sampling area was not remove as samples dried and shrank in size. Samples were serially diluted (1:10) in 0.1% buffered peptone with rifampicin (100 ppm) and plated on APC Petrifilm. All plates were

incubated 48 h at 37°C for enumeration based on 25 cm^2. Counts were log transformed for statistical analysis and reporting.

Water activity, pH, and moisture to protein

Water activity and pH were conducted for all three Experiments, moisture to protein was conducted for Experiments 2 and 3. Water activity was measured using an Aqualab water activity meter (Aqualab 4TE, Pullman, WA) after each timepoint. Surface samples were excised to approximately 4 mm, then chopped and placed into the water activity meter, while whole samples were chopped and placed into the meter. Meat pH was taken immediately after water activity using a 1:10 dilution method in DI-H_2O using a Hanna Instruments Edge pH meter with a general-purpose glass body bulb probe (Hanna Instruments, Smithfield, RI).

Samples collected for moisture to protein were chopped and frozen in liquid nitrogen before being powder homogenized using a Waring blender (Waring Commercial, Stamford, CT). Moisture to protein ratio (M:P) was determined from samples using a forced air oven (Fisher Scientific). One ± 0.1g from each sample was weighed in duplicate and placed into aluminum pans. Pans were then placed into the oven at 90ºC for 48 h. After drying, samples were moved to a desiccator and allowed to equilibrate and cool for 10 min. Percent moisture was calculated as *% moisture = ((wet sample weight - dry sample weight)/wet sample weight) x 100*.

Triplicate powdered-homogenized samples weighing approximately 0.2 g were weighed into aluminum foil. Crude protein was determined using a nitrogen auto-analyzer (Leco FP-528 Nitrogen analyzer, Leco Company, St Joseph, MI) to quantify N content which was multiplied by 6.25 to be expressed as a crude protein.

Statistical analysis

Experiments were conducted with multiple replications and each replication was performed as separate experiment using separately prepared inoculum, plating media, and slices. All data were analyzed using PROC GLM (SAS v9.4, Cary, NC) and means were separated using the LSmeans PDIFF option with Tukey adjustments at $\alpha < 0.05$ for pairwise comparisons. Experiment 1 had the fixed effect of time of sampling with slice and replicate as the random terms. In a second step, plating method (APC vs spread plating) as a fixed effect, was also analyzed across timepoints to compare the two methods. Experiment 2 was a 3 x 3 x 6 factorial with percentage salt and vinegar and time (or A_w) as fixed effects with slice and replicate as random terms. In Experiment 2 the percentage salt and vinegar were analyzed independently and were not different ($P > 0.10$), therefore, salt and vinegar were reanalyzed as combined treatments (9 treatments as fixed effects) with time. Experiment 3 was analyzed with treatment (percentage salt, vinegar, and LA exposure) and time (or A_w) as fixed effects with slice and replicate as random terms. For all experiments, if a treatment by time or plating method by time (Experiment 1) was considered significant ($P < 0.05$) data were reanalyzed within timepoint.

RESULTS

Experiment *1*

There was a time effect ($P < 0.01$) for surrogate *E. coli* populations (**CFU;** Figure 2). Inoculation counts did not differ ($P = 0.41$) from PS but were greater than all other time periods ($P \leq 0.02$). Post salt counts did not differ ($P = 0.64$) from PR but had greater counts than all other sampling times ($P < 0.01$). Post rinse did not differ ($P = 0.83$) from PB, but both periods had greater counts than all other remaining time points ($P < 0.01$), which did not differ from each other ($P \geq 0.35$). Overall, there was greater than a 5-log reduction by d 14 (5.97 log) that

sustained for the remainder of the drying periods (d 21 and 27; 6.32 and 5.69 log, respectively) and storage period (d 280; 6.35 log). There was a plating technique × time interaction for between APC and spread plating ($P < 0.05$; Figure 3) so data were reanalyzed by sampling timepoint. After INOC, PS, and PB, surrogate *E. coli* counts were similar within timepoint for APC and spread plating ($P \geq 0.07$). However, PR, d 14, and d 21 spread plating had greater counts than APC plates ($P \leq 0.02$). Even though counts were greater for spread plating than APC during the drying phase, samples, regardless of plating technique, achieved a 5-log reduction by d 14 (5.32 log for spread plate) and maintained through d 21 (5.35 log for spread plate; d 27 and 280 samples were not subjected to spread plating).

There was a time effect ($P < 0.01$; Figure 4) for surface water activity (SA_w), whole water activity (WA_w), surface pH (SpH), and whole pH (WpH). Surface A_w and WA_w showed similar trends where INOC, PR, and PB were not different from each other ($P \geq 0.64$) and were greater than all other time periods ($P < 0.01$). Remaining timepoints were different from each other for SA_w and WA_w and decreased, as expected, from d 14 to d 27 of drying ($P < 0.01$). Post rinse had a greater WpH and SpH ($P < 0.01$; Fig 4 c and d) than all other time points, likely as a consequence of residual salt and seasonings in the slices. Whole and SA_w both decreased ($P < 0.01$) after brining compared to PR but were similar to the raw inoculated samples ($P \geq 0.80$). Once samples entered the drying phase the pH of both WA_w and SA_w increased from PB samples ($P < 0.01$) and remained constant from d 14 through d 27 ($P \geq 0.90$). Drying phase samples were also similar to INOC ($P \geq 0.80$) for both WA_w and SA_w. Although comparisons are not shown, it is noteworthy that for both water activity and pH there were no differences ($P \geq 0.10$) between whole and surface samples within sampling time point.

Experiment 2

Initial analysis showed that there was not salt or vinegar main effect, nor any two- or three-way interactions with time ($P \geq 0.30$), therefore, salt and vinegar combinations were reanalyzed as total treatment combinations with time. There was not a treatment by time interaction or treatment main effect for surrogate *E. coli* counts ($P \geq 0.34$; Figure 5). There was a processing time main effect ($P < 0.01$) where surrogate *E. coli* counts sequentially decreased ($P < 0.01$) as sampling time increased from INOC through A_w 0.75. Looking at the time effect in regard to meeting USDA recommendations there was a 3.72 log CFU/cm^2 reduction from INOC after the samples reached A_w 0.80 ($P < 0.05$) and a 5.02 log CFU/cm^2 reduction from INOC to A_w 0.75 ($P < 0.01$). Examining the individual treatments (Figure 5), all treatments were able to meet a 2-log reduction by A_w 0.80 but only four of the nine treatments, those containing 1.7% NaCl + 6% vinegar, 2.2% NaCl + 4% vinegar, 2.2% NaCl + 6% vinegar, and 2.7% NaCl + 2% vinegar met a 5-log total process reduction after reaching A_w 0.75 at 5.13, 5.26, 5.78, and 5.03 log, respectively.

There was no treatment main effect for WA_w, SA_w, WpH, and SpH ($P \geq 0.35$; Table 1), but there was a time main effect for WA_w, SA_w, WpH, and SpH ($P < 0.01$; Table 2). Raw, PS, and PB were not different for WA_w ($P \geq 0.91$) and had greater values compared to 0.80 A_w and 0.75 A_w ($P < 0.01$), which did not differ ($P = 0.11$). Raw, PS, and PB were not different for SA_w ($P > 0.37$) and had greater values than 0.80 A_w and 0.75 A_w ($P < 0.01$), while 0.80 A_w was greater ($P < 0.01$) than 0.75 A_w. Whole sample pH was similar for INOC, PS, and PB samples ($P \geq 0.55$) but INOC and PS WpH was greater than 0.80 and 0.75 A_w samples ($P < 0.01$). Post brine WpH samples were lower than PS, as expected, but similar to 0.80 and 0.75 A_w. Sample surface pH increased from INOC to PS ($P < 0.01$) and then decreased PB ($P < 0.01$) compared to INOC

and PS. Post brine SpH remained similar to samples collected at A_w 0.80 and 0.75. There was not a treatment by time interaction ($P = 0.94$) or treatment main effect for moisture loss ($P \geq 0.35$) but there was a time main effect ($P < 0.01$; data not shown). Post rinse moisture loss (0.71%) was not different ($P = 0.07$) from PB (1.72%). However, moisture loss increased and was greater ($P < 0.01$) than PR and PB after reaching 0.80 (61.16%) and 0.75 (63.04%) A_w which were also different from each other ($P < 0.01$).

Experiment 3

There was not a treatment by time interaction ($P = 0.83$) for surrogate *E. coli* populations; however, there were main effects for both treatment and time ($P < 0.01$; Figure 6). Comparing treatment effects, T1 did not differ from all other treatments (3.71 log CFU/cm^2; $P > 0.08$); however, T3 (3.82 log CFU/cm^2) had greater surrogate *E. coli* populations compared to T2 and T4 (3.54 and 3.48 log CFU/cm^2, respectively; $P < 0.02$), which did not differ from each other ($P = 0.93$). Across all treatments, INOC (6.28 log CFU/cm^2) and PLAS (6.01 log CFU/cm^2) were similar ($P = 0.34$) to each other while INOC had greater ($P = 0.02$) surrogate *E. coli* counts than PS (5.85 log CFU/cm^2) which were similar to PLAS ($P = 0.88$). Although there was not a difference between INOC and PLAS, when separating out treatments that did not receive lactic acid (T1 and T3) to those that did (T2 and T4) the average reduction of T1 and T2 was only 0.09 log CFU/cm^2 while the average reduction of T2 and T4 was 0.46 log CFU. Surrogate *E. coli* populations continued to decrease PB ($P = 0.03$) and then from PB to A_w 0.85 (0.87 log CFU/cm^2; $P < 0.01$). Surrogate *E. coli* populations after A_w 0.75 (0.48 log CFU/cm^2) were less than A_w 0.85 ($P = 0.03$) while populations at A_w 0.80 (0.51 log CFU/cm^2) were similar ($P \geq 0.07$) to both A_w 0.85 and 0.75 counts. Examining the individual treatments (Figure 6) in relation to a trend or their ability meet the USDA guidelines for a 5-log reduction of *E. coli* in RTE beef

products all 4 treatments regardless of salt, vinegar, or lactic acid treatment were able to exceed a 5-log reduction by A_w 0.85 and sustain those reductions through A_w 0.75.

There was not a treatment by time interaction for Experiment 3 water activity ($P = 0.99$) or pH ($P = 0.20$), nor was there a treatment main effect for water activity ($P = 0.25$) or pH ($P = 0.37$; Table 3). However, there was a time main effect for both A_w and pH ($P < 0.01$; Table 4). Whole sample A_w was similar between INOC and PLAS ($P = 1.00$) and then decreased after PS ($P < 0.01$). Post brine A_w was similar to INOC, PLAS, and PB ($P \geq 0.15$) followed by a consecutive decrease at each remaining time point (A_w 0.85, 0.80, and 0.75; $P < 0.01$). Whole sample pH was similar between INOC, PLAS, and PS ($P \geq 0.28$). Post brine pH was similar to PLAS ($P = 0.11$), but lower than INOC and PS ($P < 0.01$). Sample pH at A_w 0.85 was similar to PLAS and PB pH ($P \geq 0.12$) and then continued to decrease as drying increased so that A_w 0.80 and 0.75 pH was similar to that of PB and A_w 0.85 ($P \geq 0.39$), but not PLAS ($P \leq 0.02$). The lower pH was expected PB and likely continued to be lower due to residual acid on the slice surface due to spraying 5% acidity vinegar on the slices for 5 d at the start of the drying period.

There was not a treatment by time interaction for percent moisture loss ($P = 0.99$), but there were treatment and time main effects ($P < 0.01$; data not shown). Percent moisture loss was measured at each processing step and then every two days throughout the drying phase on A_w 0.75 samples for consistency. Overall, T2 samples had greater percentage moisture loss (38.3%) than T1 (36.4%) and T4 (36.1%; $P \leq 0.01$) over the course of processing and drying but were similar to T3 (37.7%; $P = 0.81$). All other treatments (T1, T3, and T4) were similar to each other ($P \geq 0.12$). Cumulative moisture loss after PS and PB were similar to each other and not different from 0 (raw or INOC sample) at 2.6 and 2.4%, respectively ($P \geq 0.78$). Once the biltong slices entered the drying phase percent moisture loss increased, as expected, at each sampling point

from d 2 (15.4%) through d 12 (53.0%; $P < 0.01$) with the greatest 2-day change occurring between PB and d 2 (Δ13.0%). The rate of two-day moisture loss decreased, as expected, as time increased. Percent moisture loss from d 12 (53.0%), 14 (55.0%), and 16 (57.0%) were similar to each other ($P \geq 0.07$) at a rate of 1.0%/day compared to 4.2%/d between d 0 of drying through d 12. Cumulative moisture loss after d 18 (58.5%) and 20 (59.8%) was similar to each other ($P = 0.99$), d 14 and d 16 ($P \geq 0.07$), and d 22 ($P \geq 0.16$). On d 22 samples had a cumulative moisture loss of 62.9%, which was similar to the cumulative moisture loss recorded on d 18 and 20 ($P \geq 0.16$). The rate of drying from d 16 to 22 remained constant with that of d 12 through 16 at 1.0%/d.

DISCUSSION

Experiment 1

The objective of this study was to evaluate non-thermal, common practices used during the manufacture of biltong to evaluate their ability to be validated to reduce surrogate *E. coli* populations to a degree to meet USDA guidelines for a RTE shelf stable beef product (USDA, 2021). Traditional South African biltong does not traditionally rely on a thermal processing step for lethality, resulting in difficulty to achieve a 5-log reduction for pathogenic *E. coli* (O157:H7 and non-O157 STEC). The current study (Experiments 1, 2, and 3) used *E. coli* surrogates (BAA-1427, 1428, 1429, 1430, and 1431) that have been validated for *E. coli* O157:H7 under a myriad of conditions commonly used in beef processing (Marshall et al., 2005; Niebuhr et al., 2008; Cabrera-Diaz et al., 2009; Keeling et al., 2009). Through the various studies it was reported that at least 2 of the 5 surrogates (not always the same ones) were at least as resistant as *E. coli* O157:H7 and non-O157 STEC if not more resistant, making them ideal candidates as

surrogates. Furthermore, the surrogates BAA-1427 through 1431 have been approved by the USDA for in-plant validation (USDA, 2021). The surrogate strains used were transformed to be antibiotic resistant (rifampicin) to minimize cross contamination, aid in isolation of the surrogates, and enhance enumeration. The antibiotic transformation of surrogates also allowed for the plating on APC Petrifilm and TSA instead of selective media, which could reduce the growth and enumeration of injured cells.

Experiment 1 parameters were based on processing standards used by small Georgia manufactures. After 14 d total processing time, parameters were able to achieve a surrogate *E. coli* 5-log reduction and continue a reduction in counts throughout the remainder of the drying phase. The presence of organic acids, salt, and a reduced A_w are important factors for controlling potential destruction of pathogens and preventing microbial growth. The current study found decreasing moisture content and A_w caused an inhibition of bacterial growth. Fontana (2007) demonstrated that 0.85 is the border line A_w limit for most pathogens, including *E. coli.* Reducing the A_w to 0.85, which was achieved at approximately d 14, helped to keep the pathogens from reproducing overall. Experiment 1 also demonstrated when sampling a biltong product throughout processing timepoints, APC Petrifilm or TSA spread plating can be used. Although there were differences between APC and spread plating, samples were still able to meet a 5-log reduction after d 14. During Experiment 1, vinegar and salt levels were increased, and more inert ingredients were added. Experiment 2 was modified to include only functional ingredients at reduced levels to give a base for producers to understand. Overall, the current study found that when using traditional biltong spices and ingredients, as well as drying to a A_w of 0.85 or below can achieve a five-log reduction of surrogate *E. coli*.

Experiment 2

Experiment 2 and Experiment 3 parameters were based in part on a study by Karolenko et al. (2020), where a 5-log reduction of *Salmonella* was achieved without the use of a heat lethality step using minimal amounts of vinegar and salt. While the previous study was conducted focusing on *Salmonella* reductions, the U.S. FSIS stated destruction of *Salmonella* will indicate destruction of most other pathogens, including *E. coli* (Appendix A, 1999). After drying to A_w 0.77, all samples met greater than a 2-log reduction. Further drying to A_w 0.75 with the NaCl and vinegar concentrations used in Experiment 2 were only able to meet a 5-log reduction for 3 of the 9 treatments (1.7% NaCl +6% vinegar, 2.2% NaCl + 4% vinegar, and 2.2% NaCl + 6% vinegar). Of the treatments that achieved a 5-log reduction, there was no exact cause on why these treatments achieved the reduction compared to others. Due to this, the current study found that using such little amounts of vinegar and NaCl made no repeatable difference in *E. coli* reductions. In agreeance with the current study conclusions, Naidoo and Lindsay (2010) demonstrated salt levels greater than 20% were required to prevent growth of *Staphylococcus aureus* and *Listeria monocytogenes*, which is not feasible for biltong production. Salt, spices, and presence of organic acids as individual ingredients were not sufficient to prevent growth of *Staphylococcus aureus* and *Listeria monocytogenes* (Naidoo and Lindsay, 2010). After the completion of Experiment 2, a third Experiment was created to focus on moderate concentration of NaCl and vinegar for the effects seen in Experiment 2 with and without an additional treatment of an organic acid prior to curing.

Experiment 3

Experiment 3 was established to test for a total process that could achieve a 5-log reduction for surrogate *E. coli* using functional ingredients (NaCl and vinegar) at levels considered to be low to moderate with and without LA (3%) treatment of the raw meat. It was

expected that treatments 2 and 4 (receiving LA) would have a pronounced decrease in surrogate *E. coli* populations post exposure which differed from treatments 1 and 3 (no LA). Although there was a decrease in surrogate *E. coli* (avg 0.45 log CFU/cm2) for treatments 2 and 4 compared to treatments 1 and 3 the reduction was not enough to reach significance. While LA application did not have an immediate impact, treatments 2 and 4 had lower overall surrogate *E. coli* populations than treatment 3 with treatment 1 being intermediate. Cutter and Siragusa (1994) examined efficacy of organic acid concentration to control *E. coli* O157:H7. In agreement with the current study, the 1994 study indicated organic acids alone may not be sufficient to be considered a single microbiological safety hurdle, but they were beneficial as part of a HACCP approach to enhance microbiological safety of further processed meat products. The 5-log reduction was met for all treatments by the time the samples achieved an A_w of 0.85 and were able to maintain the reductions through A_w 0.75, meeting the USDA-FSIS guidelines for RTE beef products without applying a thermal processing step and adhering to traditional curing practices.

CONCLUSIONS

Overall, the current study showed that it is possible to achieve a five-log reduction of acid adapted surrogate E. coli without the use of a heat lethality step. With the addition of salts, vinegars, organic acids, and perhaps most importantly, drying to a A_w of 0.85 and below, these reductions were achieved. More work should be conducted with pathogenic *E. coli* 0157:H7 and other STEC strains for further validation.

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Table 1. Experiment 2 whole and surface water activity (A_w) and pH by treatment.

	Treatments[1]										
	1a	1b	1c	2a	2b	2c	3a	3b	3c	SD	*P*-value
A_w											
Whole[2]	0.88	0.88	0.89	0.86	0.87	0.86	0.87	0.86	0.86	0.01	0.336
Surface[3]	0.88	0.88	0.89	0.87	0.87	0.86	0.86	0.86	0.87	0.01	0.352
pH											
Whole[2]	5.55	5.57	5.51	5.55	5.55	5.50	5.56	5.52	5.53	0.02	0.758
Surface[3]	5.50	5.52	5.51	5.52	5.50	5.47	5.55	5.50	5.49	0.02	0.864

[1]1 = 1.7%, 2 = 2.2%, and 3 = 2.7% salt (NaCl); a = 2%, b = 4%, and c = 6% vinegar. Salt and vinegar were included based on a percentage of individual slice weight.

[2]Whole A_w and pH was a slice of the entire biltong including both surfaces and the interior.

[3]Surface was a slice of just the surface approximately 4 mm thick.

Table 2. Experiment 2 whole and surface water activity (A_w) and pH by sampling timepoint.

	Time						
	Inoculation	Post Salt	Post Brine	A_w 0.80	A_w 0.75	SD	*P*-value
A_w							
Whole[1]	0.98[a]	0.97[a]	0.98[a]	0.77[b]	0.75[b]	0.05	<0.001
Surface[2]	0.99[a]	0.97[a]	0.98[a]	0.78[b]	0.75[c]	0.05	< 0.001
pH							
Whole[1]	5.59[ab]	5.68[a]	5.51[bc]	5.49[c]	5.46[c]	0.12	< 0.001
Surface[2]	5.54[b]	5.69[a]	5.42[c]	5.44[c]	5.48[bc]	0.17	< 0.001

[abc]Means within a row without a common superscript differ ($P < 0.05$).

[1]Whole A_w and pH was a slice of the entire biltong including both surfaces and the interior.

[2]Surface was a slice of just the surface approximately 4 mm thick.

Table 3. Experiment 3 water activity (A_w) and pH by treatment.

	Treatments[1]					
	T1	T2	T3	T4	SD	*P*-value
A_w[2]	0.91	0.91	0.90	0.90	0.095	0.251
pH[2]	5.45	5.38	5.38	5.35	0.243	0.373

[1]T1: No lactic acid dip, 2% NaCl, 2.5% vinegar; T2: 3% lactic acid dip, 2% NaCl, 2.5% vinegar; T3: No lactic acid dip, 3.5% NaCl, 5% vinegar; T4: 3% lactic acid dip, 3.5% NaCl, 5% vinegar.

[2]A_w and pH was a slice of the entire biltong including both surfaces and the interior.

Table 4. Experiment 3 water activity (A_w) and pH by time point.

	Time[1]								
	INOC	PLAS	PS	PB	A_w 0.85	A_w 0.80	A_w 0.75	SD	*P*-value
A_w[2]	0.99[a]	0.99[a]	0.97[b]	0.98[ab]	0.85[c]	0.79[d]	0.76[e]	0.043	> 0.001
pH[2]	5.66[a]	5.48[ab]	5.68[a]	5.27[bc]	5.28[bc]	5.22[c]	5.11[c]	0.622	> 0.001

[abcde]Means within a row without a common superscript differ ($P < 0.05$).

[1] Time points for sampling included Inoculation (INOC), Post lactic acid dip (PLAS), Post Salt (PS), and Post Brine (PB).

[2]A_w and pH was a slice of the entire biltong including both surfaces and the interior.

Figure 1. Carcass ink markings on slices for sampling.

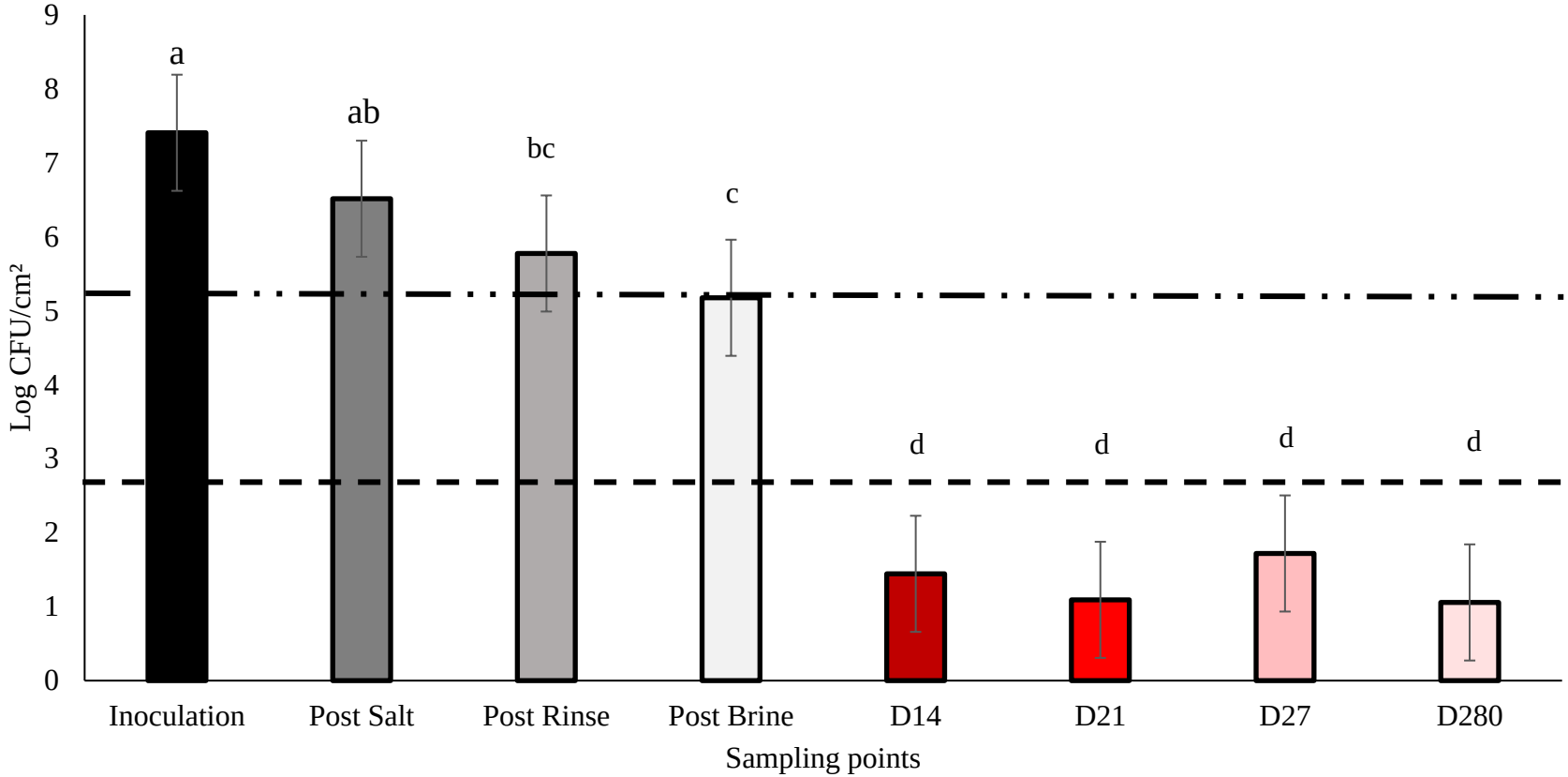

Figure 2. Experiment 1 surrogate *E. coli* populations from post inoculation to day 280 of drying. Horizontal line (----) refers to point of 5-log reduction from the inoculation level. Bars without a common superscript differ ($P < 0.05$).

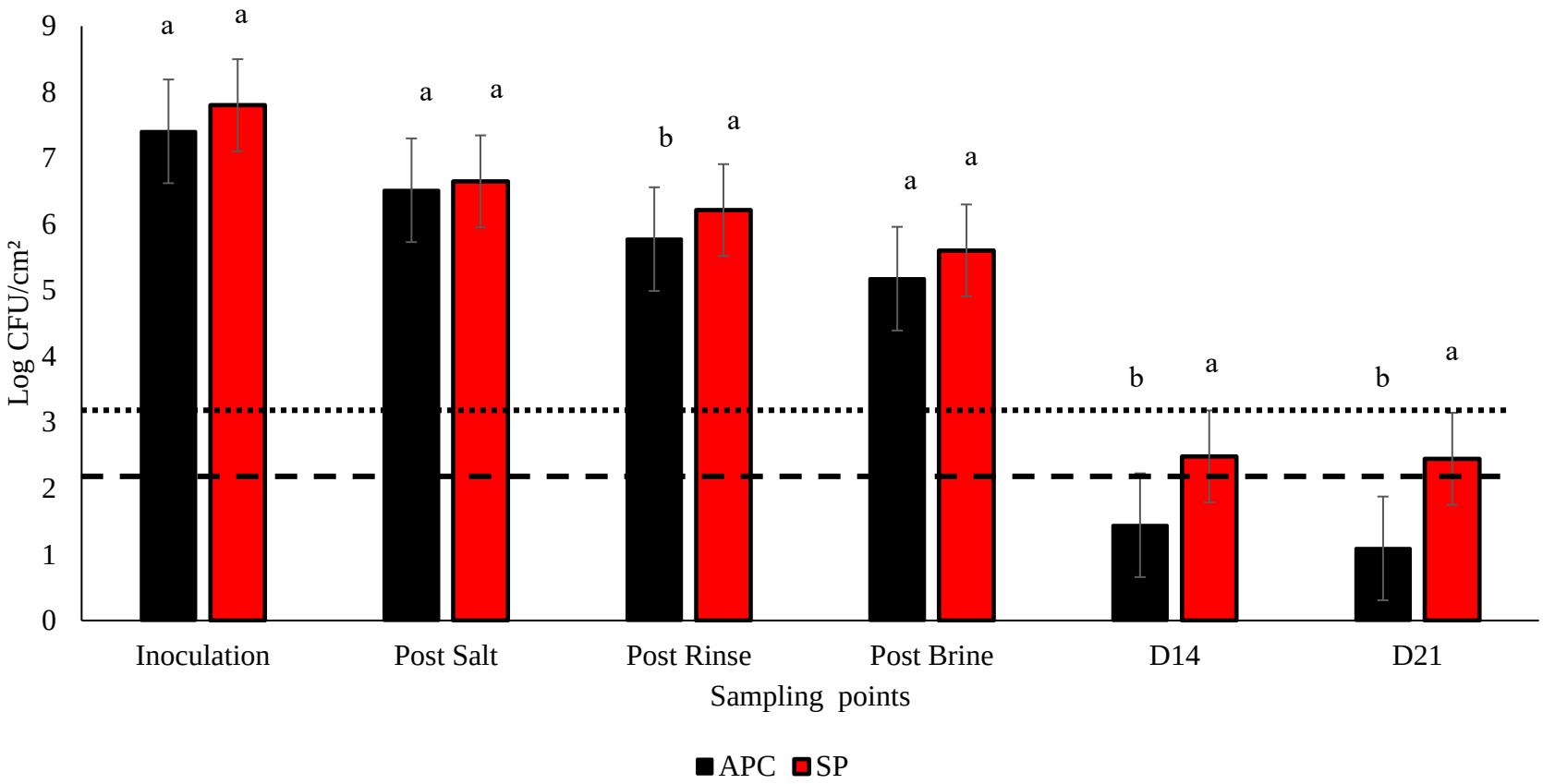

Figure 3. Experiment 1 aerobic plate count (**APC**) and spread plate (**SP**) surrogate *E. coli* populations from post inoculation through 21 days of drying to A_w 0.75. Horizontal lines refer to point of 5-log reduction from the inoculation level (--- SP;**- - -** APC). Means within sampling time periods without a common superscript differ ($P < 0.05$).

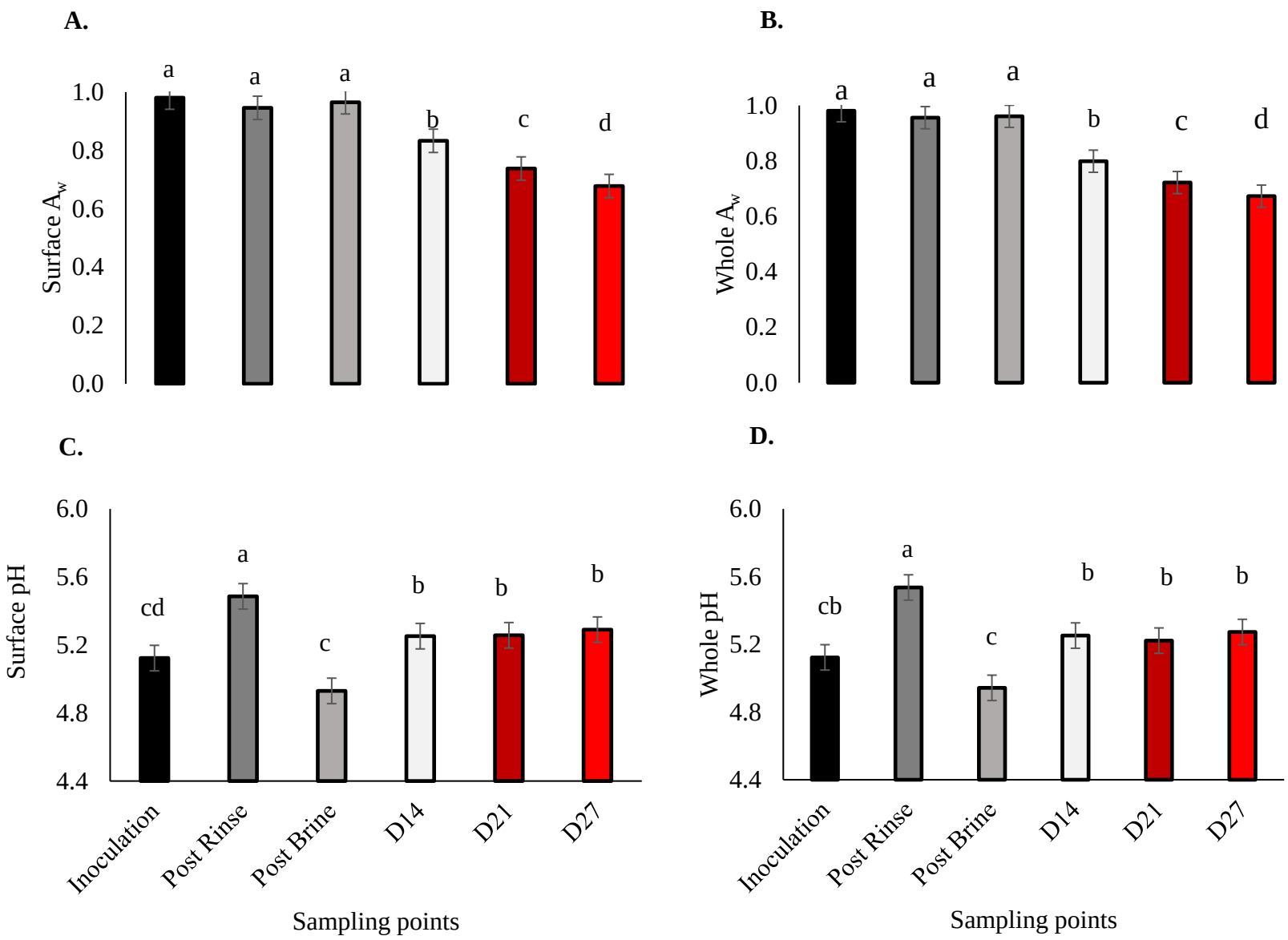
A.
Surface A_w
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
b
c
d
B.
Whole A_w
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
b
c
d
C.
Surface pH
6.0
5.6
5.2
4.8
4.4
cd
a
c
b
b
b
Inoculation
Post Rinse
Post Brine
D14
D21
D27
Sampling points
D.
Whole pH
6.0
5.6
5.2
4.8
4.4
cb
a
c
b
b
b
Inoculation
Post Rinse
Post Brine
D14
D21
D27
Sampling points

Figure 4A. Experiment 1 surface water activity (A_w) post inoculation, post salt, post rinse, post brine, d 14 drying, d 21 drying, and d 27 drying. Surface was a slice of just the surface approximately 4 mm thick. Bars without a common superscript differ ($P < 0.05$).

Figure 4B. Experiment 1 whole water activity (A_w) post inoculation, post salt, post rinse, post brine, d 14 drying, d 21 drying, and d 27 drying. Whole A_w was a slice of the entire biltong including both surfaces and the interior. Bars without a common superscript differ ($P < 0.05$).

Figure 4C. Experiment 1 surface pH post inoculation, post rinse, post brine, d 14 drying, d 21 drying, and d 27 drying. Surface was a slice of just the surface approximately 4 mm thick. Bars without a common superscript differ ($P < 0.05$).

Figure 4D. Experiment 1 whole pH post inoculation, post rinse, post brine, d 14 drying, d 21 drying, and d 27 drying. Whole A_w was a slice of the entire biltong including both surfaces and the interior. Bars without a common superscript differ ($P < 0.05$).

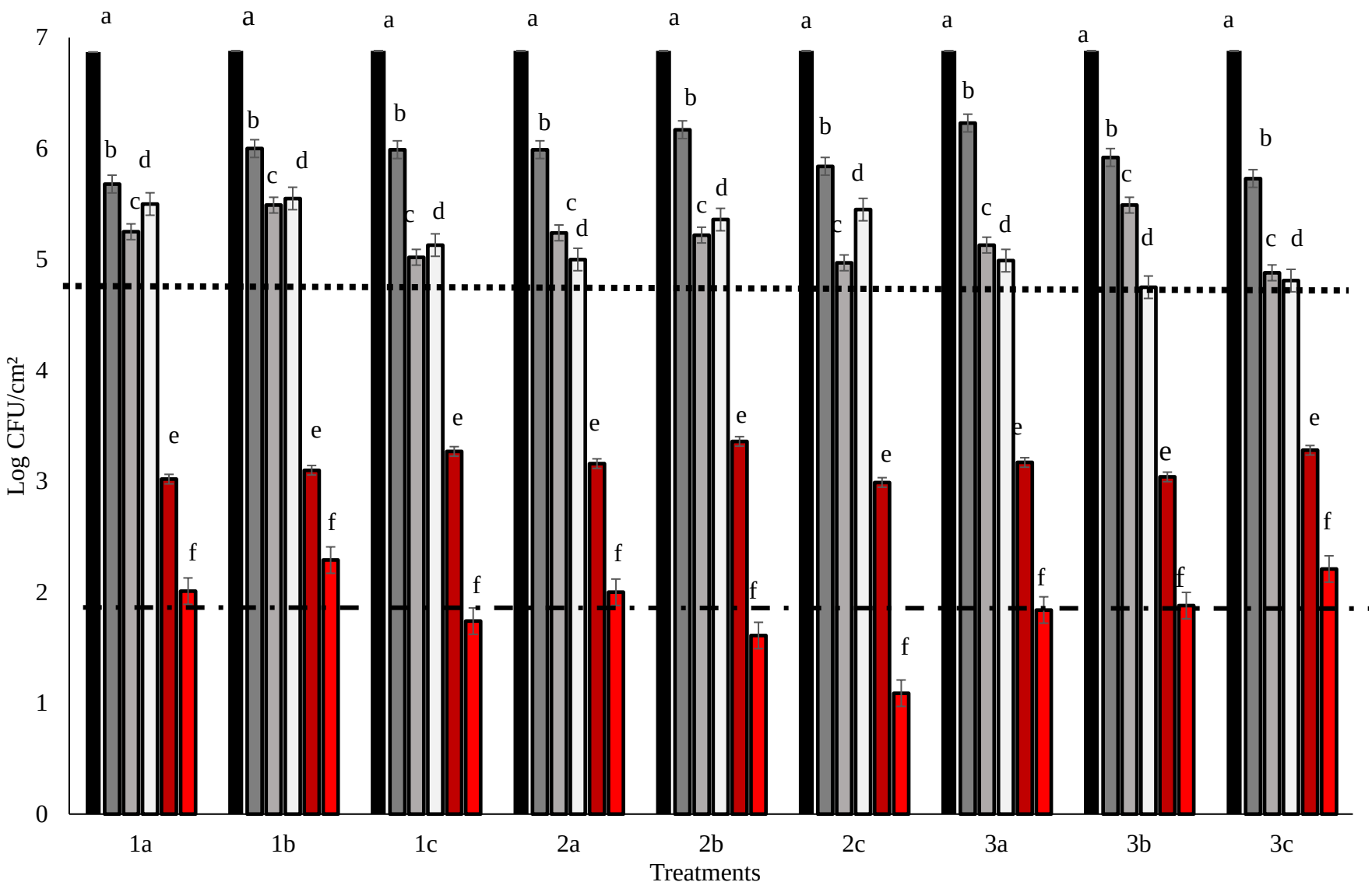
Log CFU/cm²
0
1
2
3
4
5
6
7
1a
1b
1c
2a
2b
2c
3a
3b
3c
Treatments
Inoculation
Post Salt
Post Rinse
Post Brine
Aw 0.80
Aw 0.75

Figure 5. Experiment 2 surrogate *E. coli* populations post inoculation, post salt, post rinse, post brine, 0.80 water activity (A_w), and 0.75 A_w. Treatments: 1 = 1.7%, 2 = 2.2%, and 3 = 2.7% salt (NaCl); a = 2%, b = 4%, and c = 6% vinegar. Salt and vinegar were included based on a percentage of individual slice weight. Horizontal lines refer to 2-log (·····) and 5-log (- · - · -) reduction in regard to inoculation level. Treatment by time interaction ($P = 0.94$), treatment main effect ($P = 0.35$), time main effect ($P < 0.01$). Superscripts show differences for effect of sampling time. Timepoints without a common superscript differ ($P < 0.05$).

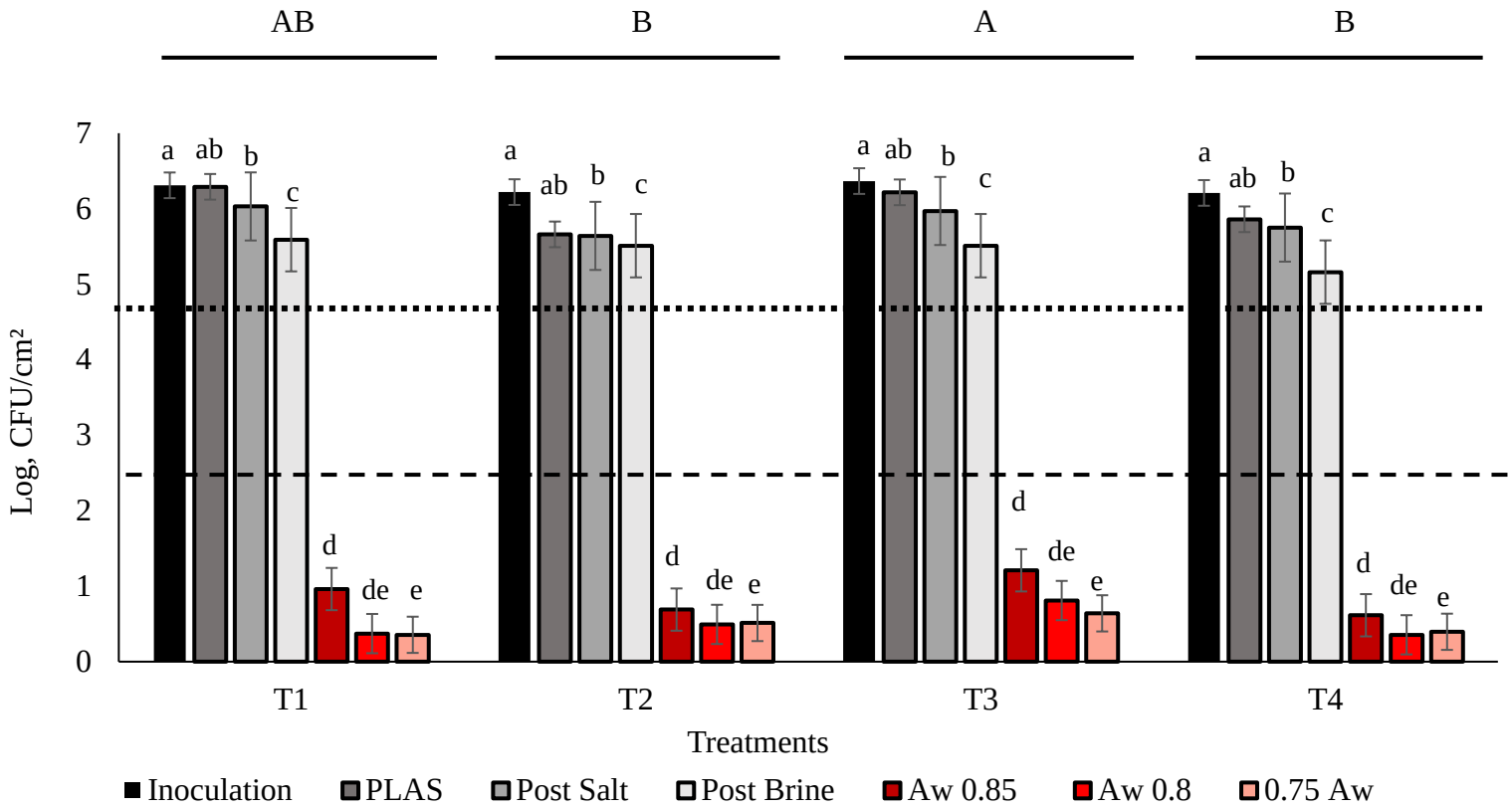

Figure 6. Experiment 3 surrogate *E. coli* populations by treatment and sampling time (inoculation, post lactic acid (PLAS), post salt, post brine, 0.85 water activity (A_w), 0.80 A_w, and 0.75 A_w. Treatments: T1: No lactic acid dip, 2% NaCl, 2.5% vinegar; T2: 3% lactic acid dip, 2% NaCl, 2.5% vinegar; T3: No lactic acid dip, 3.5% NaCl, 5% vinegar; T4: 3% lactic acid dip, 3.5% NaCl, 5% vinegar. Horizontal lines refer to 2-log (·····) and 5-log (-----) reduction in regard to inoculation level. Treatment by time interaction (P = 0.83),

treatment main effect ($P < 0.05$), time main effect ($P < 0.05$). Capital superscripts denote differences among treatments, lower case superscripts denote differences among sampling times, means without a common superscript differ ($P < 0.05$)

CHAPTER 4

CONCLUSIONS

This research shows that minimal levels of salt, vinegar, and lactic acid can achieve a five-log reduction in surrogate *E. coli* when combined with the hurdle of decreasing A_w. It was possible to achieve a five-log reduction both with increased levels of salt and vinegar with no lactic acid hurdle. It was also possible to use decreased levels of salt and vinegar, as long as a lactic acid dip was employed. While neither salt, vinegar, or lactic acid level had immediate impacts on the reduction of *E. coli*, there were long term effects that could have led to the death of the bacteria by the A_w of 0.85. This study demonstrated at least a 2-log reduction in all experiments using different processes, ingredients, and formulations, while using acid adapted cultures with decreased sensitivity to acidic environments. More work should be conducted with pathogenic E. coli 0157:H7 and other STEC strains for further validation.