MICROBIAL VALIDATION FOR BILTONG PROCESSING TO ACHIEVE 5-LOG REDUCTION OF *LISTERIA MONOCYTOGENES, E. COLI* O157:H7 AND *STAPHYLOCOCCUS AUREUS*.

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Abstract: **'**Biltong' is a South African style dried beef product that is marinaded and dried at moderate heat but still must accommodate USDA-FSIS safety concerns for process validation by demonstrating a 5-log pathogen reduction. Our objective was to achieve a 5-log reduction of *Listeria monocytogenes, E. coli* O157:H7 and *Staphylococcus aureus* without a heat lethality step and evaluate the individual contribution of marinade components (spice, salt, vinegar) to the overall reduction on inoculated beef. Beef was cut into small 'steaks' (0.75-in x 2-in x 3-in) and inoculated with a 4-serovar mixture of acid-adapted *L. monocytogenes*, *E. coli* O157:H7 or *S. aureus* and spread on the surface of the beef. Beef pieces were vacuum tumbled with either a mixture of spice, salt and vinegar, or just individual marinade components, and marinaded for 30 minutes. Beef was then dried in a temperature-controlled humidity oven for 10 days (75°F/23.9°C; 55% RH). All trials were performed in duplicate replication (with triplicate samples at each time point; n=6) and RM one-way ANOVA to determine significant differences ($p < 0.05$). The combination of spice, salt, and vinegar resulted in (>5-log) reduction for *L. monocytogenes* with drying period of 8 days. The greatest reduction of *L. monocytogenes* from individual marinade components was exhibited by vinegar (>5-log) followed by salt (4.68-log) and spice (4.1-log). Combined ingredients of marinade also resulted in reduction of (>5-log) with drying period of 4 days for *E. coli* O157:H7. Salt and vinegar when treated individually lead to similar reduction of (5.48-log) and (5.49-log) followed by spice (4.11-log) for *E. coli* O157:H7. *S. aureus* achieved (>5-log) reduction using combined components of the marinade after 8 days of drying and staphylococcal enterotoxin (SEA, SEB) was not produced in the meat through 10 days of drying. Water activity for 10-days of drying was 0.79. This is the first published work achieving (>5-log) reduction of *L. monocytogenes, E. coli* O157:H7 and *S. aureus* with biltong, thereby validating this process for USDA-FSIS approval that is sought by many upscale food stores to ensure product safety.

Keywords: Biltong, 5-log reduction, *Listeria monocytogenes, E. coli* O157:H7, *Staphylococcus aureus,* staphylococcal enterotoxin.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER I

INTRODUCTION

Meat products that are cured, fermented, and dried are considered a microbial safe product because of low pH, low water activity and the presence of salt (Mhlambi, Naidoo, & Lindsay, 2010). Biltong is a popular choice for consumers as a dried and ready to eat meat product common to South Africa. It has also expanded in many countries namely the United States, Australia, the United Kingdom, and Portugal. Generally, raw meat is prone to contamination of microorganisms from to the source (live animals) and potential for environmental contamination during slaughter and post-slaughter fabrication. Biltong beef has high nutritional quality and protein content. Therefore, the processing of biltong beef is crucial to safety as the product is dried but not cooked (Matsheka et al., 2014).

Traditionally, biltong is cut into lean strips and marinated with spices that include coriander and black pepper, salt, and vinegar (50 grain or 100 grain red wine vinegar) followed by drying the beef pieces at an ambient temperature (23.9 °C / 75°F) and humidity (~55% RH) for 4-10 days (C. E. Karolenko et al., 2020). Addition of salt that acts as a curing agent helps in preservation of the meat by binding to water, drawing it out of the meat, and influences the efficacy of drying period

(Jones, Arnaud, Gouws, & Hoffman, 2017). Microbial growth is inhibited by vinegar that helps in maintaining low pH (during marination; afterwards low pH is equilibrated by the beef protein), and a level of humidity contributes to low water activity. Production of biltong as a home industry is a major concern associated with the safety of the product. Pathogens like *Listeria monocytogenes, E. coli* O157:H7 and *Staphylococcus aureus* are recognized as a potential microbiological hazards in biltong (Burnham, Hanson, Koshick, & Ingham, 2008).

Similar dried beef products, such as production of American-styled beef jerky involves an elevated heat treatment to obtain a recommended >5-log reduction of pathogen, which makes processing different from biltong. Beef jerky utilizes techniques such as heating, curing, smoking, and drying to obtain a safe product while pathogen reduction for biltong is dependent on the components of the marinade and drying at an ambient temperature at low humidity due to the absence of heat lethality step. Manufacturing of beef jerky in the United States follows United States Department of Agricultural Food Safety and Inspection Service (USDA-FSIS) compliance guidelines. The guidelines state that the relative humidity should be maintained at 90% or above during the production of the beef product by using sealed oven or uninterrupted steam injection (USDA-FSIS, 2014)

Both, biltong, and beef jerky processes must involve sufficient pathogen reduction and obtain microbial safe product. One must also validate the process to demonstrate acceptable pathogen reduction. Because there is no heat lethality step involved with biltong processing, the USDA-FSIS has allowed two alternative processes for achieving a validated process for biltong. In one alternative process, each lot of ingredients must be tested to verify the absence of a 'pathogen of concern' (i.e., *Salmonella*) before use and

demonstrate a >2-log reduction of pathogen by the process. In a second alternative process, if the entire process achieves >5-log reduction, then the process lethality is sufficient enough that pathogen testing of ingredients for the pathogen of concern is not required. This latter >5-log reduction process is the preferred method because pathogen testing of ingredients is cumbersome and expensive and if one forgets to test an ingredient it can lead to the recall of the whole process.

Note: USDA-FSIS does not like to use the wording that "a 5-log reduction is required", they prefer 'it is recommended' because the other alternative process that is available does not require a 5-log reduction. Therefore, a biltong manufacturer is not 'required' to demonstrate a 5-log reduction (i.e., they can use the 2-log process + pathogen testing).

The first published biltong process to achieve >5-log reduction of *Salmonella* without a heat lethality step was recently demonstrated (C. E. Karolenko, Bhusal, Nelson, & Muriana, 2020). For manufacturers of biltong, the USDA-FSIS just needs to have a process validated with a single pathogen of concern (i.e., *Salmonella*). However, numerous retail/supermarket clients of those processors have indicated that they want additional validation with other likely meat-associated pathogens. Therefore, this study was done to provide additional validation with *Listeria monocytogenes*, *E. coli* O157:H7 and *Staphylococcus aureus* for the biltong process that was previously described to provide >5-log reduction of *Salmonella*.

CHAPTER II

REVIEW OF LITERATURE

Prevalence of Pathogens on Raw Beef

E. coli O157:H7

E. coli O157:H7 is a rod-shaped, Gram-negative and facultatively anaerobic bacterium (Ali, Eldin, Moghazy, Tork, & Omara, 2014). The organism is known as one of the important causes of foodborne disease. Eating undercooked ground beef and drinking raw milk have been contributing reasons for outbreaks with this organism. An important reservoir of *E. coli* O157:H7 is the intestinal tract of cattle and often young dairy cattle have been a primary source of *E. coli* O157:H7 leading to foodborne infection if meat derived from this is contaminated and consumed raw or with insufficient heating. Different control measures like heating the food product prior to consumption or following good manufacturing practices during processing of foods of animal origin help to prevent infections caused by the organism (Doyle, 1991). *E. coli* O157:H7 is responsible for causing hemorrhagic colitis and hemolytic uremic syndrome by producing shigatoxin, which is also known as verocytotoxin (Wells et al., 1991). Cattle have been noted as one of the primary reservoirs of *E. coli* O157:H7,

where it may be carried on the hide or be present in the rumen from which it can be excreted in the feces. The presence of the organism on the hide is due to different sources of contamination that includes feces from other animals, soil, and lairage.(Kennedy Jr, Williams, Brown, & Minerich, 2006). Hiko et al. (2008) in their study looked for the prevalence of *E. coli* O157:H7 in retail raw meat products of Ethiopia that includes beef, sheep, and goat. Among all the products, beef was more commonly contaminated with *E. coli* O157:H7 (8%) than sheep or goat meat (2-2.5%) (Hiko, Asrat, & Zewde, 2008). Comparing the prevalence of *E. coli* O157:H7 on raw beef in the slaughter-house and retail shops, higher percentage of occurrence of the organism was observed in retails shops (14.6%) followed by the slaughter-house (6.3%) (Bekele, Zewde, Tefera, Feleke, & Zerom, 2014). For the raw beef samples studied from the markets of South China, high rate of prevalence of *E. coli* O157:H7 was found to be present (13.32%) among the 68 samples collected (Zhang et al., 2015). Prevalence of *E. coli* O157:H7 was determined on the ground beef samples in Seattle, USA with a total of 1750 samples of ground beef were tested for *E. coli* O157:H7. Among 1750 samples, 20 samples (1.1%) tested positive for *E. coli* O157:H7 (Samadpour et al., 2006) High rate of contamination with *E. coli* O157:H7 on beef have been reported from different parts of the world; 31% in Canada, 14.7% - 22.5% in Peru and 90% in United States (Hiko, Asrat, & Zewde, 2008).

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive, non-spore forming, facultative psychrotroph present in soil, ground water, plants, and on vegetation. The organism is isolated from animals such as sheep, cattle, goat and poultry, humans, food products like (Ready-to-eat meats and dairy products) and food processing plants including slaughter and smoke

houses (Letchumanan et al., 2018). In humans, the organism causes listeriosis that preferentially effects pregnant women, infants, the elderly and the immunocompromised (Graves et al., 2005). *Listeria monocytogenes* has a high tolerance to salt concentrations (up to 10% NaCl) (Letchumanan et al., 2018) and can survive but not grow at pH < 4.3 and A_w<0.93 (Islam, Husna, Islam, & Khatun, 2016). Markedly, the prevalence of the organism in the food environment may not be proportional to the organism in the food product. (Robert L Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017). The entry of *L. monocytogenes* in the food chain is easy as it can remain in the environment for longer period of time due to its ability to survive harsh conditions (Islam et al., 2016). According to studies done by Islam et al. (2006), with 12 raw beef samples for the prevalence of *L. monocytogenes* in Bangladesh, the percentage of prevalence of the organism was found to be 16.66 % and had high contamination rate when compared to the studies done for the presence of the organism on chicken and chevon (Islam et al., 2016). In another study, the incidence of *L. monocytogenes* was studied from the domestic and imported foods in Korea with a total of 1537 samples were examined, and 122 samples (7.9%) were contaminated with *L. monocytogenes*. The rate of contamination for beef was (4.3%), pork (19.1 %) and chicken (30.2%). However, the beef had low rate of contamination when compared to that of other countries like Germany (45.8%), United states (58%) and Japan (37.2%)(Baek, Lim, Lee, Min, & Kim, 2000). The incidence of *L. monocytogenes* contamination in beef, pork, and chicken was examined in five markets of Makurdi metropolis, Nigeria. A total of 93 raw beef samples were examined for the presence of *L. monocytogenes* and the rate of contamination was 6.5% (Peter, Umeh, Azua, & Obande, 2016). A total of 512 ground beef samples were tested in Seattle, USA for the prevalence of *L. monocytogenes* and 18

samples (3.5%) were positive for *L. monocytogenes* (Samadpour et al., 2006) .

Staphylococcus aureus

Staphylococcus aureus is a Gram-positive bacterium often present in the nasal passages of animals and humans. The organism gets involved in the various human opportunistic infections like urinary tract infection, pneumonia, toxic shock, and heart valve infections. The main root of infection is through blood stream or direct contact (Adzitey, Ekli, & Abu, 2019). Some strains produce toxic shock syndrome, staphylococcal enterotoxins (SEs) such as SEA through SEI except SEF, exfoliate toxins like ETA and ETB which affects the immune system causing gastro intestinal problems along with biological effects leading to discomposure to humans (Adzitey et al., 2019; Dinges, Orwin, & Schlievert, 2000). Adzitey et al. (2009) tested 18 raw beef samples in Ghana for the presence of *Staphylococcus aureus* and recorded 0 % prevalence of the organism (Adzitey et al., 2019). The retail raw meat in Nigeria was examined by considering 75 raw beef samples and found the presence of *Staphylococcus aureus* on 21 samples with a high isolate rate of 28% due to insufficient sterilization of work station and utensils along with poor work practices in the processing stage by meat handlers (Adesiji, Alli, Adekanle, & Jolayemi, 2011). Local and imported beef meat was examined in Egypt and high rate of prevalence was recorded from local beef meat (33%) when compared to imported beef meat (13 %). The difference in the prevalence rate includes factors like geographic locations, methodologies used that include sampling season, sample type, sample size, sampling techniques, packaging, and handling practices (Osman, Amer, Badr, & Saad, 2015).

Conditions in Dried Beef as Inhibitors for Pathogens

Salt

Addition of salt has been useful in dried meat processing such as biltong; it enhances safety and quality of the product. Salt added during the processing of meat to generate low moisture levels builds a selective pressure that inhibits Gram-negative bacteria except for a few xerotolerant Gram-positive bacteria, yeast, and mold that may survive and proliferate (Gurtler, Doyle, & Kornacki, 2014). NaCl is considered an effective agent for food preservation against foodborne and spoilage microorganisms; it has a high impact on the microbial safety and quality of foods. Terms such as, 'salt tolerant', 'salt resistant', 'facultative halophile' or 'obligate halophile' are used to describe the degree of microbial tolerances to salt. Foodborne pathogens such as *L. monocytogenes* are 'salt tolerant' and *S. aureus* are 'salt resistant'(Taormina, 2010). Salting modifies the myofibrillar protein solubility and swelling of muscle fibers that leads to changes in muscle protein and its texture. (Jones et al., 2017). Addition of salt for the processed, dried meats helps to impart flavor and preserve the meat. Salt draws the water out from the meat and decreases the water activity and limits the water for microbial growth (Taormina & Sofos, 2014).

Low water activity

Water activity is considered a better indicator of microbial stability when compared to moisture content. Water activity is defined as "the vapor pressure of a food divided by the vapor pressure of the pure water at the same temperature, or the equivalent relative humidity/100". It is considered the 'free, available water' for microbial growth whereas moisture content includes 'bound water' that is not available for microbial growth. The

presence of sodium chloride influences the water activity as it binds water. Low levels of A_w lead to the inhibition of microbes (inhibition of growth) which is related to the turgor pressure in a cell caused by the water activity of the internal environment and the water activity in the external environment (Gurtler et al., 2014). Below certain water activity values, microorganisms do not grow and the water required for microorganisms should be defined in terms of water activity (Scott, 1957). Generally, bacteria, yeast, and molds require a minimum water activity of 0.90, 0.87, and 0.80 respectively for growth (Troller, 1977). Different ranges of A_w are required and specified for growth of different organisms. This range includes maximal, optimal, and minimal levels of water activity (Scott, 1957). Most bacteria can grow quickly at A_w 0.99, however, as water activity falls, bacterial growth decreases and below 0.6 the food does not support microbial growth (Corry, 1973).

Low pH

The growth of microorganisms is generally affected by the pH of the food/growth medium, and this is similar for dried beef. The addition of vinegar alters the pH of the medium by imparting an antibacterial effect, which is due to acetic acid, polyphenol, and melanoidin content. Using organic acids can result in destruction of microbes such as *L. monocytogenes*, *E. coli* O157:H7, *Salmonella.* (Jones et al., 2017). Greater levels of inactivation of microorganisms is caused by using acidified marinades during drying when compared to non-acid marinades (Nummer et al., 2004). Hence, the pH of the meat is altered by adding vinegar that denatures the surface of meat pieces (Jones et al., 2017). Different types of vinegar such as brown spirit vinegar or apple cider vinegar are generally used in the production of dried beef like biltong (Jones et al., 2017).

Processing of Beef Jerky

Beef jerky is a cooked and dried meat product that involves heating of the product to obtain a preferred dryness, texture, and a self-stable product (Buege, Searls, & Ingham, 2006). Jerky has been identified as the oldest dried meat product preferred as a snack food in North America by consumers (Calicioglu, Sofos, Samelis, Kendall, & Smith, 2003). Earlier techniques used for processing beef jerky would involve sufficient lethality by boiling beef strips in the marinade before cooking and drying and heating the strips after drying using an oven. But now the above-mentioned method is not followed because of the detrimental effects on the sensory characteristics to the product. To develop validated guidelines for heating and drying of beef jerky various variables have been considered like the thickness of the beef jerky strips, components of the marinade, conditions of marination, heating and drying conditions in oven or smokehouse (Buege et al., 2006).

Beef jerky mainly includes heat lethality step that is important to achieve the recommended 5-log reduction of the foodborne pathogen of concern, such as *salmonella*. Under the guidelines of USDA-FSIS, beef jerky is manufactured with the relative humidity of 90% or above by using sealed oven or steam that is continuously injected (USDA-FSIS, 2014).

It is preserved by using techniques such as salting and drying. And it is a shelf stable product due to low moisture protein ratio (0.75:1) and rich in nutrients due to the presence of high protein levels and iron along with low fat content. The low water activity (< 0.70) makes it microbiologically safe product (Calicioglu et al., 2003). Processing of beef jerky mainly includes techniques like heating, curing, smoking, drying and does not rely on the

usage of vinegar for obtaining a microbial safe product (Carr et al., 1997).

Studies done on processing of beef jerky includes approaches like (a) boiling the marinated meat followed by drying; (b) marination and heating at high temperature followed by drying; (c) acid dip treatment followed by marination and drying (Harrison, Harrison, Rose-Morrow, & Shewfelt, 2001); (d) boiling of the meat followed by marination and drying (Calicioglu, Sofos, Samelis, Kendall, & Smith, 2002). The recipes used are dependent on the thickness of the meat, marination techniques, temperature, time, type of ingredients used, and type of drying process used (for example, oven or food dehydrator, high or low drying temperature) (Calicioglu et al., 2003).

Some different forms of jerky are made using whole muscle and constructed from ground meat. Beef strips are soaked in liquid marinade for the whole muscle jerky and before drying or dipping the meat in 5% acetic acid solution, acidified sodium chlorite or calcium sulfate the beef strips are pre-heated in the marinade to 71.1 °C. On the other hand, ground and formed jerky is combined with dried spices and the batter is shaped into strips. The meat after seasoning is dehydrated for both whole muscle and ground and formed beef jerky to make it a shelf stable product. According to FSIS guidelines, the shelf stability of the product mainly depends on sufficient lethality and usage of water activity over moisture to protein ratio as a measure of shelf stability.

However, USDA requires a jerky manufacturing process to achieve a 5-log reduction in *Salmonella* for adequate lethality (Borowski, Ingham, & Ingham, 2009). According to studies done by Calicioglu et al. (2002) different marinades are used to treat beef jerky for inactivation of *E. coli* O157:H7. Beef slices are treated with (a) traditional marinade

at pH 4.3, (b) modified marinade (pH 3), (c) dipping the beef slices in the 5% acetic acid solution (pH 2.5) for 10 minutes followed by traditional marinade, (d) dipping the meat slices in 1% Tween 20 solution (pH 6.6) for 15 minutes followed by 5% acetic acid solution for 10 minutes and marination with traditional marinade. Traditional marinade was prepared for 1 kg of meat included soy sauce (60 ml), Worcestershire sauce (15 ml), black pepper (0.6 g), garlic powder (1.25 g), onion powder (1.5g) and hickory smoked salt (4.35 g). The modified marinade was prepared for 1 kg of meat included milder soy sauce (120 ml) with ethanol as a preservative up to 4.7-5%, Worcestershire sauce (30 ml), black pepper (0.6 g), garlic powder (1.25 g), onion powder (1.5g), hickory smoked salt (4.35 g), food grade sodium-L-lactate of 60 % preparation (3.6 ml) and the pH was adjusted to 3 using 16 ml of glacial acetic acid. The meat pieces were refrigerated at 4 °C for 24 hours and dried at 60 °C for 10 hours in the dehydrators (Calicioglu et al., 2002).

Processing of Biltong

Biltong is an uncooked, ready-to-eat, dried meat product of South Africa (Dzimba, José de Assis, & Walter, 2007). It has been produced for many years in South Africa and has gained popularity both locally and internationally (Jones et al., 2017). The processing of biltong is unique and peculiar because of using different formulations and methods of preparation, which have gained high demand with expanding economic value. The product is a popular healthy snack with high protein and low carbohydrate content, and flavored with spices and salt. Additional ingredients such as sugar, nitrite, and saltpetre may be added which gives a red color to the product (Dzimba et al., 2007).

The general flow of the production process includes a series of steps that includes meat

preparation using fresh or thawed meat, salting, spicing, and drying (Jones et al., 2017). Some processors may rinse the beef rounds before or after trimming. Biltong beef is generally cut into long strips by hand or by using machines that have rotating circular blades following the general dimensions with a width/thickness of 2.5-5 cm and a length of 25-40 cm. However, the excess fat present on the meat is trimmed away because fat will generally take more time for the beef to dry and may cause rancidity. Some biltong processes store biltong in marinade at a cold temperature (4-8°C) for 6-12 hours and then the strips are flipped and stored for another 6-12 hours before drying (Van Tonder & Van Heerden, 1992). Other processes have a shorter time in the marinade (30 min to several hours). While mixing biltong with salt, spice, and vinegar different methods are used. For example, the meat pieces are dipped in the spices followed by vinegar or dipped in the vinegar and drained to remove the excess liquid and then dipped into the dry spices. Biltong is spiced using coriander as a prominent spice and black pepper. Around 2.5-4% of salt is added to the biltong that gives an acceptable taste with a wide range of moisture content but up to 4 % of the salt content can be used that helps in preservation of the meat and increases the shelf life of the product by decreasing the water activity and making it less available for the growth of microbes (Jones et al., 2017). Vinegar is added at a commercial level with some studies showing the approximate levels of addition up to 2% of (50- or 100 grain) red wine vinegar (Caitlin E Karolenko, Arjun Bhusal, Jacob L Nelson, et al., 2020), 3% (Van den Heever, 1970) and 6% of vinegar (Naidoo & Lindsay, 2010) .

Hence, biltong is recognized as a microbial safe product due to the usage of vinegar (helps to maintain low pH of 5.5), salt (binds to water) and drying leads to low A_w (0.77) due to

the maintenance of low humidity which inhibits the growth of microbes (Mhlambi et al., 2010). Various recipes for manufacturing biltong include beef at up to 82%–96% of total formulation, and addition of vinegar (2%-11%), salt (1.5%–8%), coriander (0.3%–2%), pepper (0.1%–1%). A few recipes may also add brown sugar (0.8%–6%), and bicarbonate (0.2%–1%) (Caitlin E Karolenko, Arjun Bhusal, Jacob L Nelson, et al., 2020). During large scale manufacturing of biltong, tumbling helps in mixing of the ingredients specially improves the meat salting (Mirade et al., 2020) that facilitate the salt to diffuse into the meat which makes it juicy and tender (Jones et al., 2017). Karolenko et al. (2020) in her studies marinated the beef pieces with spice, salt, and vinegar in a stainless steel vacuum chamber evacuated to 15 inches Hg for 30 minutes (Caitlin E Karolenko, Arjun Bhusal, Jacob L Nelson, et al., 2020). By tumbling, large quantities of meat can be handled easily, promoting uniform distribution of ingredients in less time, leading to the production of high yield of uniform product (Mirade et al., 2020; Jones et al., 2017). Traditionally, biltong is dried under ambient conditions for one or two weeks. Currently, commercial biltong is dried using various drying equipment with controlled humidity and temperature chamber units at a commercial level (Jones et al., 2017). Karolenko et al. (2020) in her studies has dried the beef samples in a temperature controlled humidity oven for 8-10 days at 23.9 °C (75 °F) and 55% RH (C. Karolenko & Muriana, 2020).

USDA Regulations for Dried Beef

Specific regulations for beef jerky processing are provided by USDA-FSIS Compliance guideline that deals with critical steps for jerky processing and the controls required at each step to ensure the product produced is safe. Since beef jerky is cooked, the lethality treatment followed by drying should be followed to manufacture a safe product.

According to the guidelines, use of antimicrobial interventions before, during and after marination of the raw meat helps in increasing the level of pathogen reduction apart from heating alone for beef jerky. Including the heat lethality step during the processing of beef jerky leads to the destruction of pathogens present on or in the product making it safe for consumption(USDA-FSIS, 2014).

During the production of beef jerky, the relative humidity should be maintained at 90% or above by using the sealed oven or continuously injected steam (USDA-FSIS, 2014). Since biltong does not involve high heat and high humidity step in the processing, the processor must demonstrate to USDA-FISIS a sufficient pathogen lethality. To achieve USDA-FSIS process validation for biltong there are two options available. One option is to perform testing of every lot of edible ingredients to ensure the absence of pathogen and demonstrate a 2-log reduction of pathogen of interest developed by the 'Blue Ribbon Task Force' circa 1996 for *E. coli* O157:H7 (Nickelson, 1996). Another option is to demonstrate a 5-log reduction of pathogen by the process. Note: The 5-log reduction of pathogen is not considered as a 'requirement' by USDA-DSIS because there is an alternative 2-log process that can be performed along with pathogen testing of ingredients. According to (FSIS) jerky is dried to a Moisture-protein-ratio (MPR) of 0.75:1 or below. The values obtained for MPR indicate the degree of product drying and they do not necessarily indicate the product stability or microbial safety because they do not consider availability of the water. A shelf stable product is obtained with a low water activity, which also addresses the mold growth. USDA-FSIS require the water activity (A_w) of 0.85 or less for the products stored in the aerobic conditions that helps in preventing the mold growth. If the product is exposed to an-aerobic conditions like vacuum package, the

A_w critical limit can be 0.91 or low. Low water activity levels prevent the growth of microorganism and enterotoxin formation if *Staphylococcus aureus* is present as it can tolerate low water activity. Certain processing aids are used to improve the pathogen reduction before or after trimming the beef.

In the United States, the addition of vinegar to an equal level of acetic acid does not make vinegar and acetic acid as equivalent ingredients. Acetic acid is considered as a processing aid when applied on the meat products and is regulated according to USDA-FSIS 'Safe and Suitable Ingredients List' for meat and poultry (USDA-FSIS, 2018). The federal labeling requirements have defined the processing aid as "substances that are added to a food for their technical or functional effect in the processing but are present in the finished food at insignificant levels and do not have any technical or functional effect in that food" (Post et al., 2007) and does not required to be labelled. Acetic acid being considered as a processing aid has restricted use of levels and time of contact. Use of acetic acid is limited to a concentration of (< 5%) and treatment time of (30-60 seconds). The processing aid renders and affects during the processing and do not affect the final product. If the processing aid is used beyond the restricted time and concentration limits, it might cause a residual effect on the product, and it will no longer be considered as a processing aid but will be considered as an ingredient. On the contrary, use of vinegar is covered by US–FDA regulations (Administration, 1995). Vinegar is a Generally Recognized as a Safe substance (GRAS) with no restriction of treatment on time or concentration limit. However, when vinegar is used in the process, it must be mentioned on the ingredient label, and one must specify the type of vinegar used.

CHAPTER III

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, Phenotypic Confirmation, and Antibiotic Resistance

Bacterial cultures used in this study included 4 strains each of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Staphylococcus aureus* (Table 1). Strains of *L. monocytogenes* included Scott A-2 (serotype 4b), V7-2 (serotype 1/2a), 39-2 (retail hotdog isolate), and 383-2 (ground beef isolate) (Muriana, Quimby, Davidson, & Grooms, 2002). These strains are resistant to streptomycin (100 ug/ml; Sigma-Aldrich, St. Louis, Mo.) and rifamycin S/V (10 ug/ml; Sigma-Aldrich) and were plated on tryptic soy agar (TSA; Difco, Becton Dickenson, Sparks, MD) containing these antibiotics for enumeration of inoculated cultures recovered from biltong beef.

Strains of *E. coli* O157:H7 included ATCC 35150, ATCC 43894, ATCC 43889, and ATCC 45756. These strains are all resistant to 5 µg/ml novobiocin and 2.5 ug/ml rifamycin S/V (Sigma-Aldrich) and enumeration of these strains from inoculated were conducted on TSA containing these antibiotics.

Strains of *S. aureus* and the staphylococcal enterotoxins (SE's) they produce included

ATCC 8095 (SEA), ATCC 13565 (SEA), ATCC 14458 (SEB), and ATCC 51740 (SEB). These strains are resistant to clindamycin (5 ug/ml; Sigma-Aldrich) and were plated on TSA containing this antibiotic.

Bacterial cultures were grown in tryptic soy broth (TSB, BD Bacto BD211825, Franklin Lakes, NJ, USA) in 9 mL tubes at 30 ºC (*L. monocytogenes*) or 37 ºC (*E. coli*, *S. aureus*). Cultures were maintained for storage by centrifugation (6000× g, 5 °C) of 9 mL of fresh, overnight cultures and cell pellets were resuspended in 2–3 mL of fresh sterile TSB containing 10% glycerol. Cell suspensions were placed into glass vials and stored in an ultra-low freezer (−80 °C). Frozen stocks were revived by transferring 100 µL of the thawed cell suspension into 9 mL of TSB, incubating overnight at 37 °C, and sub-cultured twice before use. Microbial enumerations were carried out by making 10-fold dilutions in 0.1% BPW and plating in duplicate on tryptic soy agar (TSA, BD Bacto; 1.5% agar).

Table 1. List of strains used as challenge organisms for biltong processing in this study.

Organism	Strain Designation	Culture Collection Designation	Source/Reference
L. monocytogenes	Scott A-2	PMM 264	Clinical isolate
L. monocytogenes	V7-2	PMM 266	Clinical isolate
L. monocytogenes	39-2	PMM 39	Retail hotdogs
L. monocytogenes	383-2	PMM 383	Retail ground beef
E. coli O157:H7	ATCC 35150	PMM 407	Human feces
E. coli O157:H7	ATCC 43889	PMM 1111	Human feces
E. coli O157:H7	ATCC 43894	PMM 405	Human feces
E. coli O157:H7	ATCC 45756	PMM 715	JB Luchansky, USDA-ARS
S. aureus (SEA)	ATCC 8095	PMM 323	JB Luchansky, USDA-ARS
S. aureus (SEA)	ATCC 13565	PMM 318	Ham, enterotoxin illness
S. aureus (SEB)	ATCC 14458	PMM 319	Human feces, diarrhea
S. aureus (SEB)	ATCC 51740	PMM 678	Margarine

Bacterial cultures used for inoculation of biltong beef were 'acid adapted' by growing them in media augmented with glucose prior to use according to Wilde et al. (Wilde, Jørgensen, Campbell, Rowbury, & Humphrey, 2000) and as modified by Karolenko et al. (Caitlin E Karolenko, Arjun Bhusal, Dhiraj Gautam, & Peter M Muriana, 2020). Individual bacterial cultures were first propagated overnight at 37 °C (*E. coli, S. aureus*) or 30 °C (*L. monocytogenes*) in 9 mL TSB (BD Bacto BD286220). These cultures were individually used to re-inoculate 250 mL centrifuge bottles containing 200 mL pre-warmed TSB containing 1% glucose (BD Bacto BD286220 + 1% glucose) which were again incubated overnight (at 37 °C or 30 °C) for approximately 18 hrs. Individual cultures in 250 mL bottles were harvested by centrifugation, resuspended with 0.1% buffered peptone water (BPW, BD Difco), mixed in equal proportions, and held refrigerated (5 °C) or on ice until use (usually within 1-2 hours).

Acid adaptation and the effects on media pH change by growth in dextrose was examined for *L. monocytogenes, E. coli* O157:H7, and *S. aureus* cultures by growing the strains in three different TSB media: TSB containing 0% glucose (BD Bacto, BD286220), 0.25% glucose (BD Bacto, BD211825), and 1% glucose (BD286220 + 1% glucose). Cultures were individually grown in triplicate replication in TSB containing 0% glucose and incubated at respective temperatures: *L. monocytogenes* at 30 °C, and *E. coli* O157:H7 and *S. aureus* at 37 °C. After initial growth in TSB media containing 0% glucose, cultures were then further re-inoculated into TSB with different replication sets and with TSB containing 0%, 0.25%, and 1.0% glucose and again incubated at the appropriate temperatures (*L. monocytogenes* at 30 °C; *E. coli* O157:H7 and *S. aureus* at 37 °C) and pH levels were recorded after 18 hrs using a pH meter.

The pathogenic strains used in this study were further confirmed by demonstrating typical phenotypic biochemical reactions on selective and differential agars. Strains of *L. monocytogenes* were examined by plating on Modified Oxford Agar (MOX, Difco, BD). Cultures were serially diluted in 0.1% BPW and plated on MOX agar medium followed by incubation at 30ºC for 24 hours. Similarly, strains of *E. coli* O157:H7 were examined by plating on CHROMagar O157 (DRG International, Springfield, NJ). Cultures were grown in tryptic soy broth at 37ºC for 24 hours. Cultures were serially diluted in 0.1% BPW and plated on CHROMagar O157 agar medium followed by incubation at 37ºC for 24 hours. Similarly, strains of *S. aureus* used in this study were examined after plating on Mannitol Salt Agar (Difco-BBL, BD Laboratories, Franklin Lakes, NJ). Cultures were grown in tryptic soy broth at 37ºC for 24 hours, serially diluted in 0.1% BPW and plated on mannitol salt agar medium followed by incubation at 37ºC for 24 hours. The various strains on respective selective and differential media were examined for typical colony morphology and biochemical reaction to the media.

The pathogenic strains used in this study were also known to have various antibiotic resistances. Select colonies chosen from the selective and differential agars were streaked on TSA containing the antibiotics to which they were resistant. Furthermore, to ensure proper enumeration on TSA-antibiotic selective media, each strain was plated on TSA with and without antibiotics and incubated at the appropriate temperatures specified earlier. Comparison of enumerations on both media would determine whether the levels of antibiotics used would be non-inhibitory to the various strains and appropriate for enumeration.

Beef Handling, Fabrication, and Inoculation

Boneless beef bottom round (outside round, flat; select grade or ungraded) was purchased from a broker through a local meat processor (Ralph's, Perkins, OK). The original sources of beef included Greater Omaha Packing Co. (Omaha, NE) and Creekstone Farms Premium Beef LLC (Arkansas City, KS) (Figure 1). After receipt of beef, it was stored in commercial coolers at the R.M. Kerr Food and Ag Products Center meat pilot plant until needed (i.e., used within 1-2 weeks if not immediately). Beef was initially trimmed of excess fat, sliced into strips of 0.75-in, and then cut into small pieces of 2-in wide, 3-in long, and 0.75-inch thick. After bottom rounds were trimmed and cut, beef was placed on trays, wrapped in plastic bags, and maintained at 4 °C until processed (i.e., the next morning).

Inoculation of mixed strains of cultures (i.e., preparation was described earlier) was performed as follows: a) the appropriate amount off beef pieces for a particular trial were placed on foil-lined trays; b) ~150 ul of the inoculum mixture was applied by micropipette onto the surface of each beef slice; c) the inoculum was rubbed over the surface with a double-gloved finger by another person who was assisting; d) the pieces were then turned over and the process repeated on the other side; d) the tray(s) of inoculated beef were then placed in a refrigerator (5 °C) for at least 30 min to allow attachment (Figure 1).

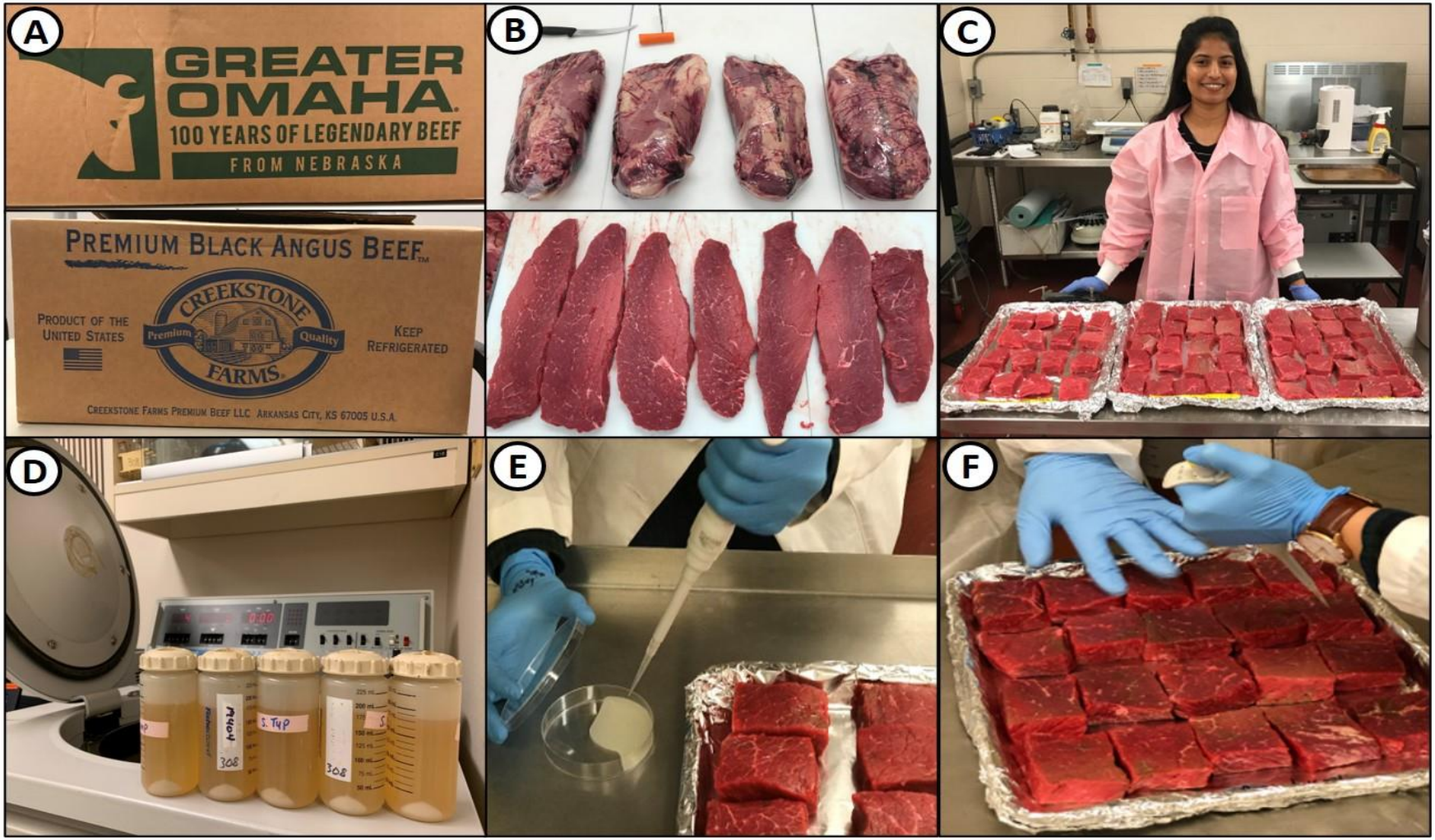

Figure 1. Biltong processing 1: (A) boxes of bottom rounds from processors; (B) bottom rounds in vacuum packaging and sliced after trimming; (C) further cutting of sliced biltong beef into small biltong beef pieces; (D) recovery and concentration of inoculum strains; (E) pipetting inoculum onto beef pieces; (F) 'gloved finger' spreading off inoculum on beef.

Marination of Inoculated Beef Pieces

Inoculated beef pieces that were held at refrigeration temperature for attachment were then subjected to a 30 min process of marination whereby the marinade mixture was added to pre-chilled stainless steel tumbling containers. A basic marinade mixture was previously determined was taken as an average of 8-10 biltong recipes found on the internet and was comprised of beef (92%), vinegar (4%), salt (2.1%), coriander (1.1%), and black pepper (0.8%). Since each non-beef component was based on the beef weight, and for each trial we obtained the weight of the total inoculated beef pieces that were going to be processed to determine the amount of spices and vinegar to add (Figure 2). These were then mixed with a whisk in a pre-chilled tumbling chamber and the inoculated beef pieces were then also added. The tumbler chamber cover with greased gasket was placed on top and a vacuum of 15-in Hg was established with a small vacuum pump; the tumbler was set to rotate/tumble for 30-min using a Biro VTS-43 tumbler (Biro, Marblehead, OH, USA) (Figure 2)

Drying of Marinated Beef Pieces

After marination, beef pieces were hung in a humidity oven (Hotpack Model #435315, SP Industries, Warminster, PA, USA) set at 75°F, 55% RH, using paper clips to hang beef pieces from the bars across the top and middle of the humidity chamber. Thermocouple temperature probes (x4) connected to a handheld temperature recorder were run into the chamber; 2 were used to record chamber temperature and 2 were inserted into 2 separate beef pieces at different locations to record internal beef temperature. An additional handheld humidity monitor was used to record both chamber humidity and temperature.

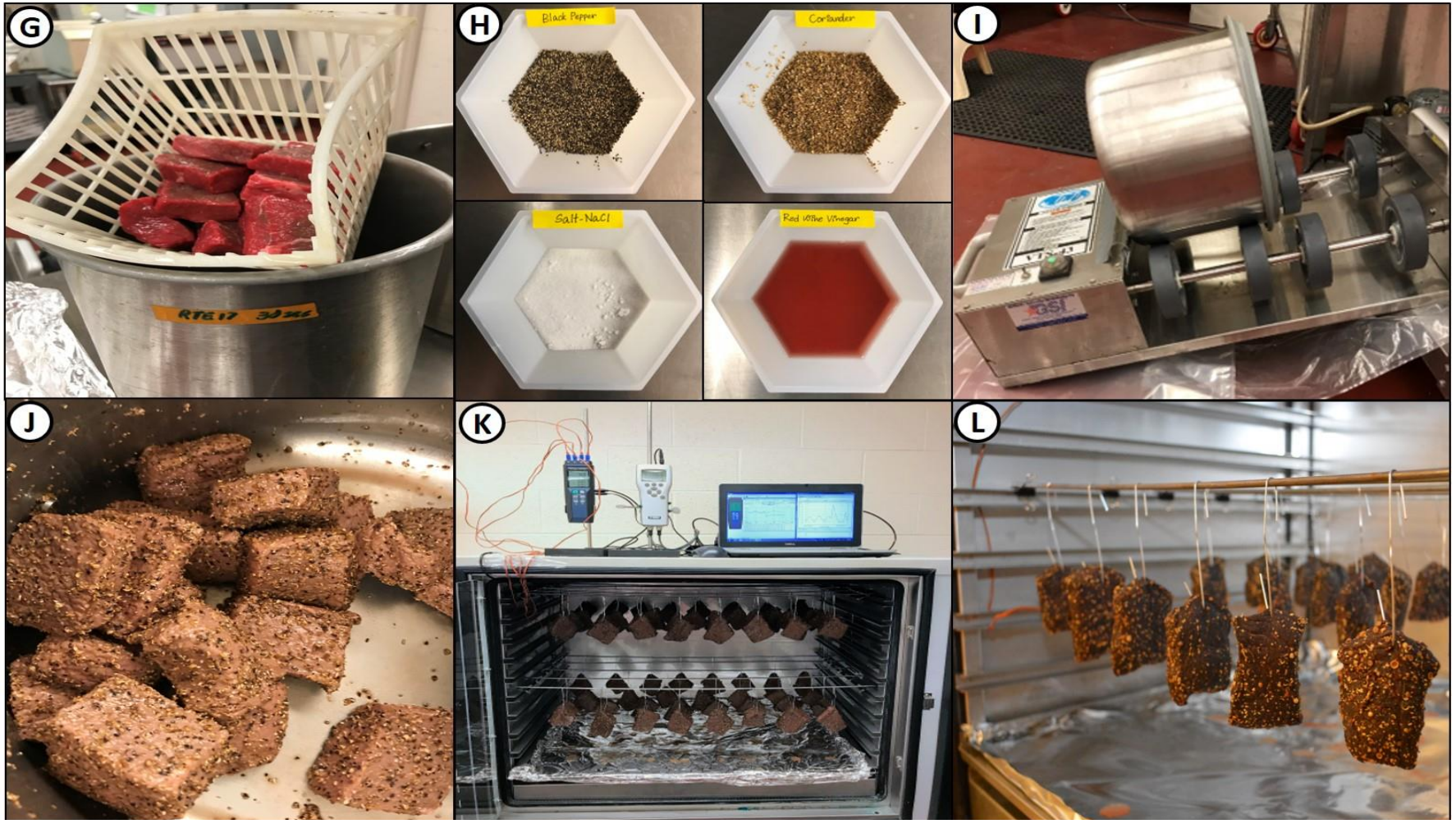

Figure 2. Biltong processing 2: (G) dip treatment of inoculated pieces in antimicrobial or water; (H) black pepper, coriander, salt, and vinegar for marinade; (I) vacuum tumbling of biltong beef in marinade; (J) spiced biltong beef after marination; (K) humidity oven with hanging biltong beef and handheld temperature and humidity monitors; (L) biltong beef on lower level (one has temperature probe inserted).

Beef Sampling, Microbial Enumeration, and Water Activity Testing

Acid adapted cultures were used to desensitize the cultures to acidic treatment during processing and plated on TSA containing antibiotics instead of enumerating on selective/differential agars which are often known to be inhibitory to stressed cells. Samples of beef retrieved from the humidity oven were placed in 6x9-inch Whirl-pak filter-stomacher bags (Nasco, Fort Atkinson, WI) to which 100 mL of neutralizing buffered peptone water (nBPW) was added. The stomacher bag was then stomached on a paddle masticator at high power for 2 min and subsequent dilutions were then made in 0.1% BPW. Select dilutions were then plated in duplicate on TSA + antibiotics for the respective pathogen. Plates were then incubated at 30 °C (*L. monocytogenes*) or 37 °C (*E. coli*, *S. aureus*) for 48 hrs before enumeration. At late stages of drying (i.e., ≥ 6 days), samples were often enumerated by plating 0.2 mL on each of 5 plates (i.e., 1 mL total) to increase the sensitivity of detection (Figures 3, 4).

For each series of duplicate trials with each pathogen, un-inoculated beef was also subjected to the entire biltong process including marination, tumbling, and up to 10 days of drying (Figure 4). This beef was used to insert temperature probes and for use in determining water activity throughout the process. Water activity was determined by slicing beef pieces in half and placing the innermost meat portion towards the humidity sensor in the water activity meter sample chamber of the model HC2-AW-USB probe with HW4-P-Quick software (Rotronic, Hauppauge, NY). This would provide water activity measurements for the inside of the beef pieces. Vacuum-tumbling would designate such beef as ‘non-intact beef’ and subject to concern of possible enterotoxin production by internalization of *S. aureus* in vacuum-tumbled beef (Figure 3).

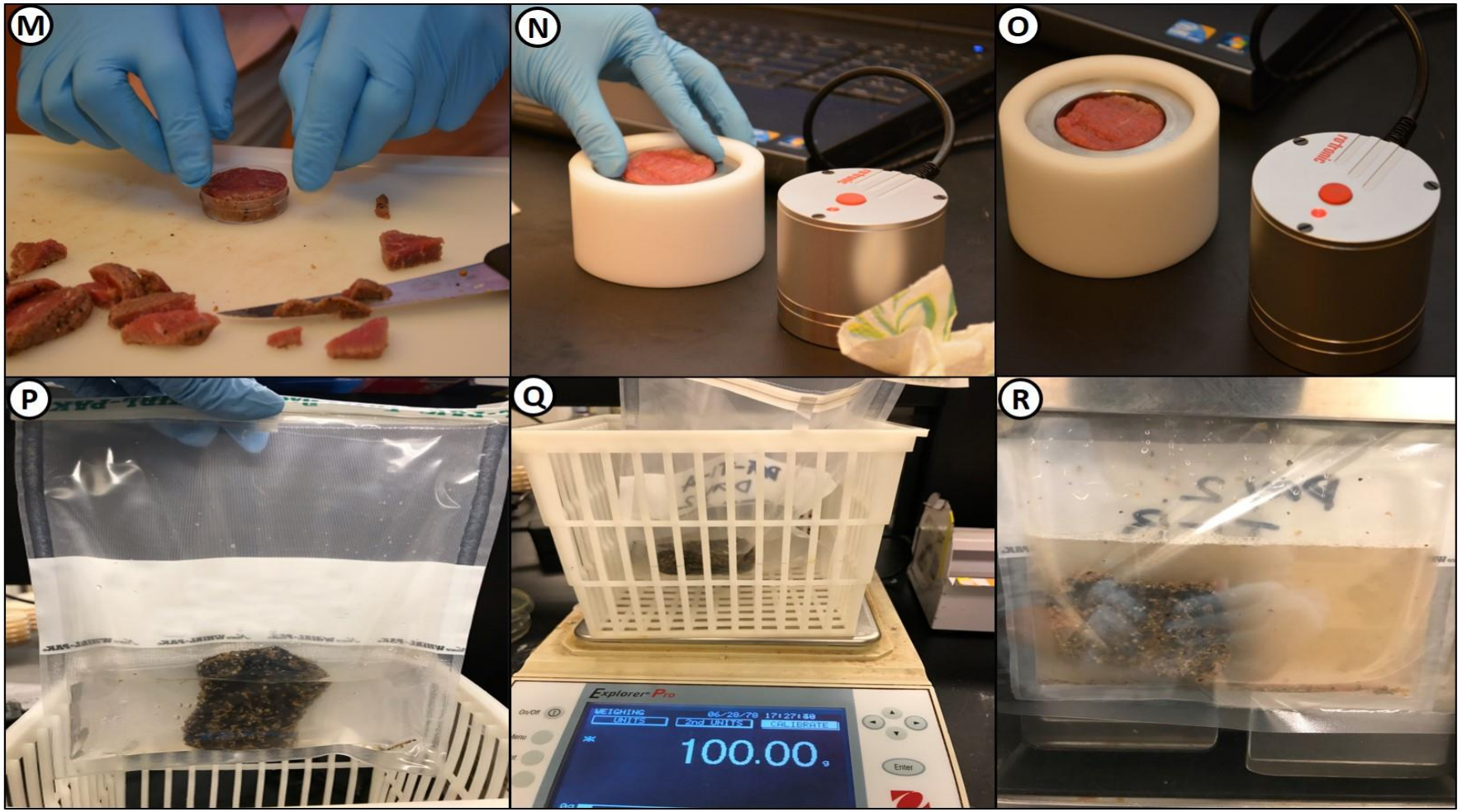

Figure 3. Biltong processing 3: (M) sectioning biltong to place in water activity cupules; (N, O) inserting water activity cupule into meter base (humidity sensor is in the cover that sits on the base); (P, Q) placement of biltong beef in 100 ml of neutralizing BPW; (R) stomaching biltong beef before microbial plating.

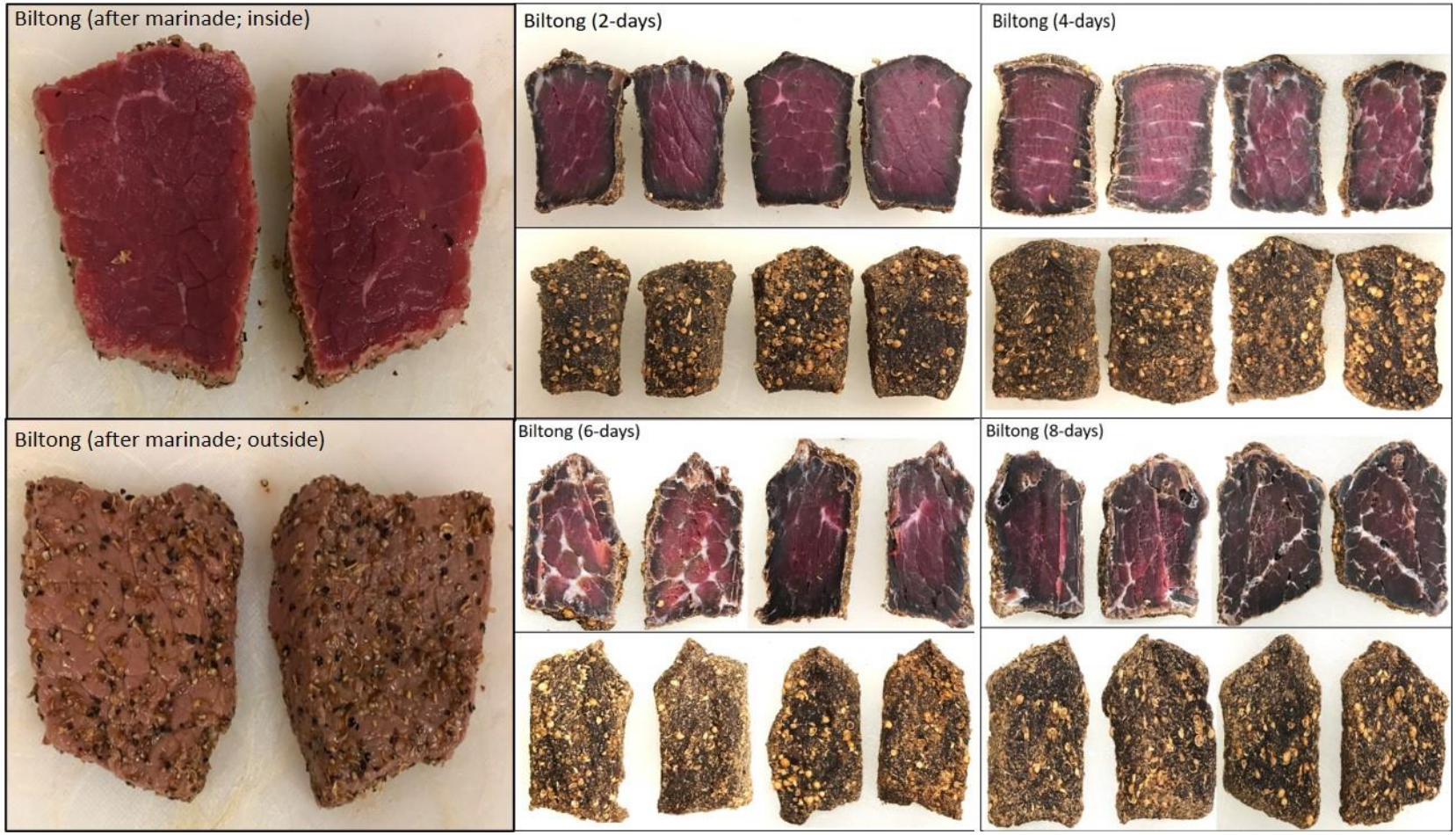

Figure 4. Biltong processing 4. Internal and external section of biltong beef after various stages of processing as indicated.

Testing for Enterotoxin Detection During Processing of Biltong for *Staphylococcus aureus*

ELISA kits for detecting SEA and SEB present in food, intestinal fluids, and liquid samples were obtained from Chondrex, Inc. (Woodinville, WA). Test samples were analyzed according to the manufacture's protocol for *S. aureus* strains producing SEA and SEB enterotoxin. Cultures included *S. aureus* ATCC 8095 (SEA), *S. aureus* ATCC 13565 (SEA), *S. aureus* ATCC 14458 (SEB), *S. aureus* ATCC 51740 (SEB). Tests performed included samples from 2 trials of *S. aureus*-inoculated biltong using the complete marinade with four samples tested for each duplicate trial replication at time points: a) after marination and b) after drying for 10 days in the temperature-controlled humidity oven.

In brief, a dilution of SEA and SEB enterotoxin standards (supplied with the kit) were prepared within a range of 0.16-10 ng/ml. The standards, samples and detection antibody were diluted in sample/standard/detection antibody dilution buffer. The assay outline included the addition of 50 ul of diluted standards or samples, and detection antibody to wells that were pre-coated with primary antibody. The plates were then incubated for 1 hour on a plate rotary shaker at room temperature. After incubation, the plates were washed 3 times using 1X wash buffer. After wash, 100 uL of TMB colorimetric substrate (Tetramethylbenzidine) was added to each well followed by an additional incubation for 25 min at room temperature. Lastly, the reaction was stopped by adding 50 uL of stop solution containing 2N sulfuric acid to each well. Plates were then read using a 450 nm passband filter on a Tecan GENios microplate reader and analyzed with Magellan software (ver. 7. 1).

Statistical Analysis

Each trial in this study was performed in duplicate replication with 3 samples tested per sampling period in each trial ($n = 6$) in accordance with validation testing criteria established by the NACMCF (Foods, 2010) and accepted by USDA-FSIS (USDA-FSIS, 2015). All replications were performed as autonomous and separate experiments using separately inoculated cultures and meat from different animals. Data are presented as the mean of multiple replications with standard deviation of the mean represented by error bars. Statistical analysis was performed using the statistics functions in Sigma-Plot ver. 13 (Systat Software, San Jose, CA). Timed data series were statistically analyzed by repeated measures one-way analysis of variance (RM-ANOVA) using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$) between treatments. Data treatments with different letters are significantly different ($p < 0.05$); treatments with the same letter are not significantly different ($p > 0.05$).

CHAPTER IV

RESULTS AND DISCUSSION

Examination of Strains on Selective Media

Examination of Strains of Listeria monocytogenes on Modified Oxford Agar Medium

The various strains of *L. monocytogenes* used in this study demonstrated black colonies on MOX agar, with concave surface appearance with recessed edges, and surrounded by a black zone confirming expected reactions and appearance of strains as *Listeria monocytogenes* (Figure 5).

Examination of Strains of E. coli O157:H7 on CHROMagar O157

CHROMagar O157 was used for differentiation and confirmation of expected appearance of *E. coli* O157:H7. Colonies *on the* CHROMagar O157 were mauve colored due the presence of chromogenic substrates in the medium and confirming the expected reactions of typical *Escherichia coli* O157:H7 strains (Figure 6).

Examination of Staphylococcus aureus on Mannitol Salt Agar

Mannitol salt agar is used as a selective and differential medium to examine individual strains of *S. aureus* used in this study. Colonies on reddish-colored mannitol salt agar were yellow colored with yellow zones due to fermentative utilization of mannitol changing the pH indicator from pink/red to yellow and confirming the expected reactions of our strains as *Staphylococcus aureus* (Figure 7).

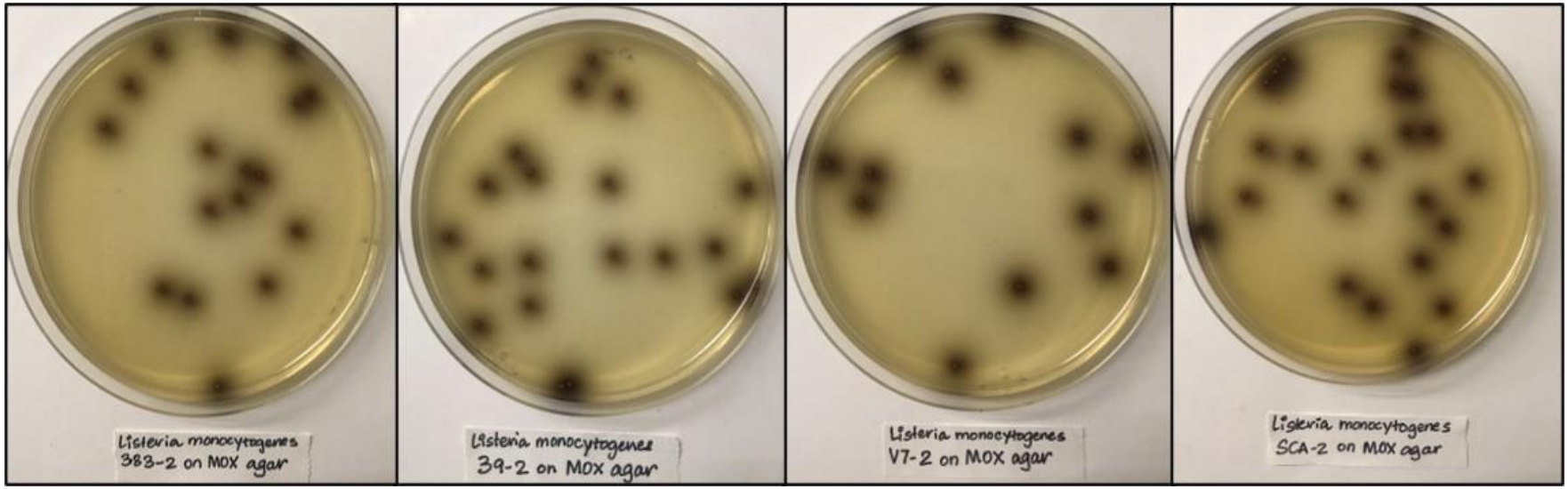

Figure 5. Examination of various strains of *Listeria monocytogenes* on modified oxford (MOX) agar medium. Cultures included *L. monocytogenes* 383-2, *L. monocytogenes* 39-2, *L. monocytogenes* V7-2, *L. monocytogenes* SCA-2.

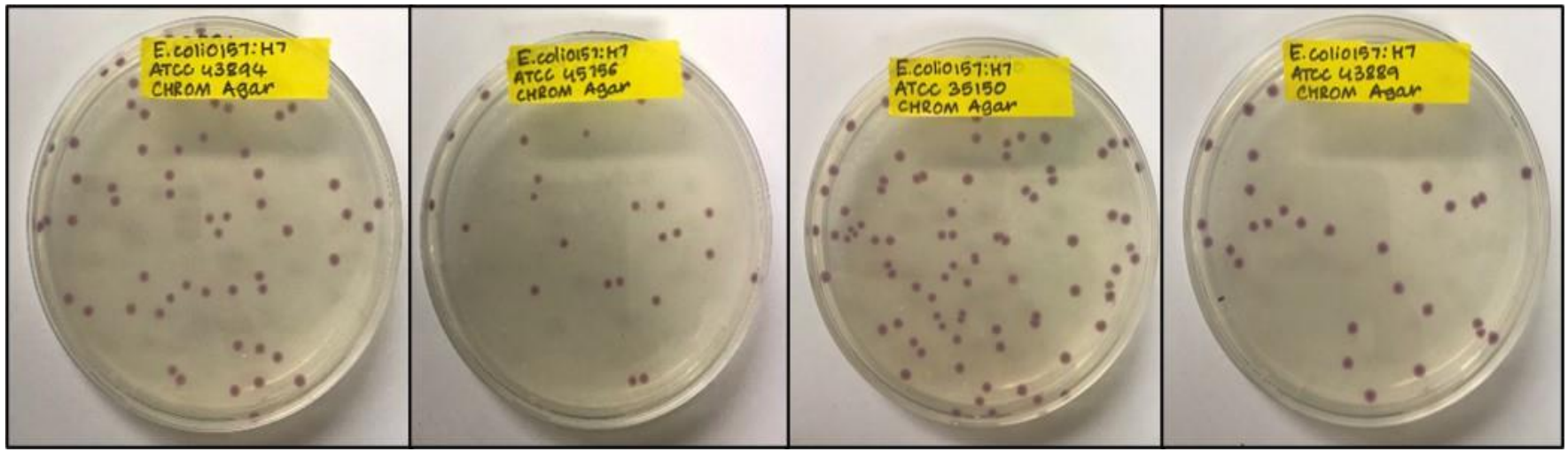

Figure 6. Examination of various strains of *Escherichia coli* O157:H7 strains on CHROM agar O157. Cultures include *E. coli* O157:H7 ATCC 43894, *E. coli* ATCC 45756, *E. coli* ATCC 35150, *E. coli* ATCC 43889.

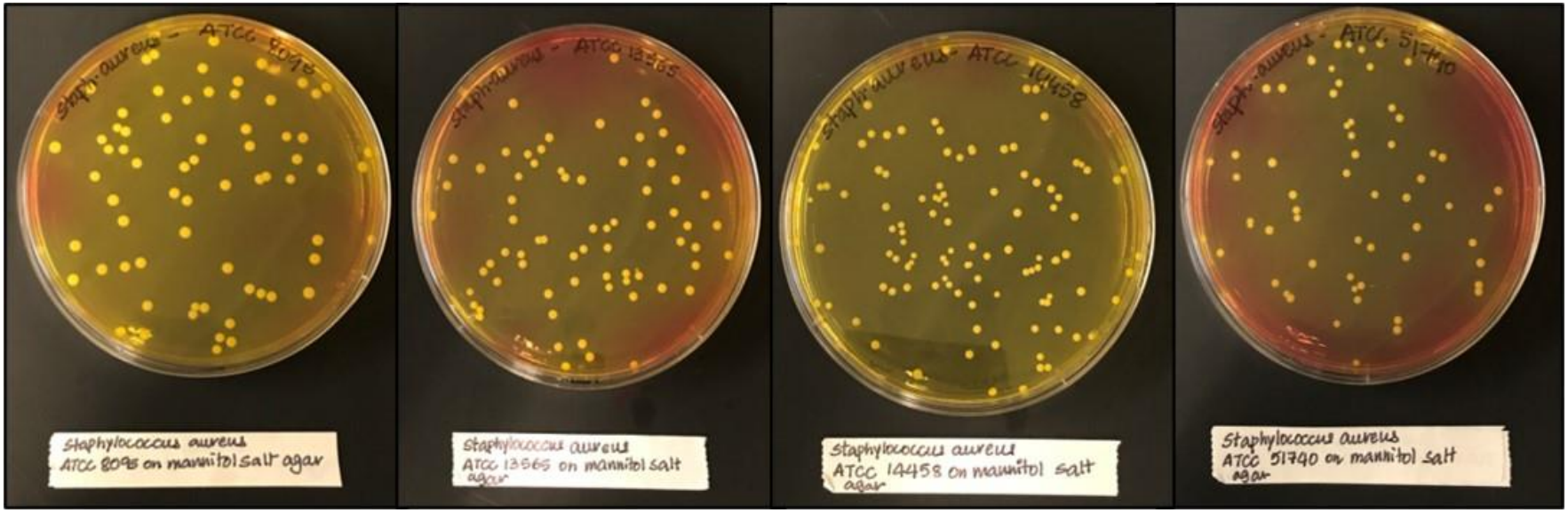

Figure 7. Examination of various strains of *Staphylococcus aureus* on mannitol salt agar. Cultures include *S. aureus* ATCC 8095, *S. aureus* ATCC 13565, *S. aureus* ATCC 14458, and *S. aureus* ATCC 51740.

Acid Adaptation of Cultures

All the cultures used in this study were subjected to acid adaptation by growing the cultures in TSB containing 1% glucose. The *E. coli* O157:H7, *L. monocytogenes*, and *S. aureus* strains used in this study as inocula for biltong beef pieces were subjected to antimicrobial intervention (i.e., vinegar in marinade suspension) during processing. Acid adaptation of *Salmonella* was first reported in the early 1990s whereby *Salmonella* pre-exposed to low pH were shown to be more resistant to acidic conditions than non-acid-adapted cells (Foster, 1991; Foster & Hall, 1990; G. J. Leyer & Johnson, 1992; G J Leyer & Johnson, 1993). Subsequently, during investigations with enterohemorrhagic *Escherichia coli* and *Listeria monocytogenes*, Buchanan et al. augmented media with glucose to allow the bacteria to ferment glucose and lower pH during growth and getting them used to an acidic environment and be 'acid-adapted' (R. L. Buchanan & Edelson, 1996; Robert L. Buchanan, Golden, Whiting, Phillips, & Smith, 1994). The ability of *Salmonella* and Shigatoxigenic *E. coli* to adapt to stressful environments has significant implications in the safety of processed foods that might be acidic or use an acidic intervention (Álvarez-Ordóñez, Prieto, Bernardo, Hill, & López, 2012; Suehr, Chen, Anderson, & Keller, 2020). Similar acid adaptation has been observed in *S. aureus* and demonstrated to provide cross tolerance to other stresses (Erdoğrul, Erb, & Toroğlu, 2006). Therefore, US regulatory agencies (USDA-FSIS, FDA) and the National Advisory Committee on the Microbial Criteria for Foods (NACMCF) have recommended the use of acid-adapted cultures when evaluating antimicrobial food processes involving acidic treatments (National Advisory Committee on the Microbiological Criteria for Foods, 2010). This ensures that the process must be robust to demonstrate the intended inhibition level and that cultures used as inoculants are

not overly sensitive to acid treatment. We have therefore adopted the practice in our laboratory that all cultures used as challenge inoculum would be acid adapted prior to use in challenge studies to preclude any suggestion that the cultures were overly sensitive to the treatments received.

The confirmation of pH effects of *L. monocytogenes*, *E. coli* O157:H7 and *S. aureus* cultures was examined by growing *L. monocytogenes* in TSB (without glucose) at 30 ºC, *E. coli* O157:H7 and *S. aureus* cultures in TSB (without glucose) at 37 ºC for 24 hours and then inoculated into TSB containing 0% glucose, 0.25% glucose, and 1% glucose and incubated at respective temperatures overnight. All cultures were separately inoculated into broth media in triplicate replication and a pH meter was used to record the pH levels of the various cultures after growth.

Acid Adaptation of Various Strains of Listeria monocytogenes

Growth of *L. monocytogenes* in TSB without glucose resulted in culture pH near neutrality (i.e., average pH 6.96) and culture pH of those grown in TSB with glucose were lower (i.e., average pH 4.99 with 0.25% glucose and an average pH 4.38 with 1% glucose) (Figure 8).

Acid Adaptation of Various Strains of E. coli O157:H7

Growth of *E. coli* O157:H7 in TSB without glucose again resulted in culture pH near neutrality (i.e., average pH 6.86) and the culture pH of those grown in TSB with glucose were significantly lower (i.e. 0.25% glucose showed an average pH of 5.81, and with 1% glucose an average pH 4.83) (Figure 9).

Acid Adaptation of Various Strains of Staphylococcus aureus

Similarly, growth of *S. aureus* in TSB without glucose again resulted in a culture pH near neutrality (i.e., average pH 6.96) and culture pH's of those grown in TSB with glucose were lower (i.e. 0.25% glucose with an average pH 5.73 and 1% glucose showing an average pH 4.75) (Figure 10).

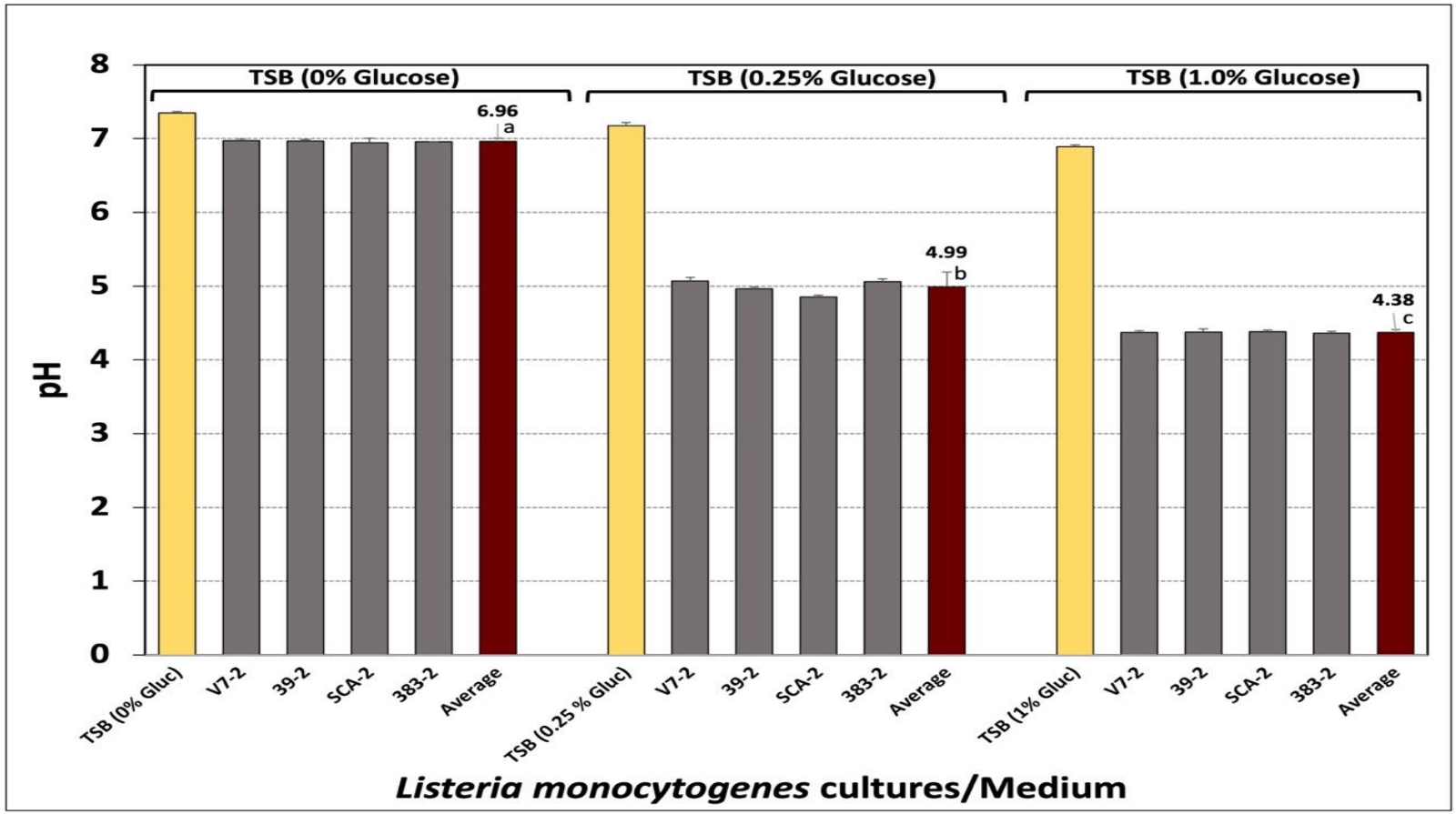

Figure 8. Analysis of the broth pH for *Listeria monocytogenes* cultures grown in TSB containing three different concentrations of glucose (0%, 0.25% and 1%) incubated at 30 ºC for 18 hours to demonstrate the effects of acid adaptation. Each set includes the data bars that represents control medium before inoculation, the four individual cultures after growth, and the average pH of four cultures. Cultures include *L. monocytogenes* V7-2, *L. monocytogenes* 39-2, *L. monocytogenes* SCA-2, and *L. monocytogenes* 383-2. Data are presented as the mean of triplicate replications and the error bars represent the standard deviation from the mean. Means (for average pH) with different letters are significantly different, as determined by one-way ANOVA using Holm-Sidak test, pairwise multiple comparisons to determine significant differences ($p < 0.05$); means with different letters are significantly different ($p < 0.05$).

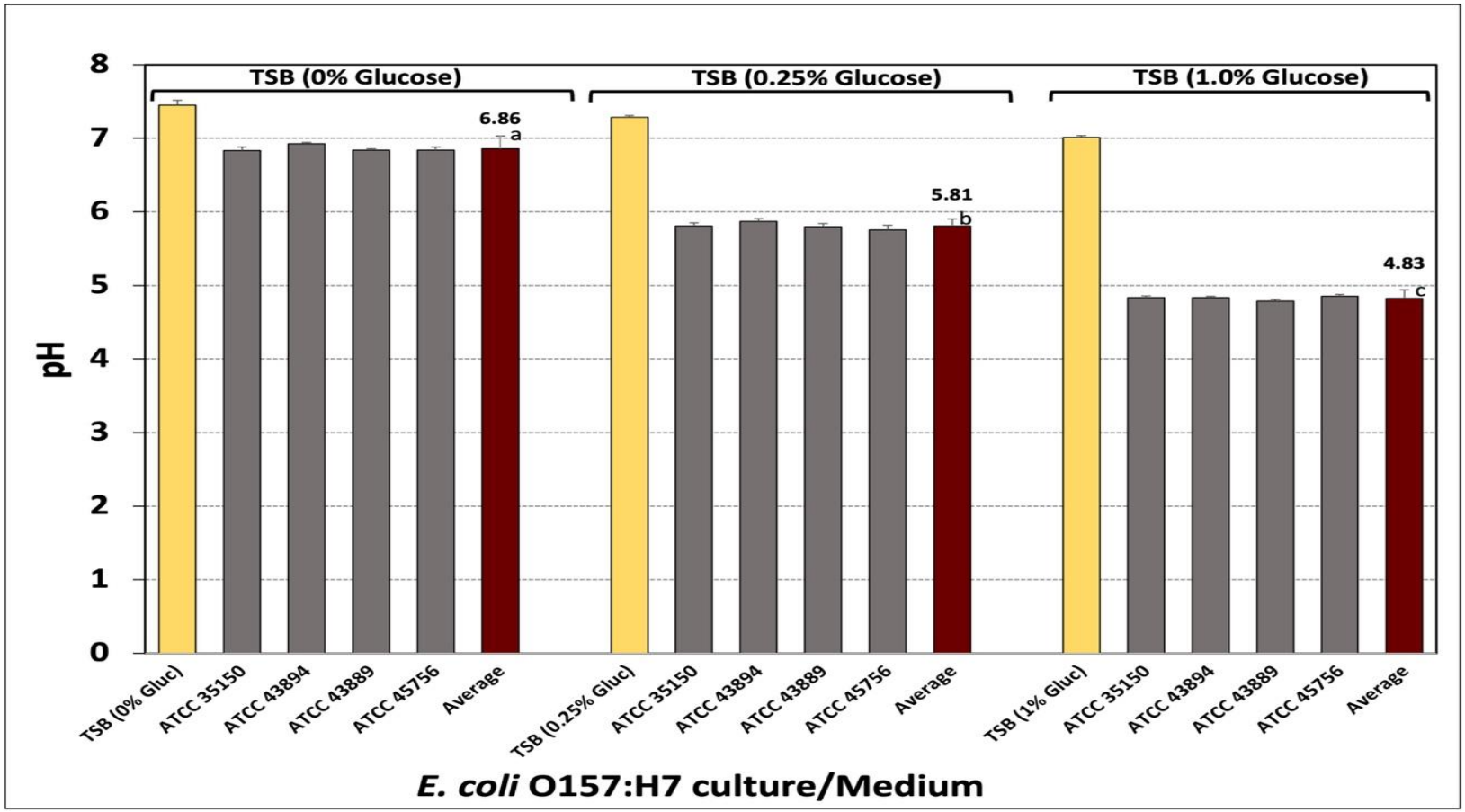

Figure 9. Analysis of the broth pH for *E. coli* O157:H7 cultures grown in TSB containing three different concentrations of glucose (0%, 0.25% and 1%) and incubated at 37 ºC for 18 hours to demonstrate the effects of acid adaptation. Each set includes the data bars that represents control medium before inoculation, the four individual cultures after growth, and the average pH of four cultures. Cultures include E. coli ATCC 35150, E. coli ATCC 43894, E. coli ATCC 43889, and E. coli ATCC 45756. Data are presented as the mean of triplicate replications and the error bars represent the standard deviation from the mean. Means (for average pH) with different letters are significantly different, as determined by one-way ANOVA using Holm-Sidak test, pairwise multiple comparisons to determine the significant differences ($p < 0.05$); means with different letters are significantly different ($p < 0.05$).

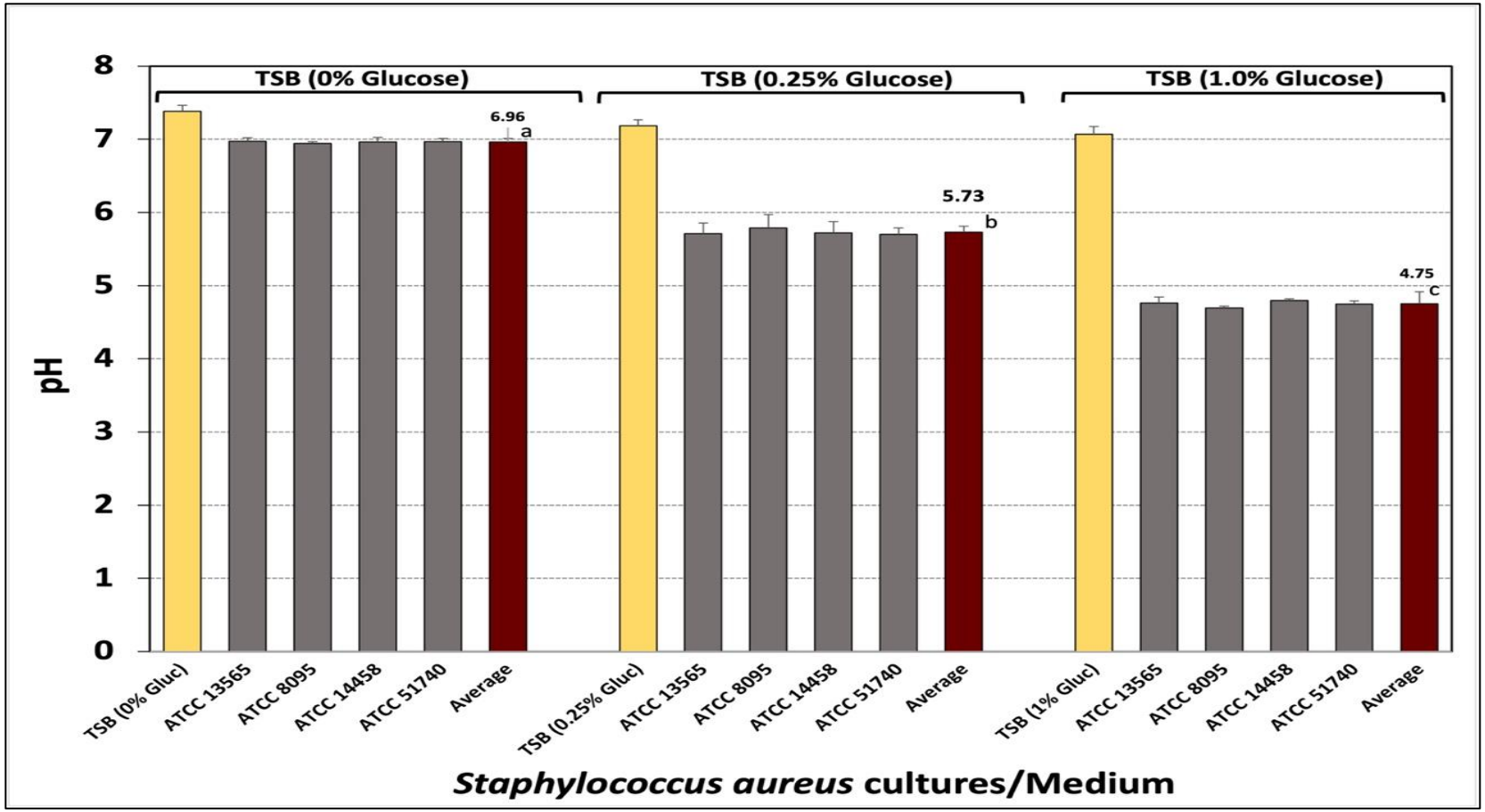

Figure 10. Analysis of the broth pH for *Staphylococcus aureus* cultures grown in TSB containing three different concentrations of glucose (0%, 0.25% and 1%) incubated at 37 ºC for 18 hours to demonstrate the effects of acid adaptation. Each set includes the data bars that represents control medium before inoculation, the four individual cultures after growth, and the average pH of four cultures. Cultures include *S. aureus* ATCC 13565, *S. aureus* ATCC 8095, *S. aureus* ATCC 14458 and *S. aureus* ATCC 51740. Data are presented as the mean of triplicate replications and the error bars represent the standard deviation from the mean. Means (for average pH) with different letters are significantly different, as determined by one-way ANOVA using Holm-Sidak test, pairwise multiple comparisons to determine the significant differences ($p < 0.05$); means with different letters are significantly different ($p < 0.05$).

Antibiotic Resistance of Cultures

Antibiotic resistance of our inoculum strains were used for their recovery from inoculated beef using generic growth media (TSA) containing antibiotics to which the bacteria are resistant. It is well known that selective and differential agars used for 'detection' of bacteria are not suitable for enumeration, especially when recovering bacteria from conditions of stress whereby the harsh selective media inhibits injured cells resulting in a falsely lower count than would be obtained otherwise. This was observed with *Salmonella* serovars enumerated after various stressful conditions on TSA, selenite cystine agar (SCA), hektoen enteric Agar (HE), and xylose lysine deoxycholate (XLD) agar whereby microbial counts obtained on HE and XLD were 1-2.5 log lower than counts obtained on TSA and SCA (C. E. Karolenko, A. Bhusal, D. Gautam, & P. M. Muriana, 2020). The inhibition of injured cells resulting in a lower count by enumeration on selective-differential media has been observed with other organisms as well including *L. monocytogenes* (i.e., on MOX agar), *E. coli* O157:H7 (on cefixime-tellurite Sorbitol MacConkey agar, CT-SMAC), and *S. aureus* (on SM-110, TGA, ETGPA, TEA, and TPEY (Crisley, Peeler, & Angelotti, 1965; Kang & Fung, 1999; Ngwa, Schop, Weir, León-Velarde, & Odumeru, 2013).

For these reasons, we have generally used intrinsic antibiotic resistance of inoculated strains to select for, and recover, challenge organisms added to food. This method is widely used (Flores, 2004; Luchansky et al., 2009; Mann & Brashears, 2006) and is supported by NACMCF as a method of selective recovery of inoculated strains from foods (National Advisory Committee on the Microbiological Criteria for Foods, 2010). However, there are situations where combinations of antibiotics are synergistically inhibitory to strains even

when the strains are individually resistant to each antibiotic without inhibition (Bollenbach, 2015; Singh & Yeh, 2017; Yilancioglu, 2019). Therefore, it is necessary to demonstrate that there is no inhibition of strains when selected on combinations of antibiotics by comparing plate counts enumerated on generic media (TSA) vs media containing antibiotic (TSA+ab).

Antibiotic resistance for *L. monocytogenes, E. coli* O157:H7 and *S. aureus* strains was confirmed by growing the strains in TSB without any antibiotics and the cultures grown were plated on TSA alone and TSA with selected antibiotics (TSA+ab). Individual strains of *L. monocytogenes* were enumerated on TSA alone and on TSA containing streptomycin sulphate (100 ug/ml) and rifamycin s/v (10 ug/ml). After enumeration on both sets of agar media, we did not observe any significant effect on the comparison of enumeration between these two-plating media with all strains tested (Figure 11).

Individual strains of *E. coli* O157:H7 were also enumerated on TSA alone and TSA containing novobiocin (5 ug/ml) and rifamycin s/v (2.5 ug/ml). Likewise, no significant difference was observed between non-selective and antibiotic-based selective plating (Figure 12).

Similarly, individual strains of *S. aureus* were enumerated on TSA alone and on TSA containing clindamycin phosphate (5 ug/ml). Again, no significant difference was observed between the selective and non-selective media used (Figure 13).

This demonstrates that the various sets of strains are resistant to the respective antibiotics used and the antibiotic resistance of the strains facilitates the use of antibiotics in the media to quantitatively recover the strains present on the food products.

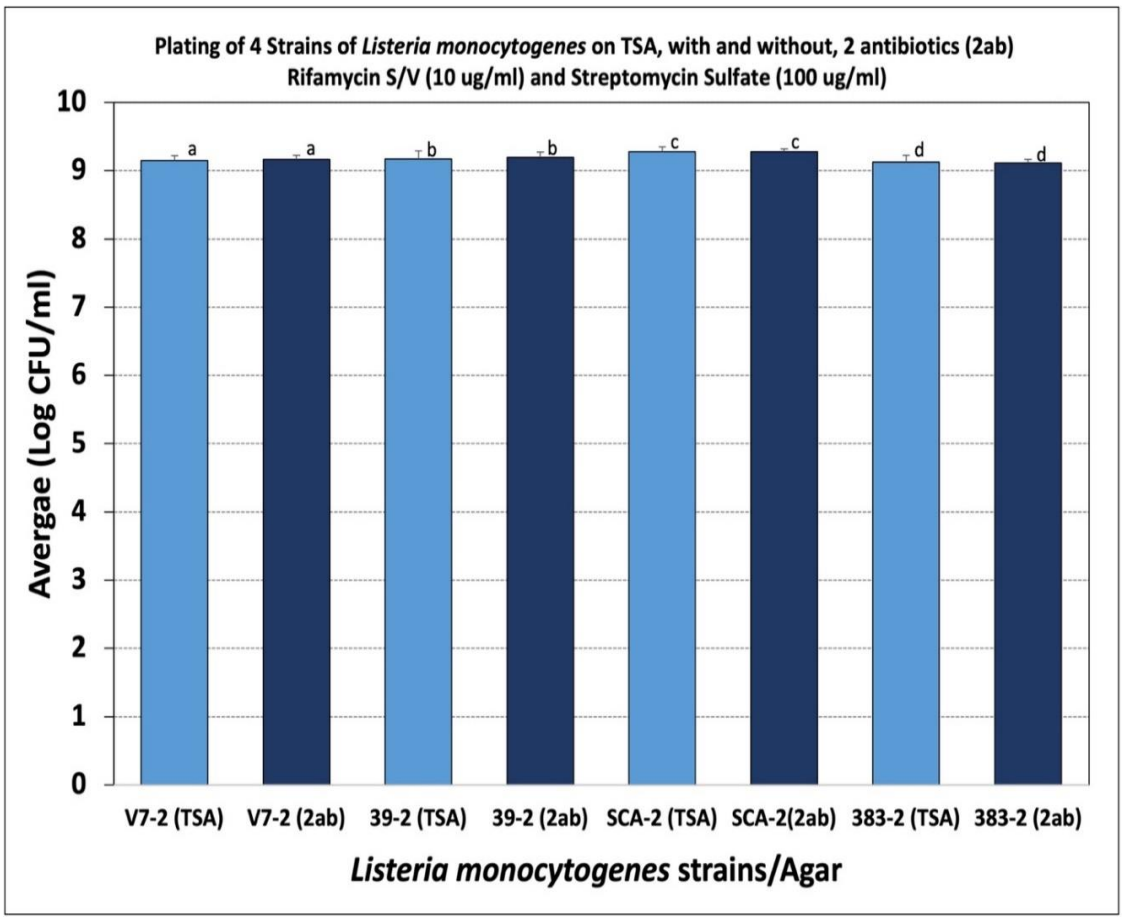

Figure 11. Quantification of antibiotic resistance for *Listeria monocytogenes* on TSA without antibiotics as a control vs. TSA with streptomycin sulphate (100 ug/mL) and rifamycin s/v (10 ug/mL). Cultures include *L. monocytogenes* V7-2, *L. monocytogenes* 39-2, *L. monocytogenes* SCA-2, and *L. monocytogenes* 383-2. Cultures were grown in tryptic soy broth without antibiotics for 18 hours at 30 ºC. Cultures were serially diluted in 0.1% BPW and plated on TSA with antibiotics. Data are presented as the mean of triplicate replications and the error bars represent the standard deviation from the mean. Significant differences ($p < 0.05$) were only determined for the same strain on the two sets of media; data represented with the same letters for the same strain are not significantly different ($p > 0.05$) as determined by one way analysis of variance (ANOVA) using the Holm-Sidak test pairwise comparisons to determine significant differences.

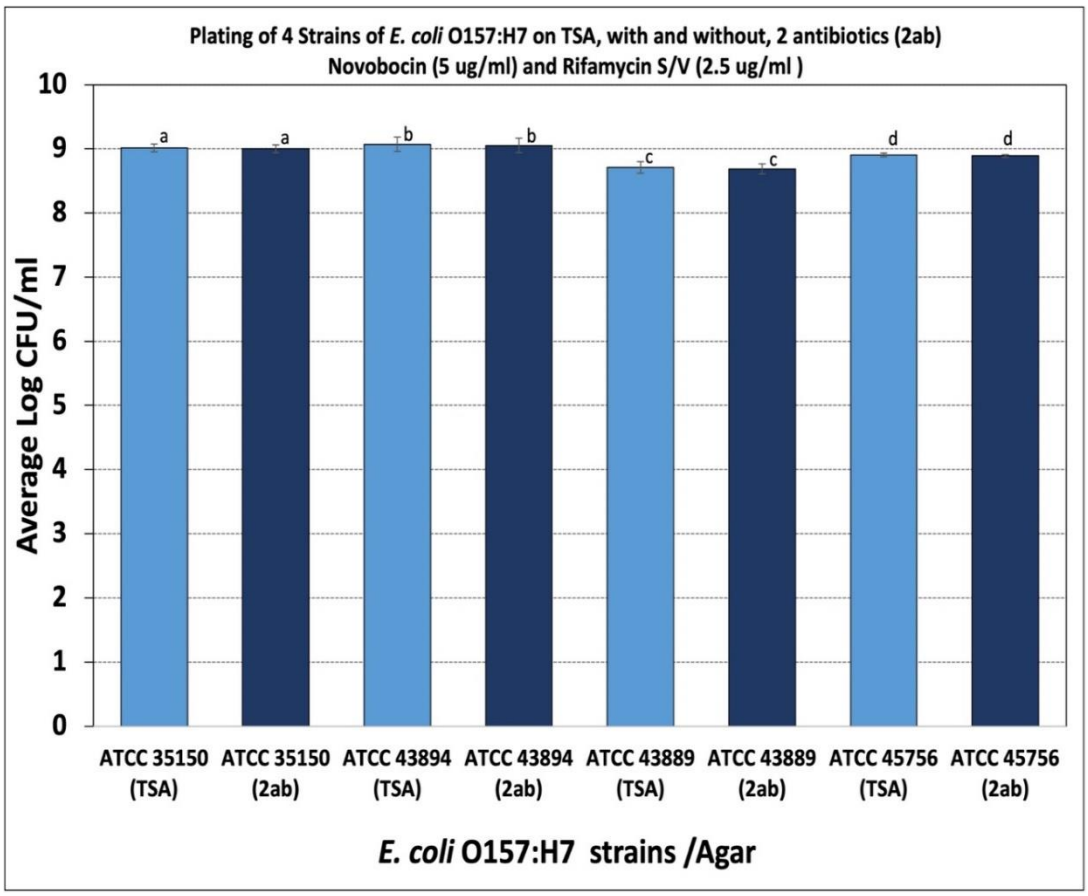

Figure 12. Quantification of antibiotic resistance for *E. coli* O157:H7 on TSA without antibiotics as a control vs. TSA with novobiocin (5ug/mL) and rifamycin s/v (2.5 ug/mL). *E. coli* ATCC 35150, *E. coli* ATCC 43894, *E. coli* ATCC 43889 and *E. coli* ATCC 45756. Cultures were grown in tryptic soy broth without antibiotics for 18 hours at 37 ºC. Cultures were serially diluted in 0.1% buffered peptone water and plated on TSA with antibiotics. Data are presented as the mean of triplicate replications and the error bars represent the standard deviation from the mean. Significant differences ($p < 0.05$) were only determined for the same strain on the two sets of media; data represented with the same letters for the same strain are not significantly different ($p > 0.05$) as determined by one way analysis of variance (ANOVA) using the Holm-Sidak test pairwise comparisons to determine significant differences.

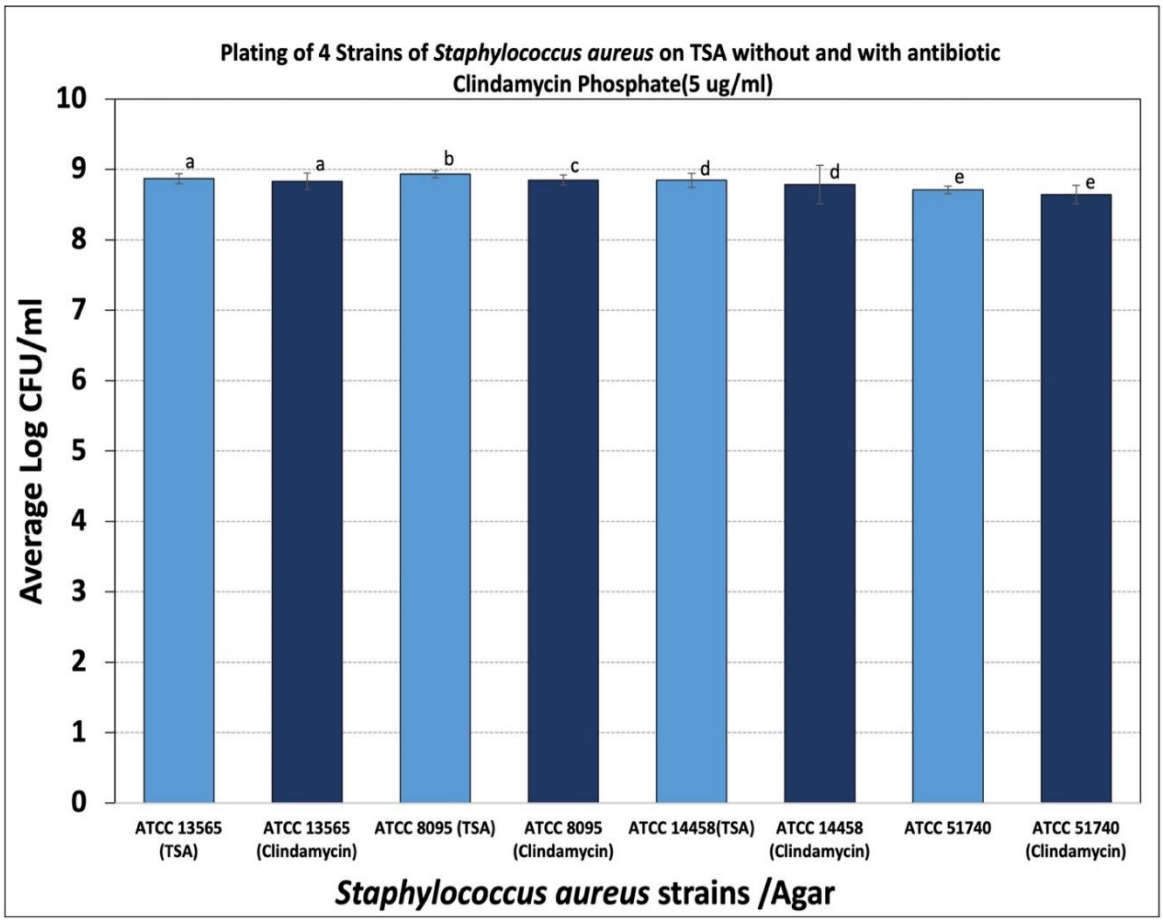

Figure 13. Quantification of antibiotic resistance for *Staphylococcus aureus* on TSA without antibiotics as a control vs. TSA with clindamycin phosphate (5 ug/mL). *S. aureus* ATCC 13565, *S. aureus* ATCC 8095, *S. aureus* ATCC 14458 and *S. aureus* ATCC 51740. Cultures were grown in tryptic soy broth without antibiotics for 18 hours at 37 ºC. Cultures were serially diluted in 0.1% buffered peptone water and plated on TSA with antibiotics. Data are presented as the mean of triplicate replications and the error bars represent the standard deviation from the mean. Significant differences ($p < 0.05$) were only determined for the same strain on the two sets of media; data represented with the same letters for the same strain are not significantly different ($p > 0.05$) as determined by one way analysis of variance (ANOVA) using the Holm-Sidak test pairwise comparisons to determine significant differences.

Biltong Marination

Evaluation of the Individual and Combined Components of the Biltong Marinade on the Reduction of Listeria monocytogenes

Data for biltong marinated in spice (black pepper, coriander), salt, and vinegar resulted in >5-log reduction of *L. monocytogenes* in 6 days of drying and >6-log reduction by 10 days of drying (Figure 14). Data for biltong processing of inoculated beef using individual components of the marinade (spice, salt or vinegar) showed greatest reduction of *L. monocytogenes* exhibited by vinegar (>5-log) over a drying period of 8 days followed by salt (4.68-log) and spice (4.1-log) during 10 day drying period (Figure 15).

The conditions of drying, without any added ingredients, rendered a (4.2-log) reduction over a 10-day drying period. Treatment with vinegar alone or combined components of the marinade showed no significance difference between them ($p > 0.05$). Treatment with salt or spice alone showed no significance difference between them ($p > 0.05$). Treatment with vinegar showed a significance difference ($p < 0.05$) when compared to individual treatment with salt or spice. Among the components of the marinade, vinegar appears to play the most important role in the reduction of *L. monocytogenes.*

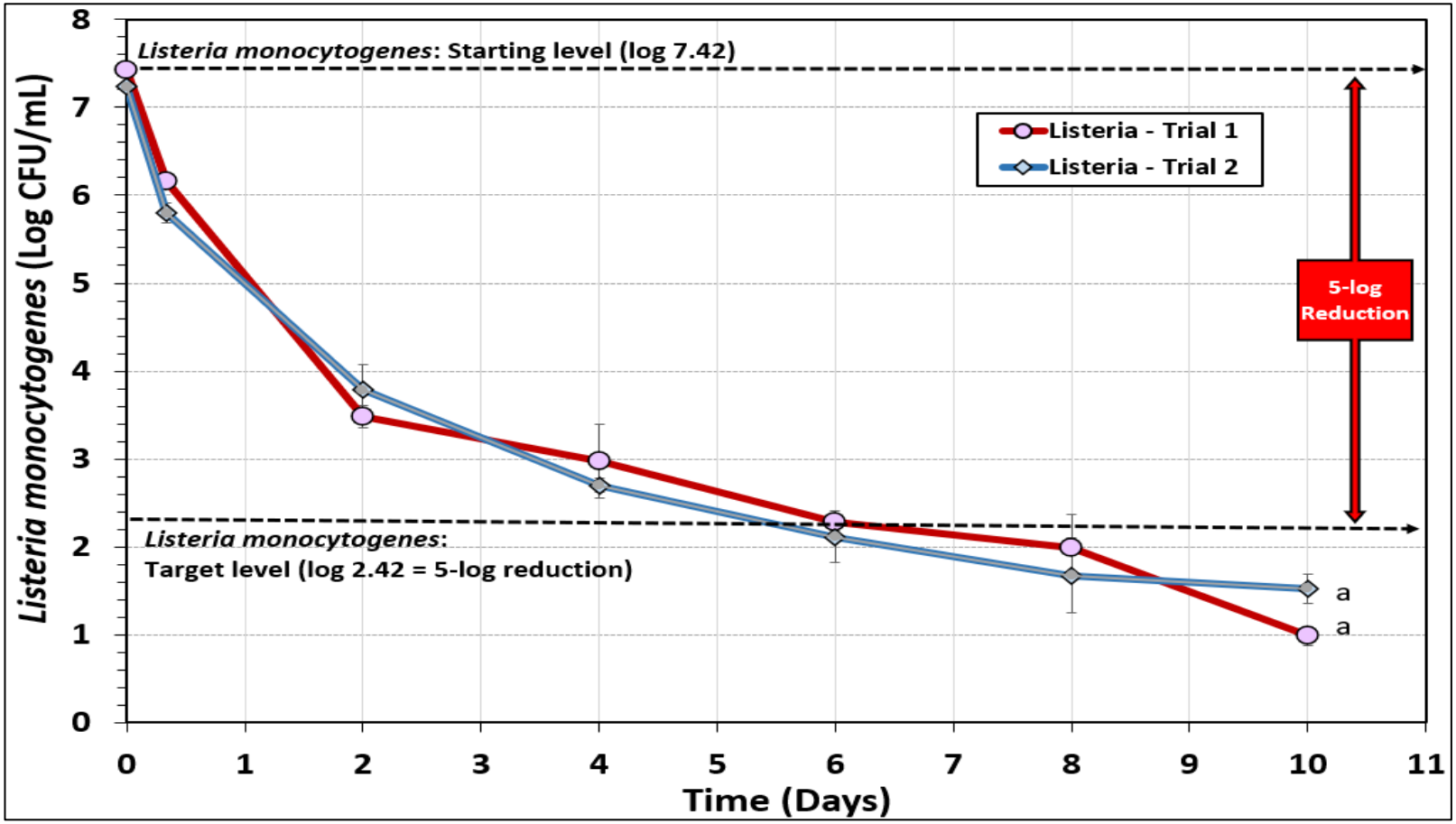

Figure 14. Biltong marination with combined components of the marinade (salt, spice, and vinegar) for the reduction of *L. monocytogenes.* After marination the beef pieces were hung and dried at 75 ºF and 55% RH for up to 10 days. A set of beef pieces without inoculation were considered as negative controls for demonstrating the efficacy of the media used against the background organisms. Trials were performed in duplicate replication (with triplicate samples at each time point; n=6) and treatments were analyzed by repeated measures one-way analysis of variance (RM-ANOVA) using Holm-Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$); treatments with the same letters are not significantly different ($p > 0.05$); treatment with different letters are significantly different ($p < 0.05$).

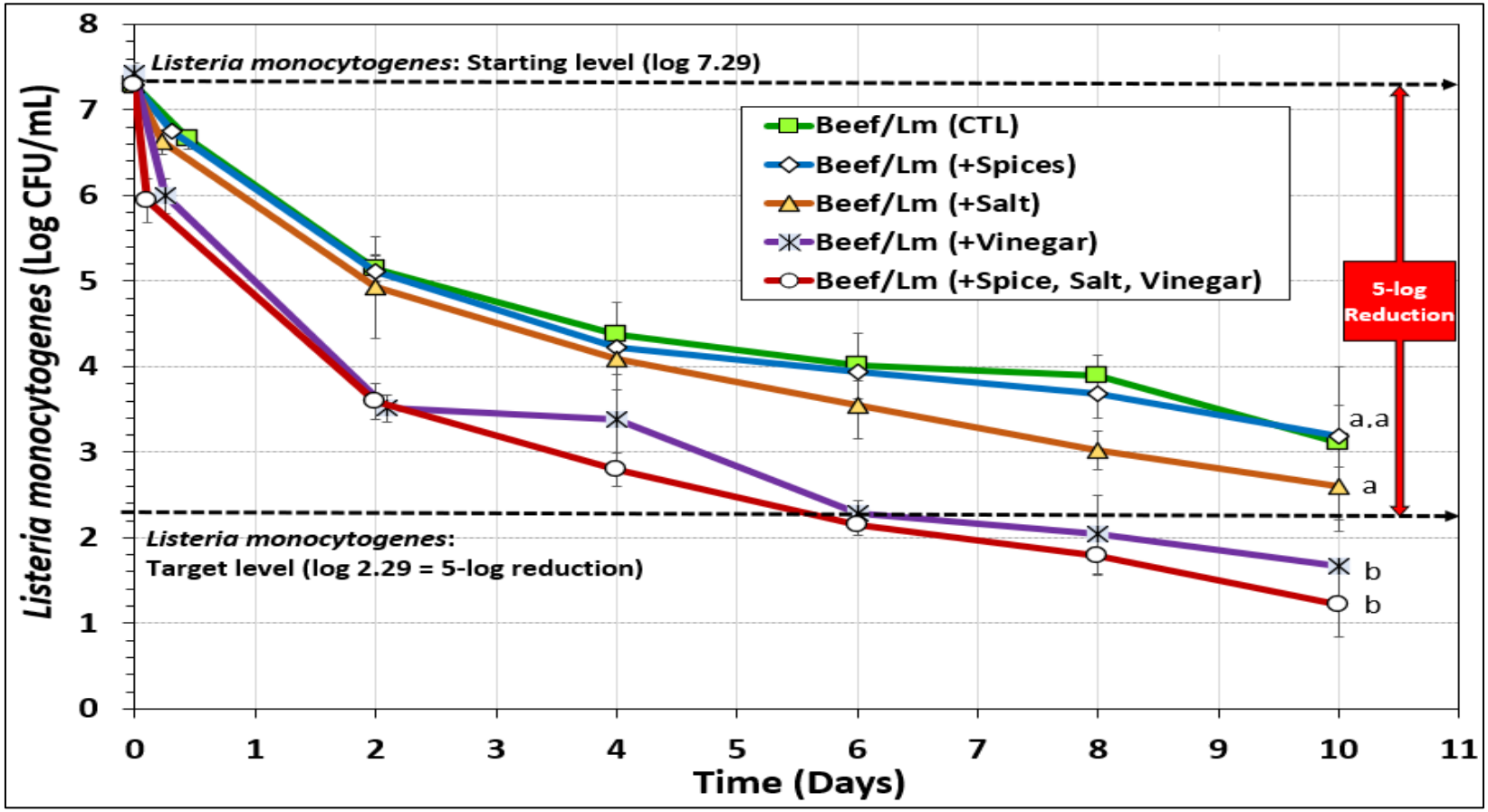

Figure 15. Individual contributions of marinade components (spice, salt, vinegar) to the overall reduction of *L. monocytogenes* during biltong processing**.** After marination the beef pieces were hung and dried at 75 ºF and 55% RH for up to 10 days. A set of beef pieces without inoculation were considered as negative controls for demonstrating the efficacy of the media used against the background organisms. Graphs of different trials were adjusted to same common point. Trials were performed in duplicate replication (with triplicate samples at each time point; n=6) and treatments were analyzed by repeated measures one-way analysis of variance (RM-ANOVA) using Holm-Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$); treatments with the same letters are not significantly different ($p > 0.05$); treatment with different letters are significantly different ($p < 0.05$).

Evaluation of the Individual and Combined Components of the Biltong Marinade on the Reduction of E. coli O157:H7

Data for biltong marinated in spice, salt and vinegar resulted in (>5-log) reduction of *E. coli* O157:H7 in 4 days of drying and (~6-log) reduction in 10 days of drying(Figure 16) and data for treatment with individual components of the marinade (spice, salt or vinegar) demonstrated similar level of reduction for treatment with salt and vinegar alone with a reduction of (5.48-log) and (5.49-log) respectively and followed by spice (4.11-log) over a drying period of 10 days (Figure 17).

The conditions of drying, without any added ingredients, rendered (3.84-log) reduction over a drying period of 10 days. Individual treatment with salt or vinegar showed no significance difference between them ($p > 0.05$). Treatment with spice alone and conditions of beef dried, without any added ingredient showed no significance difference between them ($p > 0.05$). Treatment with combined components of the marinade showed significance difference when compared with individual treatment with marinade components ($p < 0.05$). Both salt and vinegar are the key components in the marinade that resulted in (>5-log) reduction of *E. coli* O157:H7.

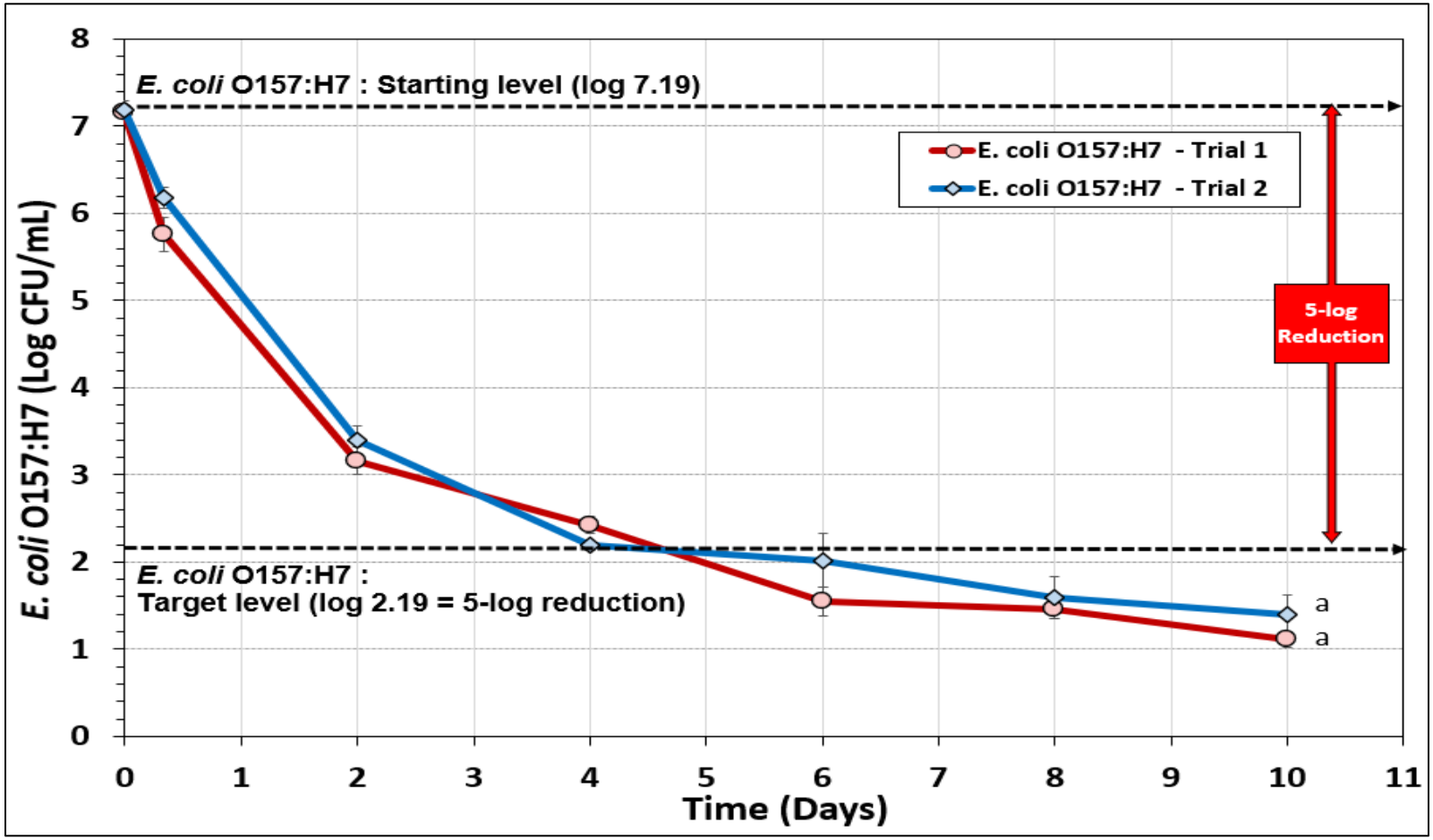

Figure 16. Biltong marination with combined components of the marinade (salt, spice, and vinegar) for the reduction of *E. coli* O157:H7. After marination the beef pieces were hung and dried at 75 ºF and 55% RH for up to 10 days. A set of beef pieces without inoculation were considered as negative controls for demonstrating the efficacy of the media used against the background organisms. Trials were performed in duplicate replication (with triplicate samples at each time point; n=6) and trials were analyzed by repeated measures one-way analysis of variance (RM-ANOVA) using the Holm-Sidak test for pairwise multiple comparisons. Trials with different letters are significantly different ($p < 0.05$); those with same letters are not significantly different ($p > 0.05$).

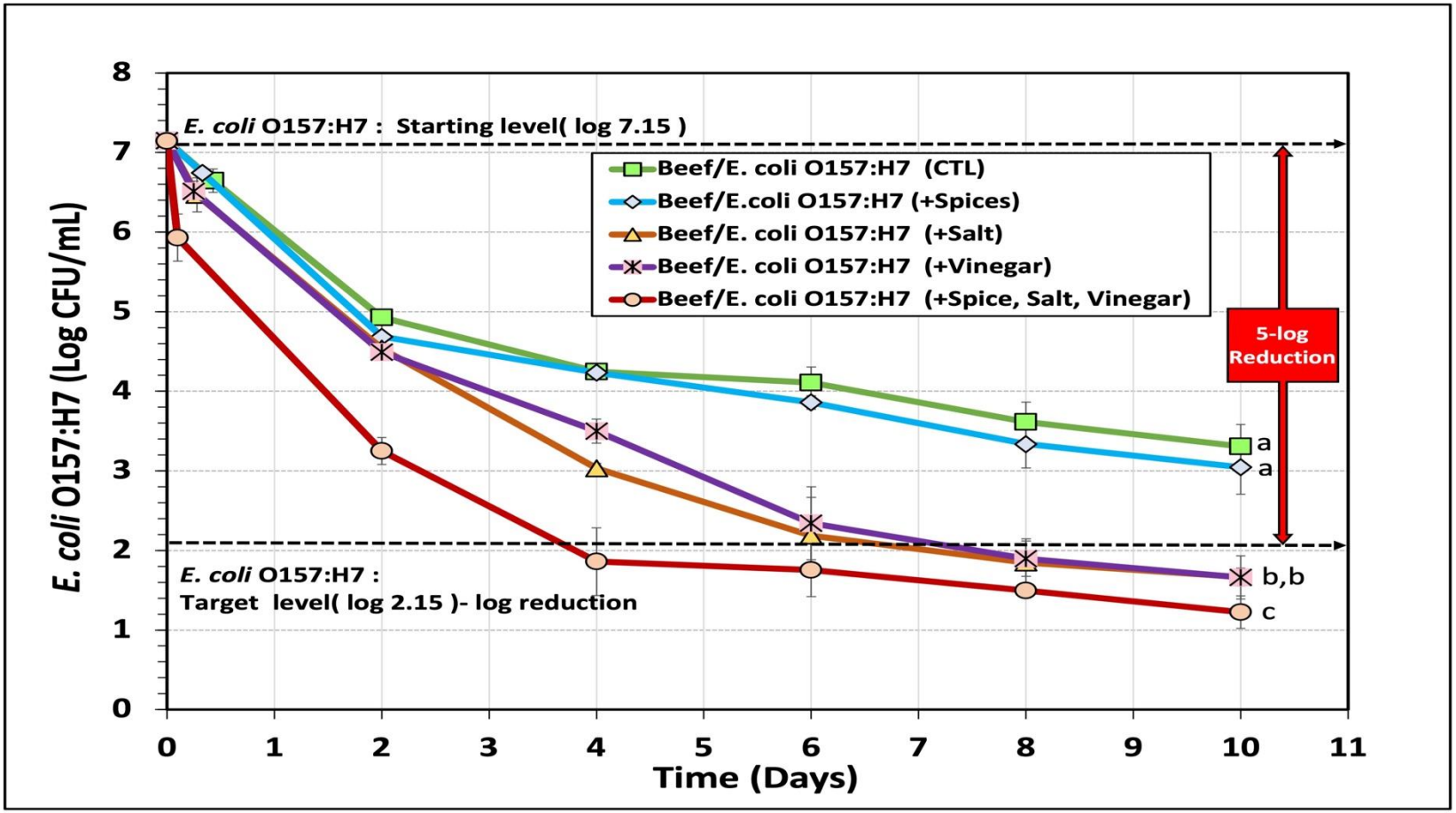

Figure 17. Individual contribution of marinade components (spice, salt, vinegar) to the overall reduction of *E. coli* O157:H7**.** After marination the beef pieces were hung and dried at 75 ºF and 55 %RH for up to 10 days. A set of beef pieces without inoculation were considered as negative controls for demonstrating the efficacy of the media used against the background organisms. Graphs of different trials were adjusted to same common point. Trials were performed in duplicate replication (with triplicate samples at each time point; n=6) and trials were analyzed by repeated measures one-way analysis of variance (RM-ANOVA) using the Holm-Sidak test for pairwise multiple comparisons. Trials with different letters are significantly different ($p < 0.05$); those with same letters are not significantly different ($p > 0.05$).

Examination of the Complete Marinade Mixture on Reduction of Staphylococcus aureus During Biltong Processing

Staphylococcus aureus is a concern on biltong that is especially subjected to vacuum tumbling. The USDA-FSIS considers beef that is vacuum-tumbled as 'non-intact' beef whereby the possibility exists that the vacuum tumbling process, although intended to help marination juices become absorbed by the meat products, may also allow entry and internalization of surface bacteria. This represents a food safety hazard if the processing does not reduce the internal water activity of the biltong beef and it is possible that *S. aureus* may produce enterotoxin internally.

Data for *S. aureus* shows that similar to *L. monocytogenes* and *E. coli* O157:H7, *S. aureus* is also reduced during biltong processing when marinated in spice, salt, and vinegar by >5-log reduction after drying for 7 days (extrapolated), 5.44-log reduction within 8 days, and 5.95-log reduction after 10 days (Figure 18).

Of the 3 pathogens tested, *S. aureus* is among the most salt tolerant and the data shows that vinegar contributes the most to lethality during biltong processing.

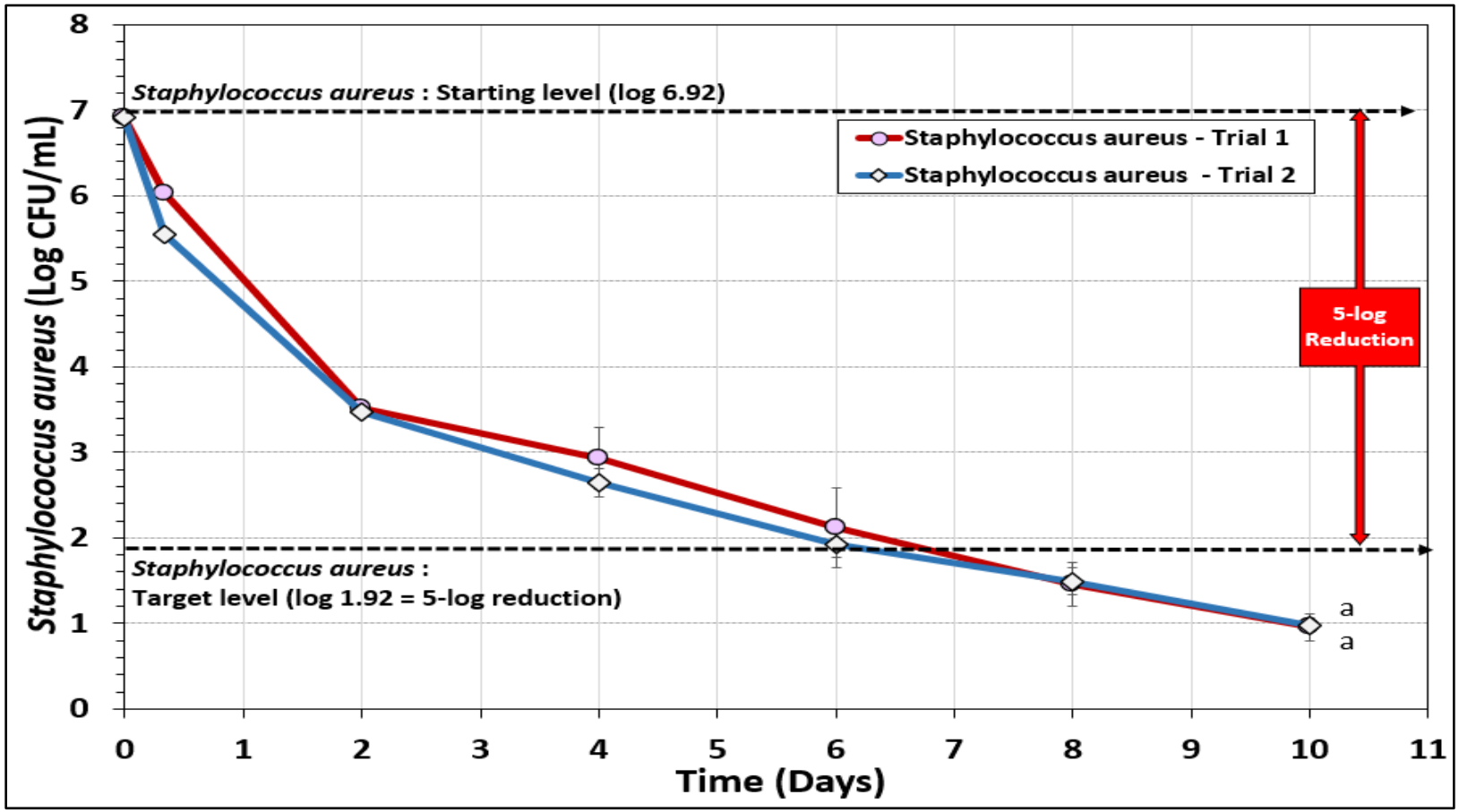

Figure 18. Biltong marination with combined components of the marinade (salt, spice, and vinegar) for the reduction of *Staphylococcus aureus.* After marination the beef pieces were hung and dried at 75 ºF and 55 %RH for up to 10 days. A set of beef pieces without inoculation were considered as negative controls for demonstrating the efficacy of the media used against the background organisms. Trials were performed in duplicate replication (with triplicate samples at each time point; n=6) and trials were analyzed by repeated measures one-way analysis of variance (RM-ANOVA) using the Holm-Sidak test for pairwise multiple comparisons. Trials with the same same letters are not significantly different ($p > 0.05$).

Temperature and Relative Humidity Measurements

The chamber temperatures is shown by 2 overlapping graphs representing 2 thermocouple temperature thermocouple probes inserted at different positions within the chamber. An additional 2 temperature probes were placed in 2 different pieces of biltong beef (un-inoculated) also placed at different positions. A humidity probe was also placed in the center to record %RH in the chamber (Figure 19). Temperature controlled humidity oven was used to dry the beef pieces at 75 ºF and 55 %RH for up to 10 days for reduction of *L. monocytogenes* (Figure 20), *E. coli* O157:H7 (Figure 21), and *S. aureus* (Figure 22) on biltong processed beef. When the temperature reached below the set point, the unit heats up the air and cycles up and down; the air temperature of the chamber varies more than the uniform temperature of the beef pieces. The temperature when reach above the set point the air in the chamber is cooled down by the refrigeration system of the unit. Also, the humidity of the chamber was measured by placing an external humidity probe inside the chamber that was set to 55% relative humidity and a temperature of 75 ºF .

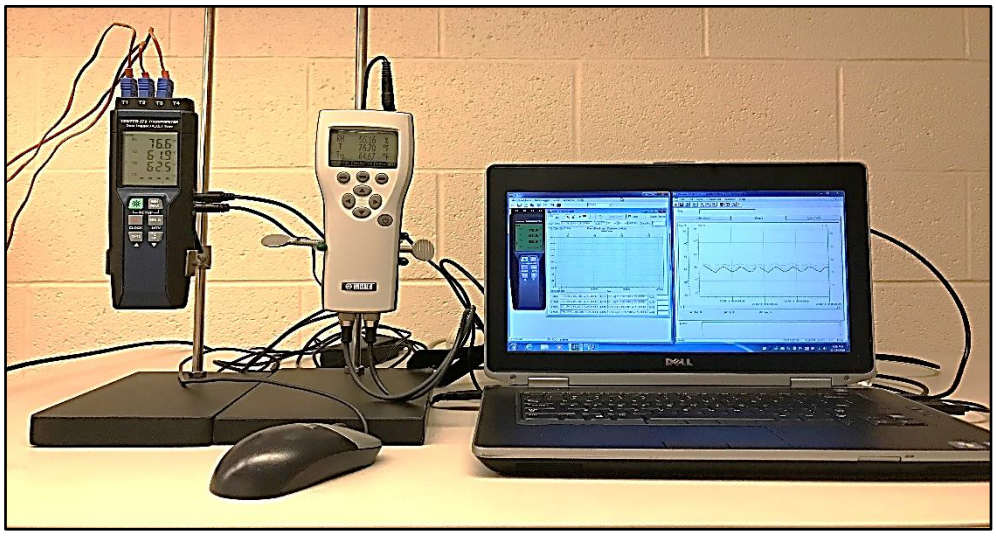

Figure 19. Handheld Centre temperature recorder and Vaisala humidity recorder with laptop for recording temperature and humidity during biltong processing.

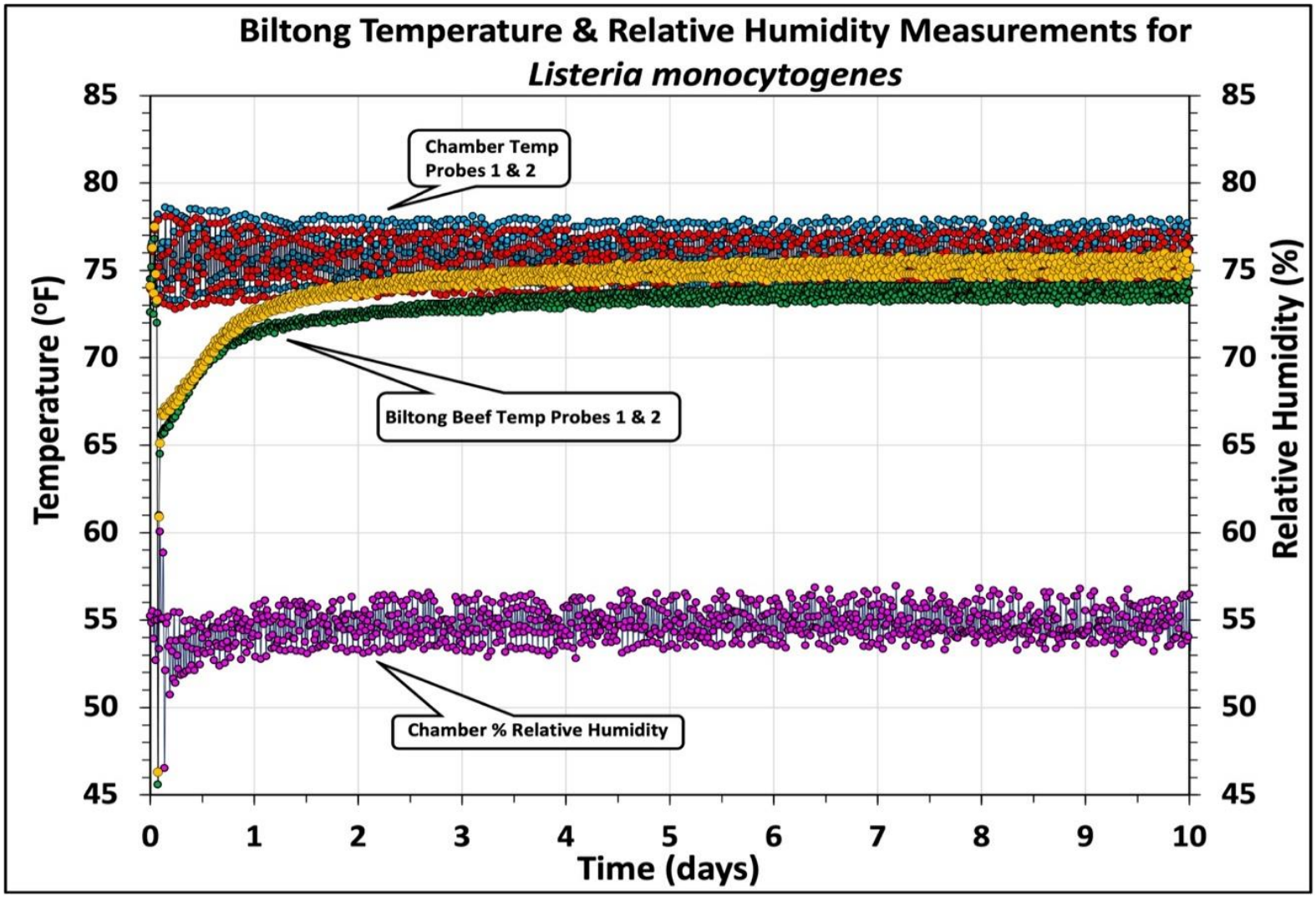

Figure 20. Temperature (°F) and relative humidity (%RH) measurements during processing of biltong during microbial reduction of *Listeria monocytogenes.*

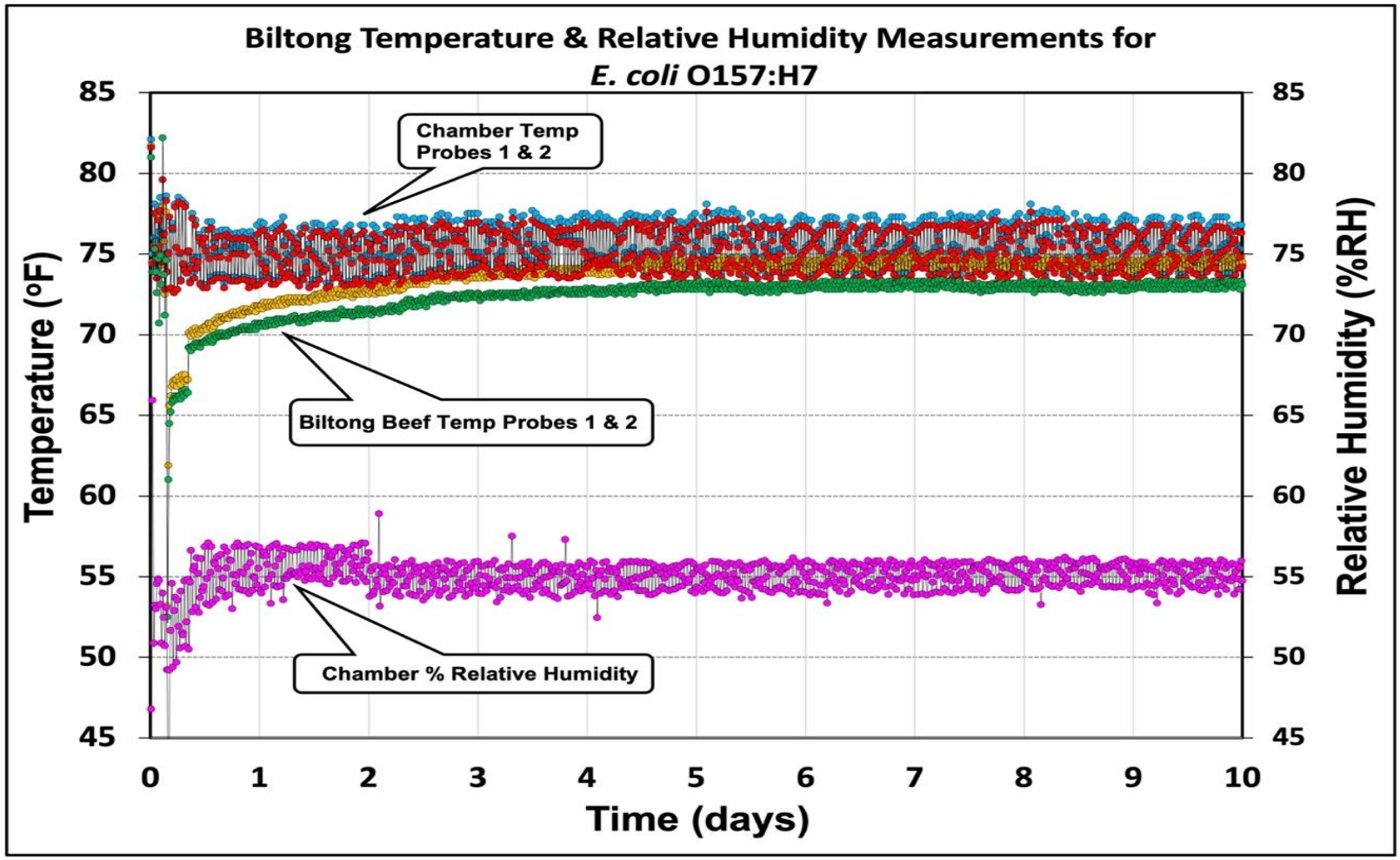

Figure 21. Temperature (ºF) and relative humidity (%RH) measurements during processing of biltong during microbial reduction of *E. coli* O157:H7.

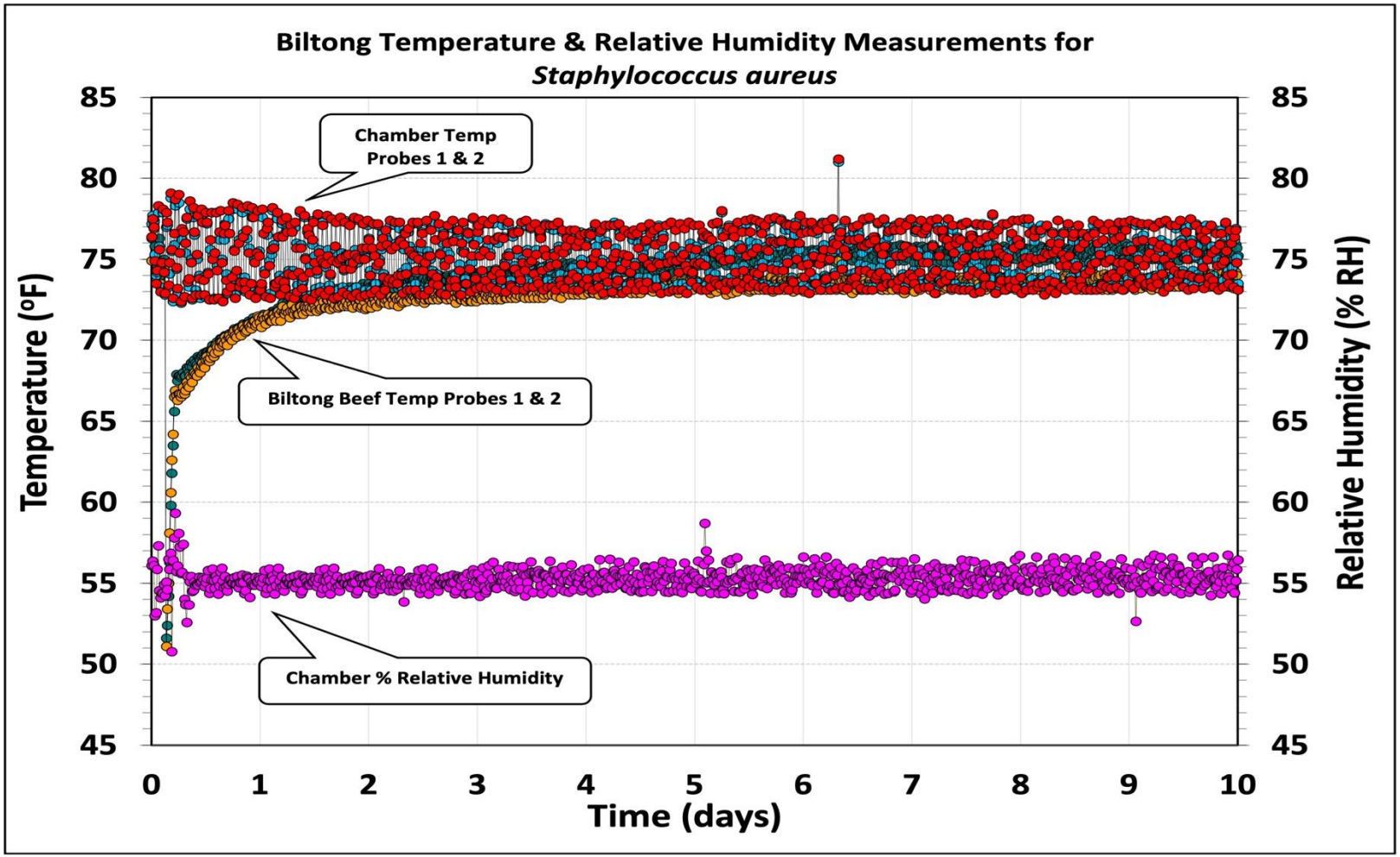

Figure 22. Temperature (ºF) and relative humidity (%RH) measurements during processing of biltong during microbial reduction of *Staphylococcus aureus.*

Water Activity Measurements for Listeria monocytogenes, E. coli O157:H7 and Staphylococcus aureus

Water activity measurements for biltong was determined by using beef samples that are un-inoculated (i.e., negative control) and marinated with spice, salt and vinegar. Water activity was recorded after marination and after 2, 4, 6, 8, and 10 days of drying at 75 ºF and 55% relative humidity. Pieces of beef were cut into half and the internal moist portion was placed upright in a cupule facing the water activity sensor meter and Rotronic USB probe chamber, laptop, and software were used to obtain the values for water activity. After 10 day drying period biltong water activity measurement for *Listeria monocytogenes* (0.79), *E. coli* O157:H7 (0.80) and *Staphylococcus aureus* (0.79) (Figure 23).

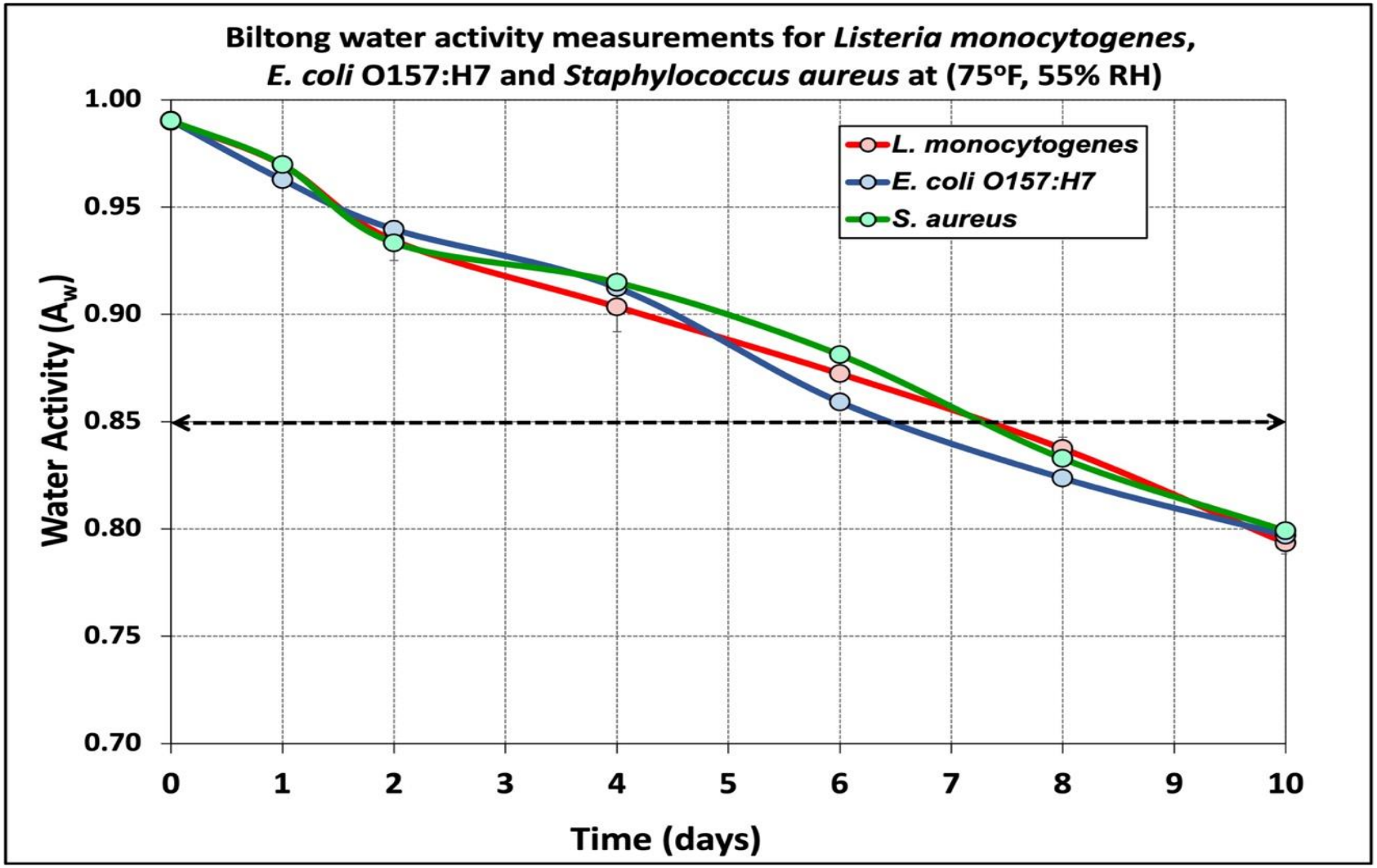

Figure 23. Water activity readings taken during biltong processing for *L. monocytogenes*, *E. coli* O157:H7 and *S. aureus* at 75 ºF and 55 % relative humidity. Each data point is the average of triplicate readings made with duplicate samples at each time point.

Detection of Staphylococcal Enterotoxin A and B During Processing of Biltong for Staphylococcus aureus

The OD values of standards were plotted against ng/ml of antibody standard dilutions and regression analysis was used to determine linearity of fit to the data for SEA (Figure 24) and SEB (Figure 25). The ng/ml of antibody in the samples was calculated from the regression curve. The SEA level detected after post marination looked higher (0.9125 ng/ml) when compared to the SEA level detected after a drying period of 10 days (0.4325 ng/ml) (Figure 24). However, analysis by one-way ANOVA indicated that there was no significant difference ($p > 0.185$) between the 0-day and 10-day SEA levels. Similarly, The SEB level detected after post marination appeared higher (1.0163 ng/ml) when compared to the SEB level detected after a drying period of 10 days (0.4325 ng/ml) (Figure 25). However, again because of the large standard deviations, the 2 data points were determined to not be significantly different by one-way ANOVA ($p > 0.287$).

Hence, from the data obtained we conclude that the staphylococcal enterotoxin was not produced during 10 day drying period. The levels of enterotoxin we detected were likely residual levels that were present from the centrifugation and concentration of 4 strains of *S. aureus* inoculum cells after overnight growth (i.e., SEA and SEB were likely produced in broth culture and residual levels were still present after washing). We had previously shown high levels of SEA/SEB were produced in broth (data not shown).

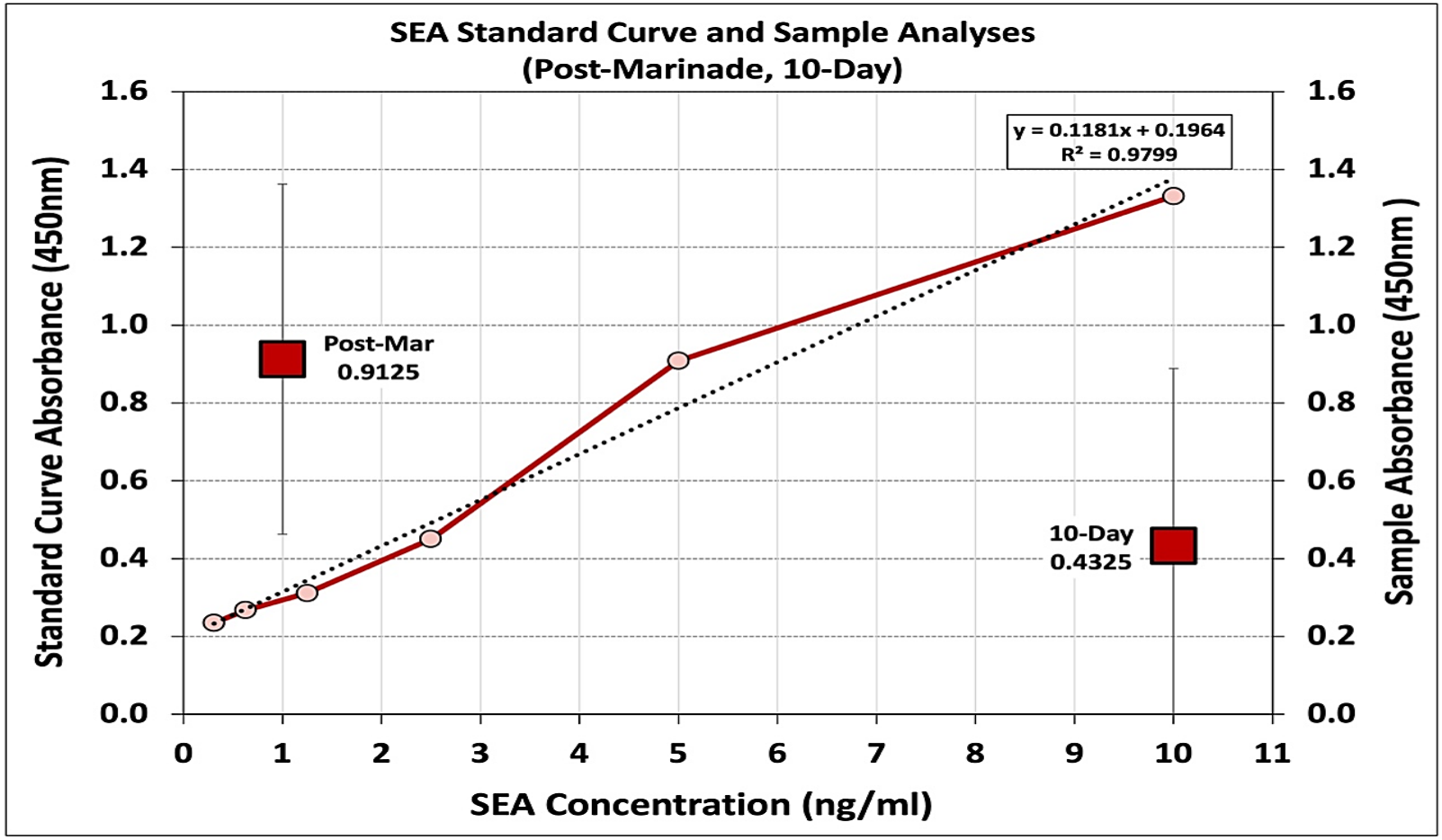

Figure 24. Detection of staphylococcal enterotoxin A (SEA) for biltong after post marination and after 10 day drying period. Cultures included *S. aureus* ATCC 13565 (SEA), *S. aureus* ATCC 8095 (SEA), *S. aureus* ATCC 14458 (SEB) and *S. aureus* ATCC 51740 (SEB). The graph includes standard curve absorbance and sample absorbance at 450 nm. Data obtained represents the average of 2 trials with four samples tested for each trial in duplicate replication after post marination and after 10 day drying period. The SEA level detected after post marination was higher (0.9125 ng/ml) when compared to the SEA level detected after a drying period of 10 days (0.4325 ng/ml). SEA was not produced in the meat through 10 days of drying (i.e., not increased).

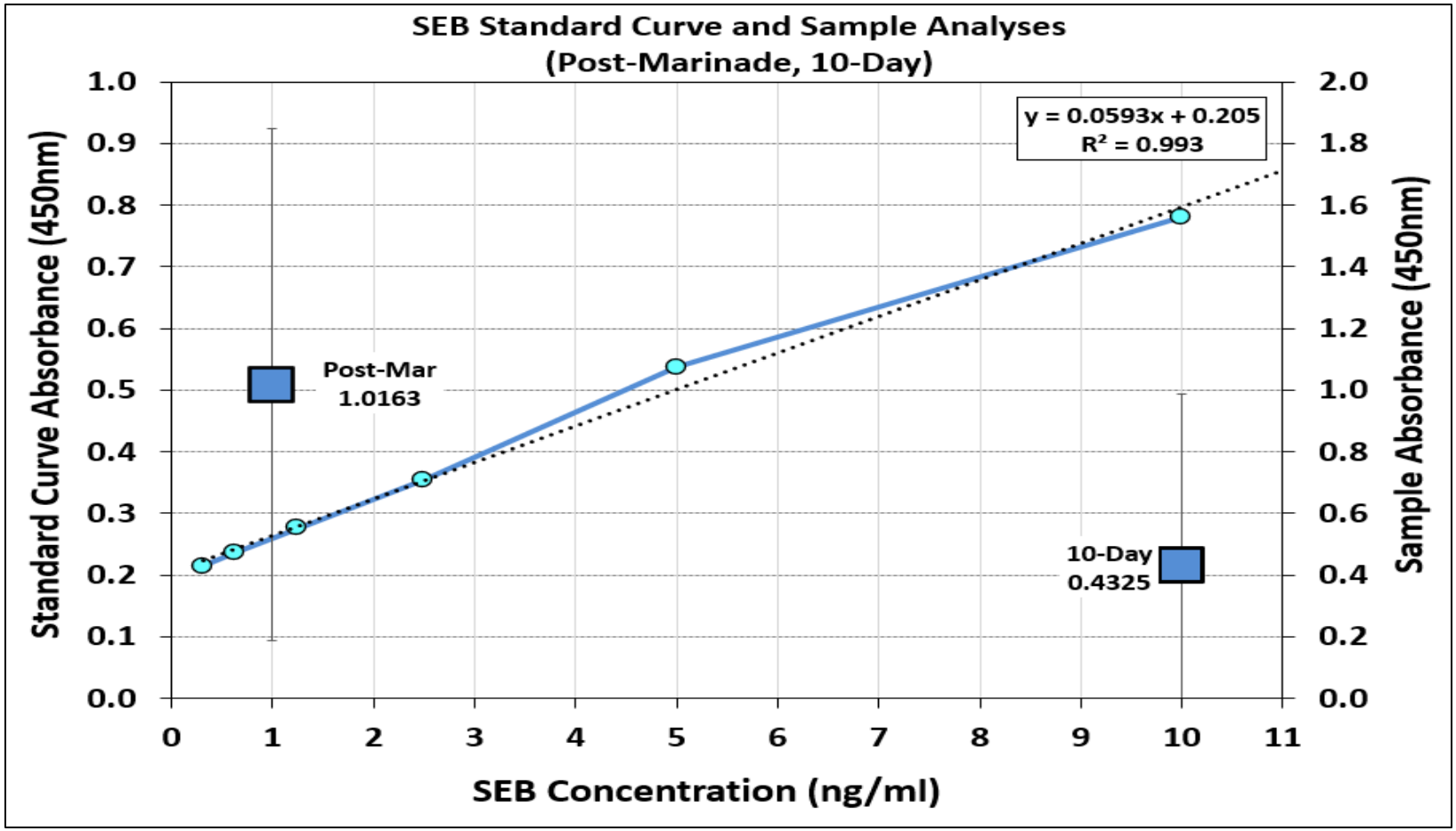

Figure 25. Detection of staphylococcal enterotoxin B (SEB) for biltong after post marination and after 10 day drying period. Cultures included *S. aureus* ATCC 13565 (SEA), *S. aureus* ATCC 8095 (SEA), *S. aureus* ATCC 14458 (SEB) and *S. aureus* ATCC 51740 (SEB). The graph includes standard curve absorbance and sample absorbance at 450 nm. Data obtained represents the average of 2 trials with four samples tested for each trial in duplicate replication after post marination and after 10 day drying period. The SEB level detected after post marination was higher (1.0163 ng/ml) when compared to the SEB level detected after a drying period of 10 days (0.4325 ng/ml). SEB was not produced in the meat through 10 days of drying (i.e., level was not increased).

CHAPTER V

CONCLUSION

A biltong process with complete marinade formulation can achieve a >5-log reduction of *Listeria monocytogenes*, *E. coli* O157:H7, and *Staphylococcus aureus* over a drying period of ~4-8 days at a temperature of 75 ºF and 55% relative humidity. Furthermore, staphylococcal enterotoxin (SEA, SEB) was not produced in the meat through 10 days of drying. By evaluating the reduction *Listeria monocytogenes E. coli* O157:H7 and *Staphylococcus aureus* by treatment with individual components of the marinade (spice, salt, or vinegar), we conclude that treatment with spice alone was not effective in the reduction of pathogens, but vinegar and salt are the key ingredients in achieving this log reduction, even when the cultures are acid-adapted and more resistant to stress conditions of the process (acid, salt, desiccation). Also, enumerating the microorganisms on the basis of CFU/ml eliminated the issue regarding the moisture loss of meat due to drying and rendered 5 log reduction of the *Listeria monocytogenes*, *E. coli* O157:H7 and *Staphylococcus aureus*. Water activity of ≤ 0.85 can be achieved by drying the product at a temperature of 75°F (23.9 °C) and 55 % RH.

The ability to manufacture and sell biltong is dependent on validation studies that demonstrate that the process a company uses can achieve the targeted reduction of the pathogen of concern. The lack of availability of validated biltong studies to small processors may be considered a ‘barrier of entry into the market’. Therefore, the data

provided herein should provide both small and large processors alike sufficient validation for biltong processing to not only achieve USDA-FSIS process approval but also satisfy particular retail merchants and supermarkets that prefer additional pathogen validations even though only a single one is required by USDA-FSIS.

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