PATHOGEN INHIBITION,

SURROGATE VALIDATION

AND MICROBIOME ANAYLSIS DURING

BILTONG PROCESSING

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Abstract: Biltong is a South African dried beef product that is made from beef that is marinated in a mixture of spices, vinegar and salt and then dried at ambient temperature and humidity. USDA-FSIS dried beef processing guidelines require beef jerky products to be cooked in excess of 90% humidity and 145°F. Since biltong processing deviates from these requirements, processors must demonstrate to the USDA-FSIS that their process can achieve a sufficient microbial reduction. There is limited data demonstrating sufficient reduction of foodborne pathogen of interest (*Salmonella*) for biltong manufacturing. The objective of this study was to identify approaches to control *Salmonella* in biltong processing. Validation studies were conducted using beef pieces (1.9-cm x 5.1-cm x 7.6-cm) inoculated with a four-serovar mixture of *Salmonella*, vacuum-tumbled in a marinade comprised of spices, 4% 100-grain red wine vinegar, and 2.2% NaCl and dried in a humidity-controlled oven for 8 days (25°C/75°F; 55% relative humidity). Microbial enumeration of surviving surrogate bacteria and evaluation of intrinsic factors (water activity, pH, salt concentration) were performed post-inoculation, post-marinade, and after 2-, 4-, 6-, and 8 days of drying. Separate validation studies were conducted using potential surrogate organisms (commercially available starter culture lactic acid bacteria and isolates obtained from biltong-processed beef) for in-plant validation of biltong processing. Further microbiome analysis of biltong manufacturing was also done to evaluate changes in the microbiome during processing. Samples were taken at each step of the biltong process. DNA extraction was performed prior to 16S rRNA sequencing and bioinformatic analysis. A 5-log reduction was achieved in all validation studies. *Carnobacterium* sp. was the only tested bacteria with similar activity to pathogens during processing and is recommended as a surrogate for biltong processing. During processing, there was an increase in observed diversity on the raw beef which diminishes after marination and drying, resulting in primarily *Latilactobacillus* sp. on the beef. We believe this is the first published report of a biltong process achieving >5.0 log10 reduction of *Salmonella* which is a process validation recommendation by USDA-FSIS for the sale of dried beef in the USA and helps to fill USDA-FSIS knowledge gaps in air-dried, shelf-stable dried beef.

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Chapter I

Introduction

Dried meats are a popular food throughout the world due to shelf stability and high protein content of the product (Taormina & Sofos, 2014). Drying and curing of meats dates back centuries as an effective way to preserve foods, particularly meat (Wentworth, 1956). Biltong is a specific dried beef product native to South Africa. It is typically made from lean beef rounds that are marinated in a mixture of traditional spices (usually coriander and black pepper), salt and vinegar prior to being dried at ambient room temperature and humidity (Jones, Arnaud, Gouws, & Hoffman, 2017). Traditional drying methods use a wooden box with a fan kept outdoors to house the beef during the drying process. Following drying, the beef is thinly shaven and stored for consumption. There is growing interest to produce biltong in the United States by manufactures who desire to produce the product under traditional processing conditions including drying under lower temperatures and relative humidity. However, given the lack of a high heat lethality step during processing, there is concern for foodborne pathogens to be present on the product making it microbially unsafe for consumers.

In the United States, dried beef products like beef jerky are manufactured under the guidance of the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS). The USDA-FSIS refers biltong manufactures to the "FSIS Compliance Guidelines for Meat and Poultry Jerky Produced by Small and Very Small Establishments"(USDA-FSIS, 2014). The guidance set forth by the USDA-FSIS requires beef jerky type products to be heated to a temperature in excess of 145°F (62.8°C) in combination with the presence of ≥90% relative humidity (USDA-FSIS, 2014, 2017). This is in stark contrast to biltong production which is dried at ambient temperature (70-80°F/ 21.1-26.7°C) and humidity (55% RH). To account for the lack of a high heat lethality or cooking step, the USDA-FSIS has two alternative compliance options for biltong manufactures. The first option requires testing of all processing ingredients prior to use for absence/presence of *Salmonella* sp. (pathogen of concern via compliance guidelines). Additionally, processors must demonstrate their process can achieve an overall ≥2-log reduction. Alternatively, manufactures can choose to demonstrate an overall process reduction of ≥5-log without any additional ingredient testing. The latter option is preferred since it eliminates expensive ingredient testing but requires a more robust manufacturing process to ensure the microbial safety of the product.

There is sufficient concern for the presence of foodborne pathogens on biltong products. Several studies assessed the prevalence of foodborne pathogens in biltong products. One study reported the presence of *Listeria monocytogenes* and enterotoxin-producing *Staphylococcus* strains on South African market available biltong samples (Naidoo & Lindsay, 2010). Other studies have detected *Escherichia coli* O157:H7, *Salmonella* and *Bacillus cereus* on commercially available biltong samples (Matsheka et al., 2014; Van Den Heever, 1970). As demand grows for specialty meat products like biltong, the USDA-FSIS acknowledges that there is insufficient data available for processes that demonstrate a sufficient 5-log reduction of *Salmonella* and other foodborne pathogens of concern including *L. monocytogenes* and *E. coli* O157:H7. Approaches to control human pathogens in dried meat products is considered a "data gap" by the USDA-FSIS and requires more studies to identify safe processes for the manufacture of biltong (USDA-FSIS, Updated 2021). This work is an attempt to understand the limits of biltong processing and bridge the data gap that exists within dried beef manufacturing.

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Chapter II

Review of Literature

Prevalence of *Salmonella* on Dried Beef Products (Beef Jerky)

Salmonella, a rod-shaped, Gram-negative foodborne pathogen, can contaminate raw beef that is used in dried beef processing by several different mechanisms (Tauxe, Doyle, Kuchenmüller, Schlundt, & Stein, 2010). Contamination may come from animal fecal contamination of the hide/carcass and transferred during slaughter/fabrication to subsequent beef subprimals (Beach, Murana, & Acuff, 2002; Sheridan, 1998) . Similarly, contamination may come from contaminated contact-surfaces during slaughter or subsequent fabrication, and/or by contaminated human contact (Wang, He, & Yang, 2018). During further processing (i.e., at the level of the biltong processor) raw beef may be additionally contaminated by introduction from process ingredients that are added to the beef, or again through unsanitary processing environments and human contact contamination (De Filippis, La Storia, Villani, & Ercolini, 2013; Wambui, Lamuka, Karuri, Matorari, & Njage, 2018) .

The prevalence of *Salmonella* on beef jerky products can usually be controlled through a high heat lethality step, the process itself, and the implementation of good manufacturing practices (GMPs) in the processing facility. Several outbreaks involving *Salmonella*-contaminated commercial beef jerky products have occurred. Several outbreaks in the 1980s involving *Salmonella* Newport and *Salmonella* Montevideo in beef jerky were the result of using frozen beef in combination with a drying temperature that was below 60°C which was insufficient to heat the cold beef properly and control *Salmonella* (Eidson, Swell, Graves, & Olson, 2000). More recently, a 2003 *Salmonella* Kiambu outbreak associated with beef jerky in New Mexico was the result of a slow drying process under low humidity conditions (1% Relative Humidity) (CDC, 1995). This outbreak highlighted the need for more regulated manufacturing guidelines to be issued by the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) leading to the establishment of the *Compliance Guideline for Meat and Poultry Jerky Produced by Small and Very Small Establishments* (USDA-FSIS, 2014).

Biltong vs Beef Jerky Processing

Biltong is a popular air-dried meat product common to South Africa. Traditionally, it is usually made from lean strips of beef that are marinated in traditional spices (coriander, black pepper), salt and vinegar (malt vinegar or red wine vinegar) and then dried at ambient temperature and humidity (Naidoo & Lindsay, 2010). This is contrast to North American style beef jerky products that are made from either whole muscle, or chopped and formed meat in a casing, and dried at an elevated temperature of ≥ 160 °F (Burfoot, Everis, Mulvey, Wood, & Betts, 2010; Harrison, Harrison, Rose-Morrow, & Shewfelt, 2001).

As a ready-to-eat (RTE) food commodity, biltong is not reheated or cooked prior to consumption and therefore sufficient control of foodborne pathogens needs to occur during processing. Since biltong does not have a high heat lethality step, the microbial safety of the product is reliant on the combination of the low pH of the vinegar, salt to absorb and draw water out of the product, and drying time to allow for the meat to achieve a low water activity (A_w) and thus prevent microbial growth (Gurtler et al., 2019; Nummer et al., 2004). The combination of multiple critical processing parameters including time, temperature, pH and water activity to control microbial growth is called hurdle technology (Mogren et al., 2018). This approach allows for a multilayered approach to ensure food safety.

USDA-FSIS Policy Regarding Biltong: A_w requirement; Non-Intact Beef

USDA-FSIS has several regulatory stances that may involve biltong. One is 'non-intact' vs 'intact beef' (USDA-FSIS, 2019). There are several 'non-intact' beef situations, each more exceedingly obscure than the previous. The first is ground beef and obviously 'non-intact' because it's ground up and what was on the outside is now on the inside. As a food safety measure, USDA-FSIS requires that ground meat hamburgers be cooked to an internal temperature of 160 °F to ensure that bacteria now existing inside the ground beef be heated to a lethal temperature (USDA-FSIS, 2021b). The second, less obvious 'non-intact beef' is blade/needle-tenderized beef that can translocate bacteria from the surface to the interior of whole muscle beef products (Hajmeer, Ceylan, Marsden, & Phebus, 2000; Heller et al., 2007; Youssef, Yang, & Gill, 2014). The third and least obvious non-intact beef is vacuum-tumbled beef. USDA-FSIS considers beef that has been marinated and vacuum-tumbled to be 'non-intact' because the vacuum process could draw bacteria into the sub-surfaces of the beef, and similar to how marinade components are drawn into the beef. Therefore, biltong beef that is vacuum-tumbled is considered non-intact and therefore must have a safety measure associated with ensuring the safety of the product.

Therefore, USDA-FSIS requires vacuum-tumbled beef to have water activity below 0.91 if kept packaged and refrigerated to ensure safety from *Salmonella*/*E. coli* O157:H7 or below 0.85 if it is considered 'shelf-stable'. The requirement for < 0.85 Aw is to prevent production of staphylococcal enterotoxins if *Staphylococcus aureus* were to be drawn inside of biltong beef during vacuum-tumbling (USDA-FSIS, 2014).

Health Aspects Associated with Biltong

Salt has historically been used for hundreds of years in food for two main purposes: flavoring and preservation. High sodium content is of particular importance for processed, dried or cured meats that traditionally rely on the addition of sodium chloride (NaCl) during processing to help flavor and preserve the meat. The use of salt in dried beef products helps to decrease in A_w by drawing water out of the meat and limiting the amount of free water available for microbial growth

(Taormina, 2010). The salt content in commercially available biltong products can range from 2 to 13% (Van Den Heever, 1970). In additional to microbial safety, salt can also influence the overall flavor, appearance, and texture of the product for customer appeal.

However, sodium intake among consumers has dramatically increased with the consumption of processed foods. High sodium intake is associated with many health issues including hypertension and cardiovascular disease (J. He et al., 1999). The recommended dietary guidelines for daily intake of sodium should be less than 2300 milligrams (mg), however the average adult normally consumes more than 3000 mg per day (Department of Health and Human Services, 2016)

Therefore, it is important for consumers to have options for food products that contain low levels of salt without compromising on the taste, texture, or microbial safety of the food. One strategy to reduce the sodium content in foods while preserving the microbiological inhibitory effects is to replace NaCl with an alternative salt such as KCl or $CaCl_2$. Cation replacement in dried beef formulation can provide for an alternative source of beneficial minerals that are traditionally lacking in modern diets so long that the microbial safety of the product is not compromised (F. J. He & MacGregor, 2008; Miller, Jarvis, & McBean, 2001).

Processing Regulations for Dried Beef Products in the United States

In the United States, beef jerky is manufactured under the purview of the USDA-FSIS. Many processors used to cite USDA-FSIS lethality performance standards for certain meat and poultry products (Appendix A) for their temperature targets during processing (USDA-FSIS, 2017). The citing of the USDA-FSIS Appendix A by many beef jerky processors was in reference to the temperature tables indicating a 6.5-log10 or 7-log10 reduction of *Salmonella* achieved when beef was processed for various times at various temperatures. However, USDA-FSIS pointed out in a subsequent updated compliance guideline, the relative humidity must be maintained above 90% throughout the cooking or thermal heating process by using a sealed oven or steam injection (USDA-FSIS, 2014).

In addition to required processing conditions, the USDA-FSIS also has other requirements for intrinsic factors related to the safety of the shelf-stable dried beef product. The final water activity of a shelf-stable product must be equal to or less than 0.85 with a moisture-to-protein ratio (MPR) of 0.75:1 or less (USDA-FSIS, 2014). The growth of pathogenic organisms (including the production of enterotoxins produced from *Staphylococcus* aureus) and helps to ensure the safety of the product. The exception to this requirement is if the dried meat product is vacuum-packaged in which case the Aw can be as high at 0.91 according the USDA-FSIS.

Validation Studies for Dried Meat Products

Processors who do not adhere strictly to USDA-FSIS guidelines (i.e., humidity) for the manufacture of beef or poultry jerky must provide validation that their process provides adequate pathogen reduction to ensure product is safe and wholesome for human consumption. The factors and parameters for consideration and designing an effective challenge study have been outlined by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) in 2014 and are encouraged for use by the USDA-FSIS (National Advisory Committee on the Microbiological Criteria for Foods, 2010). Through these validation or challenge studies, the USDA-FSIS 'Process Requirement for Biltong' states that a processor can either demonstrate a ≥ 5-log reduction of *Salmonella* or alternatively, demonstrate a ≥ 2-log reduction of *Salmonella* in addition to performing testing of every lot of edible ingredient to insure no *Salmonella* is present (Nickelson, Luchansky,

Kaspar, & Johnson, 1996). *Salmonella* is considered an indicator of lethality in validation studies since it is more heat tolerant compared to *Listeria monocytogenes* (Goodfellow & Brown, 1978). Therefore, a sufficient reduction in *Salmonella* would also validate sufficient critical processing parameters to control *L. monocytogenes* as well.

Methodology to Recover and Enumerate *Salmonella*

Microbial challenge studies of foods are often conducted using pathogens or spoilage microorganisms that are inoculated into targeted food products. After processing or some period of shelf life, the inoculated microorganisms are recovered and enumerated to determine whether the food formulation or food processing conditions inhibits (bactericidal), prevents growth (bacteriostatic), or allows survival and growth (no control) under the conditions of treatment. Such inoculates are often added to products that are not sterile and may include a background of other microorganisms from which the inoculum must be differentially enumerated. Sometimes, no selective media is required for the inoculated microorganisms if the level of inoculum is significantly higher (several orders of magnitude) than the underlying background. Enumeration of un-inoculated control samples provides proof that the background microbiota is well below the inoculum level in test samples.

The use of simple media containing one or more antibiotics for which the added strains are resistant can readily be employed to provide a sufficient 'knock down' of background organisms. Antibiotic resistance can be generated by the selective recovery of low frequency (10^6) spontaneous mutations incurred during selective pressure of high cell levels plated on antibiotic containing media. These mutational changes in DNA can eliminate target binding sites of antibiotics that normally bind to RNA polymerase (rifampin, rifamycin) or ribosomes (streptomycin, spectinomycin, gentamycin) affecting transcription or translation, respectively, and provide stable resistance to those antibiotics. Numerous examples using this basic approach are found in the published literature and is supported by the NACMCF as a method for selective recovery of inoculated strains from foods (National Advisory Committee on the Microbiological Criteria for Foods, 2010).

Alternatively, challenge organisms can also be screened for innate antibiotic resistance using commercial antibiotic discs (i.e., BD Sensi-Discs) and applied to a lawn of bacteria, incubated, and the array of antibiotics not showing a zone is an indication of resistance to that antibiotic (at the level listed on the disc). With either method of obtaining antibiotic-resistant bacteria, enumeration should be performed on both regular media and media with antibiotics to insure there is no affect on microbial enumeration elicited by the antibiotics.

More elaborate approaches have examined whether selective/differential media used for 'detection' of foodborne pathogens may also be adapted for purposes of microbial 'enumeration'. The use of such agars for differential enumeration has often lead to inaccurate underreporting of pathogenic populations because such media are harsh on injured cells and may result in significantly lower microbial counts (Gorski, 2012). Accurate enumeration can be enhanced by a variety of substances that may improve recovery of injured bacteria from stressed conditions. Sodium pyruvate, yeast extract, free radical scavengers (superoxide dismutase, catalase), cations (zinc), or diluent/media buffers may improve recovery of injured bacteria after stressed conditions (Jacobson et al., 2017). In addition to media additives, various layered agar methods (i.e., direct overlay, thin agar overlay, and agar underlay method) have also been used to improve recovery of injured cells that might otherwise be suppressed by selective media.

Use of Surrogates in Process Validation Studies

Validation studies for the processing of dried meats including biltong are commonly conducted in a testing laboratory setting with pathogenic challenge organisms (*Salmonella, Listeria monocytogenes,* STEC *E. coli, Staphylococcus* aureus) to mimic the manufacturing process. However, the strict control on processing conditions like temperature and humidity that are observed in the lab, are not always observed in commercial plants (i.e. more variability in processing conditions in a manufacturing facility). An alternative to pathogenic challenge studies is an in-plant validation study using non-pathogenic organisms which could better evaluate if the commercial process could obtain satisfactory microbial reductions under more variable conditions. An effective surrogate is defined as "a non-pathogenic species and strain responding to a particular treatment in a manner equivalent to a pathogenic species and strain" by the United States Food and Drug Administration (FDA) (FDA, 2002). While no surrogate has been identified for biltong processing, other studies have investigated the efficacy of non-pathogenic organisms for other dried beef and low-moisture products:

Surrogate Organisms Evaluated for Low-Moisture Foods

Enterococcus faecium

Enterococcus faecium NRRL B-2354 has been extensively investigated as a surrogate organism for a wide variety of low-moisture foods including pet food, dried flour and almonds (Jeong, Marks, & Ryser, 2011; Rachon, Peñaloza, & Gibbs, 2016; Verma et al., 2018). In regards to meat products, *E. faecium* NRRL B-2354 was validated as a suitable surrogate for *Salmonella* in high-pressure carbon dioxide lethality-based processing of beef jerky (Schultze, Couto, Temelli, McMullen, & Gänzle, 2020).

Pediococcus sp.

The use of *Pediococcus* spp. has been investigated as a potential surrogate for low-moisture foods. Several studies have looked at commercially available starter cultures, traditionally used for fermenting meat products, that contain species of *Pediococcus* (*P. acidilactici, P. pentosaceus,* etc.) to be used as surrogates for beef processing. In studies of thermal processing of beef jerky, Saga200 (Kerry) and Biosource (Biosource), commercially available *Pediococcus* sp. starter cultures, were used to predict the reduction of both *Salmonella* and *E. coli* O157:H7 (Borowski, Ingham, & Ingham, 2009; Buege, Searls, & Ingham, 2006). Both starter cultures had similar activity to *Salmonella and E. coli* during processing and were recommended as viable surrogate candidates for in-plant validation purposes. Other commercially available starter cultures such as Bactoferm LHP Dry, which is a mixture of P. *acidilactici and P. pentosaceus* was used to successfully evaluate beef carcass intervention treatment (drying and acid/antimicrobial) efficacy (Ingham, Algino, Ihgham, & Schell, 2010). Other non-starter cultures *Pediococcus* spp. include the specific strain *P. acidilactici* ATCC 8042. This specific ATCC strain has been successfully tested as a surrogate in thermal processing of toasted oats cereal, peanut butter and pet food and could be applicable for dried beef products (Ceylan & Bautista, 2015; Deen & Diez-Gonzalez, 2019).

E. coli Biotype I Strains (*E. coli* ATCC BAA 1427-1431)

Non-pathogenic *E. coli* strains were isolated from beef cattle hide at the Department of Animal Science at Iowa State University (Ames, IA, USA) and were tested as meat processing indicators for fresh meat treated with antimicrobial interventions (Marshall, Niebuhr, Acuff, Lucia, & Dickson, 2005). Further testing, evaluated these strains as potential surrogates for *E. coli* O157:H7 during common meat processing conditions. Keeling, Niebuhr, Acuff, and Dickson (2009) found

that at least one of the five biotype I strains tested (BAA 1427-1431) were not statistically different from *E. coil* O157:H7 for each of the common meat processing condition tested (freezing, refrigerating, fermentation, and thermal inactivation). More specifically the study found that strains BAA 1427, 1429 and 1430 were either not significantly different compared to *E. coli* O157:H7 or overpredicted the *E. coli* O157:H7 population during thermal inactivation processing of beef, adding an additional margin of safety when using the *E. coli* biotype I strains as surrogates (Keeling et al., 2009). Further research investigated the *E. coli* biotype I strains as potential surrogates for *Salmonella* as well. All five strains were validated to be used individually or collectively as process validation indicators for *Salmonella* in selected antimicrobial treatments, cold storage and fermentation processes in meat (Niebuhr, Laury, Acuff, & Dickson, 2008). The *E. coli* biotype I strains are supported for use by the USDA-FSIS to use in in-plant validation studies looking to determine process control of *Salmonella* and *E. coli* O157:H7 and have been used widespread in the food industry (Ingham et al., 2010; USDA-FSIS, 2021a).

Microbial Profile of Processed Dried Beef

Microbiome of Dried Beef

The microbiome of a food product is a compilation of the native microbial community of the food ingredients combined with the microbial community of the processing environment (Johansson et al., 2020). The initial bacterial community of the meat consists of mesophilic and psychotropics bacteria. As the meat ages, meat spoilage bacteria such as *Pseudomonas* spp., *Brochothrix thermosphacta,* and various lactic acid bacteria can increase (Dainty & Mackey, 1992; Hilgarth, Behr, & Vogel, 2018). Often, these spoilage bacteria are introduced to the meat during meat processing through contact with equipment and humans (Hultman et al., 2015; Stellato et al., 2016). Further processing of the beef introduces other process ingredients to the beef which may have their own unique bacterial communities. The addition of a marinade to the meat, which is common in dried beef processing, can increase selective lactic acid bacteria such as *Leuconostoc* spp. and *Lactobacillus* spp. in the community composition (Nieminen et al., 2012). On the final dried beef product, the predominate bacteria may include *Bacillus* spp., *Pseudomonas* spp. and *Lactobacillus* spp. (Borch, Kant-Muermans, & Blixt, 1996; Matsheka et al., 2014)

Culture Dependent vs. Culture Independent

Culture based methodology is standard within the food industry to identify bacteria from a food matrix. However, this is not always an accurate representative of the entire microbiome present. Culture dependent methodologies represent less than one percent of the entire bacteria present in complex communities like the gut microbiome (Cao, Fanning, Proos, Jordan, & Srikumar, 2017). Culture-based methods are biased towards those that are culturable, whereas a DNA-based approach can present a more accurate representation of the bacterial community (Jarvis et al., 2018). Sequencing of the 16S rRNA gene, a commonly targeted housekeeping gene present in all bacteria, can be used to profile the microbiomes of foods (D'Amore et al., 2016). This allows for all bacteria to be identified, even given limitations with DNA extraction techniques, rather than only the bacteria that are culturable.

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Chapter III

Selenite Cystine Agar for Enumeration of Inoculated *Salmonella* Serovars Recovered from Stressful Conditions During Antimicrobial Validation Studies

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Abstract: Process validation studies often require the inoculation of select foodborne pathogens into targeted foods to determine the lethality of the process or antimicrobial ingredients, and quantitative recovery of surviving inoculum bacteria helps to make those assessments. Such processes introduce various stressors on the inoculated challenge microorganisms whereby traditional selective media are too harsh to enumerate the remaining viable and injured population quantitatively. Innate antibiotic resistance of challenge organisms has often been used to establish simple selective media (i.e., Tryptic Soy Agar/TSA + antibiotics) for recovering inoculated strains, but sometimes antibiotic resistant background microorganisms are higher than desired. *Salmonella* Thompson 120, *Salmonella* Heidelberg F5038BG1, *Salmonella* Hadar MF60404, *Salmonella* Enteritidis H3527, and *Salmonella* Typhimurium H3380 were characterized for antibiotic resistance and acid adaptation in Tryptic Soy Broth containing 0%, 0.25%, or 1.0% glucose. Sodium pyruvate was evaluated for recovery after stress but no enhancing effect was observed, possibly because the strains were acid-adapted. Selenite Cystine Broth, traditionally used as a selective enrichment broth, was used as the basis for Selenite Cystine Agar (SCA) in combination with three antibiotics to which our Salmonella are resistant. Serovars of Salmonella, both individually and in mixtures, were enumerated on TSA, SCA, Xylose Lysine Desoxycholate (XLD), and Hektoen Enteric (HE) selective agars (all containing the same antibiotics) after conditions of nutrient starvation, desiccation, acid stress, and thermal stress. The data show that quantitative enumeration of our Salmonella serovars on SCA was not significantly different ($p > 0.05$) than those achieved on TSA for all tested stress categories. Levels of Salmonella enumerated on XLD and/or HE were significantly different ($p < 0.05$) than on TSA and SCA and often more than 1–2-log lower, consistent with the inhibition of injured cells. These data confirm that SCA (+ antibiotics) is a suitable selective medium for enumeration of these acid-adapted Salmonella serovars as challenge organisms recovered from various conditions of stress.

Keywords: *Salmonella*; acid adaptation; stress; Xylose Lysine Desoxycholate; Hektoen Enteric; Selenite Cystine; antibiotics; inoculum

1. Introduction

Microbial challenge studies of foods are often conducted using pathogens or spoilage microorganisms that are inoculated into targeted food products. After processing or some period of shelf life, the inoculated microorganisms are recovered and enumerated to determine whether the food formulation or food processing conditions inhibits (bactericidal), prevents growth (bacteriostatic), or allows survival and growth (no control) under the conditions of treatment. Such inoculates are often added to products that are not sterile and may include a background of other microorganisms from which the inoculum must be differentially enumerated. Sometimes, no selective media is required for the inoculated microorganisms if the level of inoculum is significantly higher (several orders of magnitude) than the underlying background. Enumeration of un-inoculated control samples provides proof that the background microbiota are well below the inoculum level in test samples.

The use of simple media containing one or more antibiotics for which the added strains are resistant can readily be employed to provide sufficient 'knock down' of background organisms. Antibiotic resistance can be generated by the selective recovery of low frequency (10^{-6}) spontaneous mutations incurred during selective pressure of high cell levels plated on antibiotic containing media (Knopp & Andersson, 2018). These mutational changes in DNA can eliminate target binding sites of antibiotics that normally bind to RNA polymerase (rifampin, rifamycin) or ribosomes (streptomycin, spectinomycin, gentamycin) affecting transcription or translation, respectively, and provide stable resistance to those antibiotics (Sun et al., 2019; Woodford & Ellington, 2007). Numerous examples using this basic approach are found in the published literature (Flores, 2004; Luchansky et al., 2009; Mann & Brashears, 2006; Muriana & Klaenhammer, 1987; Price et al., 2000), and is supported by the National Advisory Committee for the Microbial Criteria of Food (NACMCF) (NACMCF, 2010) as a method of selective recovery of inoculated strains from foods.

More elaborate approaches have examined whether selective/differential media used for 'detection' of foodborne pathogens may also be adapted for purposes of microbial 'enumeration'. The use of such agars for differential enumeration has often lead to inaccurate underreporting of pathogenic populations because such media are harsh on injured cells and may result in significantly lower microbial counts (Gorski, 2012; Pao, Kalantari, & Huang, 2006). Accurate enumeration can be enhanced by a variety of substances that may improve recovery of injured bacteria from stressed conditions. Sodium pyruvate, yeast extract, free radical scavengers (superoxide dismutase, catalase), cations (zinc), or diluent/media buffers may improve recovery of injured bacteria after stressed conditions (Jacobson et al., 2017; Martin, Flowers, & Ordal, 1976; McDonald, Hackney, & Ray, 1983; Shi, Zhang, Lan, Chen, & Kan, 2019; Williams, Ebel, Hretz, & Golden, 2018; Yan, Gurtler, & Kornacki, 2006). In addition to media additives, various layered agar methods (i.e., direct overlay, thin agar overlay, and agar underlay method) have also been used to improve recovery of injured cells that might otherwise be suppressed by selective media (D. H. Kang & Fung, 1999; Dong Hyun Kang & Fung, 2000; D. H. Kang & Siragusa, 1999; Wu, Fung, Kang, & Thompson, 2001).

The current work describes our efforts to characterize several selective media for enumeration of widely used *Salmonella* serovars that are often added as challenge inocula in process validation studies and recovered after exposure to acidic antimicrobial treatments, nutrient depletion, thermal treatment, or desiccation.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Active cultures were grown in Tryptic Soy Broth (TSB, BD Bacto, Franklin Lakes, NJ, USA) in 9-mL tubes at 37°C. Cultures were maintained for storage by centrifugation (6,000xg, 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 enumeration for all assays was carried out on Tryptic Soy Agar (TSA, BD Bacto; 1.5% agar) and plated in duplicate.

Salmonella serovars used in this study included: *Salmonella enterica subsp. enterica* serotype Thompson 120 (chicken isolate), *Salmonella enterica subsp. enterica* serotype Heidelberg F5038BG1 (ham isolate), *Salmonella enterica subsp. enterica* serotype Hadar MF60404 (turkey isolate), *Salmonella enterica subsp. enterica* serotype Enteritidis H3527 (phage type 13a, clinical isolate), *Salmonella enterica subsp. enterica* serotype Typhimurium H3380 (DT 104 clinical isolate), and *Salmonella enterica subsp. enterica* serotype Montevideo FSIS 051 (beef isolate). These are well-characterized strains that have been used in numerous research publications involving antimicrobial interventions against *Salmonella* spp (Carpenter, Smith, & Broadbent, 2011; Juneja, Eblen, & Marks, 2001; Juneja, Hwang, & Friedman, 2010; Juneja et al., 2012).

Acid adaptation of our *Salmonella* serovars was carried out according to Wilde et al. (Wilde, Jørgensen, Campbell, Rowbury, & Humphrey, 2000) in which cultures were inoculated in TSB augmented with 1% glucose prior to use (Calicioglu, Sofos, Samelis, Kendall, & Smith, 2003). Individual cultures were harvested by centrifugation, and resuspended with 0.1% buffered peptone water (BPW, BD Difco) and held refrigerated until use (5 °C). In situations where a mixed-inoculum was used, the centrifuged and resuspended individual cultures were mixed in equal proportions. All stress tests in this study were performed using acid-adapted *Salmonella* cultures in TSA containing 1% glucose as described above. The US Department of Agriculture, Food Safety Inspection Service (USDA-FSIS) 'highly recommends' the use of acid-adapted cultures when such inoculum strains would be used for stressed conditions to ensure that they are not easily overcome by acidic processing conditions.

Confirmation of pH effects of *Salmonella* grown in media containing glucose was examined in three different TSB media: TSB containing 0% glucose (BD Bacto, BD286220), 0.25% glucose (BD Bacto, BD211825), and 1% glucose (BD286220 + 1% glucose). All cultures were separately inoculated into TSB media containing 0% glucose (in triplicate replication) and incubated overnight at 37 °C; these cultures in turn, were used to inoculate different replicative sets of TSB at 0%, 0.25%, and 1.0% glucose and pH levels of the various cultures were then recorded after 18 hrs at 37 °C.

2.2. Antibiotics, Disc Assay, and Media Validation of Antibiotic Resistance

Five *Salmonella* serovars were tested for innate antibiotic resistance using BD BBL Sensi-Discs (Becton-Dickenson Laboratories, Franklin Lakes, NJ, USA) consisting of sterile paper discs impregnated with specific levels of antibiotic (Sandle, 2016). Bacterial lawns were obtained for individual *Salmonella* serovars by seeding 0.1 mL of overnight culture into 10 mL molten/tempered TSA (0.75% agar), mixed, and overlaid onto pre-poured TSA (1.5% agar) in 150-mm petri plates. When the overlay was solidified, antibiotic discs were aseptically dispensed onto the bacterial lawns and plates were incubated overnight at 37 °C. Following incubation, cultures were evaluated for resistance (no zone) or degree of susceptibility based on subjective size of the inhibitory zone (slightly sensitive, sensitive, very sensitive).

Antibiotics examined included Amikacin (30 ug), Ampicillin (10 ug), Cefazolin (30 ug), Cefotaxime (30 ug), Cefoxitin (30 ug), Cephalothin (30 ug), Chloramphenicol (30 ug), Chloramphenicol (5 ug), Ciprofloxacin (5 ug), Clindamycin (2 ug), Colistin (10 ug), Erythromycin (15 ug), Ethionamide (25 ug), Furazolidone (100 ug), Gentamicin (10 ug), Isoniazid (5 ug), Nalidixic acid (30 ug), Nitrofurantoin (300 ug), Novobiocin (5 ug), Oxacillin (1 ug), Penicillin (10 units), Piperacillin (100 ug), Rifampin (5 ug), Streptomycin (10 ug), Streptomycin (50 ug), Tetracycline (30 ug), Tobramycin (10 ug), Vancomycin (30 ug) (BD Labs).

Antibiotic resistance was confirmed on agar by plating individual cultures grown in TSB (without antibiotics) for comparative enumeration onto TSA plates, with and without individual

antibiotics. This was especially important for combinations of antibiotics to insure the absence of synergistic inhibitory activity when multiple antibiotics are combined. All assays were performed in triplicate replication.

2.3. Salmonella-Selective Agar Media Used for Enumeration of Salmonella spp. after Stressed Conditions

Four selective agar media were compared for enumeration of acid-adapted *Salmonella* serovars after various stress situations including nutrient depletion, acid stress, desiccation, and thermal stress to mimic conditions from which they may be recovered when examining various antimicrobial/processing conditions. The selective media included TSA (non-selective), Selenite Cystine Agar (SCA), Hektoen Enteric (HE), and Xylose Lysine Desoxycholate (XLD) agars. All four of these agar media contained three antibiotics: Spectinomycin (5 ug/mL), clindamycin (5 ug/mL), and novobiocin (50 ug/mL). Each of the individual *Salmonella* serovars were retrieved after consecutive passage (streak isolation) from one agar media to the other to insure clonal isolates would be tolerant of each media before growth and storage of cultures as frozen stocks.

2.4. Preliminary Studies: Evaluation of Sodium Pyruvate for Recovery of Injured Bacterial Cells

In addition to various selective media and antibiotics to which the *Salmonella* strains were resistant, sodium pyruvate (0.1%) was evaluated to determine if it enhanced the recovery of injured *Salmonella.* Optimal levels of sodium pyruvate for recovery of stressed/injured cells have been reported as low as 0.05% to as high as 1.0% (Gurtler & Beuchat, 2005; Lee & Hartman, 1989; Morishige, Fujimori, & Amano, 2013).

2.4.1. Sodium Pyruvate Following Acid Adaptation and Nutrient Starvation

Acid-adapted cultures grown in TSB (1% glucose) were centrifuged and resuspended in a reduced volume of 0.1% BPW to concentrate cells approximately 10-fold and combined in equal amounts. The mixed culture suspension was further diluted in a 10-fold dilution series with 0.1% BPW and maintained at 4 °C for 10 days to induce starvation, as per Wesche et al. (Wesche, Marks, & Ryser, 2005) and Dickson and Frank (Dickson & Frank, 1993). The stored dilutions were then plated on TSA, SCA, XLD, and HE and on the same agars containing 0.1% sodium pyruvate (i.e., TSA-SP, SCA-SP, XLD-SP, HE-SP), and incubated at 37 °C for 48 hrs before enumeration. Each of the agar media contained spectinomycin (5 ug/mL), novobiocin (50 ug/mL), and clindamycin (5 ug/mL).

2.4.2. Sodium Pyruvate Following Acid Adaptation, Salt Desiccation, and Acid Exposure

Mixed acid-adapted *Salmonella* serovars, as described above, were surface-inoculated (150 uL/side) onto ~100 gm beef pieces, vacuum-marinated (15 inches Hg) for 30 min in a vacuum tumbler (Biro VTS-43, Marblehead, OH, USA) in a biltong spice blend containing 2% salt and 3% vinegar (10% acetic acid) (% of ingredient is listed as a % of total formulation). Following marination, beef samples were stomached with 100 mL of neutralizing buffered peptone water (nBPW; Hardy Diagnostics, Santa Maria, CA, USA) from which additional 10-fold dilutions were made in 0.1% BPW. These dilutions were then plated on the same selective plates described above (with and without sodium pyruvate; all containing three antibiotics) and incubated at 37 °C for 48 hrs before enumeration.

2.5. Stress Conditions for Enumeration

Multiple selective agar media (TSA, SCA, XLD, and HE) containing spectinomycin (5 ug/mL), clindamycin (5 ug/mL), and novobiocin (50 ug/mL) were compared for enumeration of *Salmonella,* either individually or in mixture, under different stress conditions including: nutrient depletion, acid stress, desiccation, and thermal treatment.

2.5.1. Nutrient Depletion and Cell Starvation

Each of five individual *Salmonella* serovars, in triplicate replication, were grown in TSB containing 1% glucose, serially diluted in 0.1% BPW, and surface plated immediately after harvesting; at this point, these were referred to as 'fresh cells'. The *Salmonella* were plated onto four selective media (TSA, SCA, HE, and XLD), each containing three antibiotics: Spectinomycin (5 ug/mL), clindamycin (5 ug/mL), novobiocin (50 ug/mL). Plates were then incubated at 37 °C for 48 h. Nutrient starvation was assessed by maintaining the dilution tubes used for the fresh cell experiment at 4 °C and determining the bacterial populations after extended three- and six-week intervals (Figure 1A). The trials were performed in triplicate, with each dilution plated in duplicate, on TSA, SCA, HE, and XLD (all containing three antibiotics), and incubated for 48 h at 37 °C.

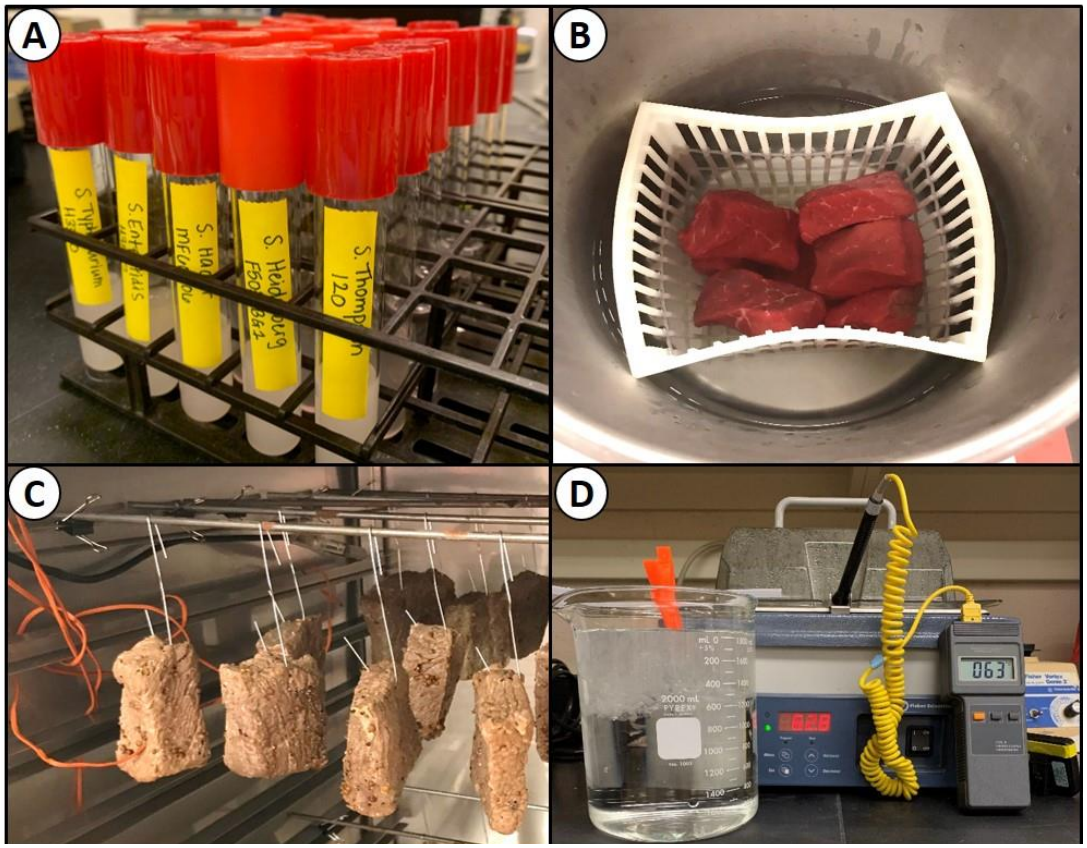

Figure 1. Stress conditions for *Salmonella* serovars: (**A**) nutrient depletion/starvation by extended refrigeration of individual *Salmonella* serovars in 0.1% BPW; (**B**) acid stress by dip treatment of *Salmonella*-inoculated beef in acidic solutions (vinegar, 5%; 3% sodium acid sulfate; 5% lactic acid); (**C**) desiccation of *Salmonella*-inoculated and spice-coated beef at 23.9 °C (75 °F) and 55% RH in a temperature-controlled humidity oven; (**D**) thermal heating of vacuum-packaged bags containing *Salmonella* inocula at 62.8 °C (145 °F) for 75 sec.

2.5.2. Antimicrobial (Acid) Stress

Intact, select grade, beef bottom-round sub-primal cuts (Ralph's Packing Co., Perkins, OK, USA) were trimmed to approximately 0.75-in thick × 2-inch wide × 3-inch long 'steaks' at the R.M. Kerr Food & Agricultural Product Center (FAPC). Beef pieces used for this experiment were vacuum-packaged fresh, stored frozen (−20 °C), and thawed immediately before use. The beef pieces were inoculated by pipette with 150 uL of the 5-serovar *Salmonella* mixture on each side, and immediately spread with a 'gloved finger'. Inoculated beef pieces were then incubated for 30 min at 4–5 °C to allow for bacterial attachment prior to use. Following incubation, the inoculated beef was dipped in white vinegar (5% acetic acid), lactic acid (5%), or sodium acid sulfate (SAS, 3%) for 30 s and excess liquid was allowed to drain before proceeding (Figure 1B). Meat samples were transferred to filter-

stomaching bags, followed by the addition of 100 mL of 1% nBPW (Hardy Diagnostics) and then stomached for 90 sec in a Masticator paddle-blender (IUL Instruments, Barcelona, Spain). Samples were withdrawn, serially-diluted with 0.1% BPW, and dilutions surface plated (0.1 mL) in duplicate, on TSA, SCA, HE, and XLD plates (each containing spectinomycin, clindamycin, and novobiocin) and incubated at 37 °C for 48 h before enumeration. All trials were performed as separate experiments in triplicate replication.

2.5.3. Desiccation and Drying

Beef pieces, as described previously, were inoculated with 150 uL of a five-serovar *Salmonella* mixture on each side that was spread with a gloved finger and held at 4–5 °C for 30 min to allow for bacterial attachment. The beef pieces were then tumbled (without vacuum) in a biltong spice mixture (2% salt) for 5 min until pieces were evenly coated. Beef pieces were then hung in a temperature-controlled humidity oven (Hotpack, Warminster, PA, USA) at 23.9 °C (75 °F) and 55% relative humidity (RH) and allowed to dry for up to 4 days (Figure 1C). Beef was sampled after inoculation (0 days), and after 2 and 4 days of drying. Beef samples were stomached with 1% nBPW and then serially diluted in 0.1% BPW. Serial dilutions were plated in duplicate (0.1 mL) on the surface of TSA, SCA, HE, and XLD (each containing spectinomycin, clindamycin, and novobiocin) and incubated for 48 h at 37 °C. Treatments were performed in triplicate replication.

2.5.4. Thermal Stress

Salmonella serovar cultures were grown in TSB (+ 1% glucose) as described earlier, harvested by centrifugation, resuspended in fresh/sterile TSB (+ 1% glucose), mixed in equal volumes, and held on

$$\% \, Injury = \frac{[(count\ on\ nonselective\ agar) - (count\ on\ selective\ agar)]}{(count\ on\ nonselective\ agar)} \text{ x } 100$$

ice until used. Then, 1.0 mL of the mixed culture was heat-sealed as a thin layer in vacuum-package bags and heated at 145 °F (62.8 °C) for 75 sec (Figure 1D). The cultures were then removed to ice water to chill for 15 min and held at room temperature for 5 min. Dilutions were then made in 0.1% BPW and surface plated (0.1 mL) in duplicate onto TSA, SCA, HE, and XLD agars (each containing spectinomycin, clindamycin, and novobiocin) and incubated for 48 h at 37 °C. All treatments, including inoculations, were performed in triplicate replication.

2.6. Bacterial Injury

The degree of bacterial sublethal injury was determined by comparing microbial counts on nonselective media (TSA) to those on selective media (XLD) for various treatments in the prior trials according to the equation described by Wesche et al. (Wesche et al., 2005) :

2.7. Statistical Analysis

Each trial was performed in triplicate replication and all replications were performed as autonomous and separate experiments using separately inoculated cultures and prepared plating media. All data were presented as the mean of triplicate replications with standard deviation of the mean represented by error bars. Statistical analysis was done using one-way analysis of variance (ANOVA) and the Holm–Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$). Data treatments with different letters are significantly different ($p < 0.05$); treatments with the same letter are not significantly different ($p > 0.05$).

3. Results

3.1. Acid Adaptation of Salmonella Cultures

Cultures were grown in TSB containing 1% glucose in order to 'acid adapt' them to low pH for all stress conditions used in this study. The pH of each of five different *Salmonella* serovar cultures was examined after growth in TSB, with and without glucose (0%, 0.25%, and 1.0% glucose). Growth in TSB without glucose resulted in spent culture broth pH near neutrality (i.e., average, pH 6.7) whereas the culture broth pH of those grown in TSB with glucose were significantly lower (i.e., 0.25% glucose, average, pH 5.8; 1.0% glucose, average, pH 4.9) (Figure 2).

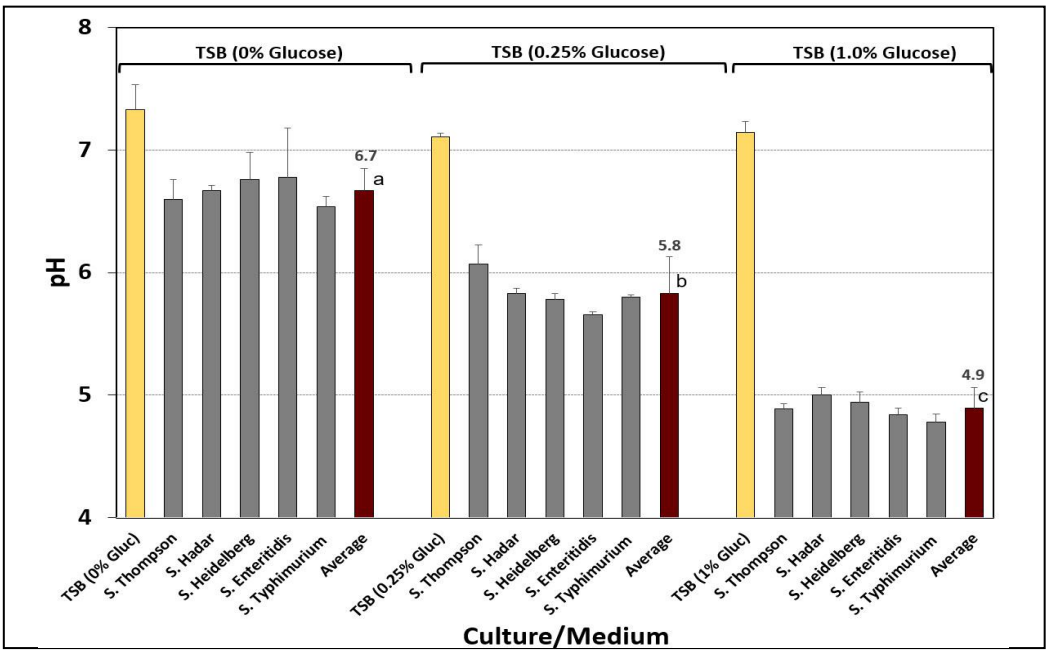

Figure 2. Analysis of spent broth pH for *Salmonella* cultures grown in Tryptic Soy Broth (TSB) containing 0%, 0.25%, or 1% glucose incubated at 37 °C for 18 hrs. The data bars in each set are pH values for the medium before inoculation, the five individual cultures after growth, and the average pH of the five cultures. Cultures include *S.* Thompson 120, *S.* Heidelberg F5038BG1, *S.* Hadar MF60404, *S.* Enteritidis H3527, and *S.* Typhimurium H3380. Data are presented as the mean of triplicate replications and 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 the Holm–Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$); means with different letters are significantly different ($p < 0.05$).

3.2. Determination of Salmonella Antibiotic Resistance (Disc Assay)

Antibiotic resistance for six *Salmonella* serovars was examined using BD BBL Sensi-Discs on lawns of individual *Salmonella* serovars and scored qualitatively for sensitivity or resistance to the antibiotic discs (Table 1; only those antibiotics for which four or more strains were resistant are shown). 'Resistance' was characterized as an antibiotic disc that showed no inhibition zone around the periphery of the disc while various degrees of sensitivity were subjectively attributed according to size of a visible inhibition zone.

Table 1. Antibiotic disc assay of select *Salmonella* serovars showing multidrug resistance (Res, resistant; Sens, sensitive; S-sens, slightly sensitive; V-sens, very sensitive)

Salmonella Serovars	Clin CC 2*	Novo NB 5	Oxa OX 1	Pen P 10	Van VA 30	Spec 10 ug	Amo AMC 30	Amp AM 10	Pip PIP 100	Str S 10
***S*. Enteritidis H3527**	Res	Res	Res	Res	Res	Res	Sens	Sens	Sens	Sens
***S*. Hadar MF60404**	Res	Res	Res	Res	Res	Res	Res	Res	Res	Res
***S*. Heidelberg**	Res	Res	Res	Res	Res	Res	Res	Res	Res	Res
***S*. Montevideo FSIS 051**	Res	Res	Res	Res	Res	Res	Sens	S-Sens	V-Sens	S-Sens
***S*. Thompson 120**	Res	Res	Res	Res	Res	Res	Res	Res	Res	Res
***S*. Typhimurium H3380**	Res	Res	Res	Res	Res	Res	Res	Res	Res	Res

Antibiotics: Clin (Clindamycin), Novo (Novobiocin), Oxa (Oxacillin), Pen(Penicillin), Van (Vancomycin), Spec (Spectinomycin), Amo (Amoxicillin), Amp (Ampicillin), Pip (Piperacillin), Str (Streptomycin).

*BD BBL Sensi-Disc product designations.

3.3. Confirmation of Salmonella Antibiotic Resistance (Plating on Agar Containing Antibiotics)

Based on the antibiotic disc assay (Table 1), 'on agar' antibiotic resistance was confirmed by growing cultures in TSB (without antibiotics) and plating the cultures on TSA alone, and on TSA containing the chosen antibiotics (Figure 3). This was done to determine if there was any antagonism that might be at play when multiple antibiotics are added in combination (Bollenbach, 2015; Ocampo et al., 2014; Singh & Yeh, 2017; Yilancioglu, 2019).

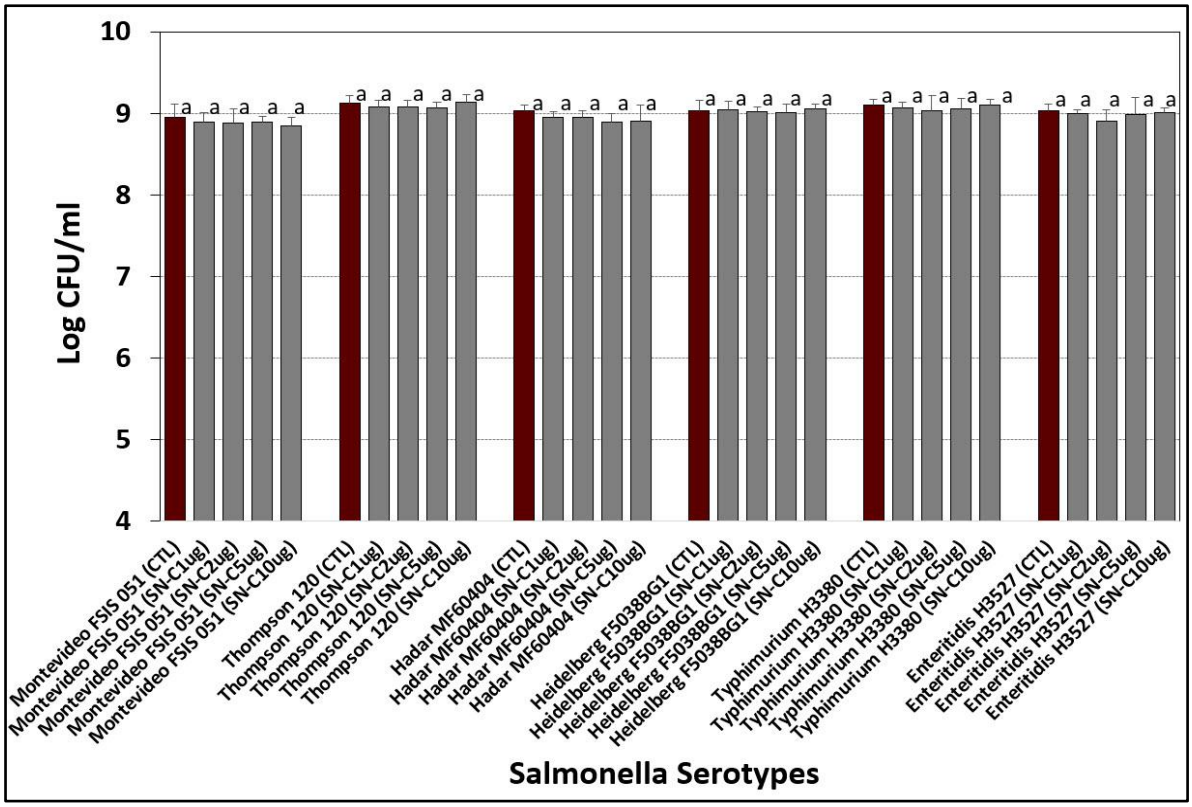

Figure 3. Evaluation of *Salmonella* serovars on TSA alone as Control (CTL) vs. TSA containing spectinomycin (S, 10 ug/mL), novobiocin (N, 100 ug/mL), and increasing amounts of clindamycin (C, 1-, 2-, 5-, and 10 ug/mL). Cultures include *S.* Montevideo FSIS 051, *S.* Thompson 120, *S.* Heidelberg F5038BG1, *S.* Hadar MF60404, *S.* Enteritidis H3527, and *S.* Typhimurium H3380. Cultures were grown 18–20 hrs at 37 °C in TSB (without antibiotics), 10-fold dilutions in 0.1% BPW, and plated on the various media as indicated. Data are presented as the mean of triplicate replications and error bars represent the standard deviation from the mean. Within the same serotype, comparisons of means with the same letters are not significantly different ($p > 0.05$) as determined by one-way ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences.

3.4. Selective Agar Media Containing Antibiotics

Based on the microbial platings of the individual *Salmonella* serovars on TSA containing spectinomycin, novobiocin, and clindamycin at 10-, 100-, and 10-ug/mL, respectively, we felt these levels, or even slightly lower in TSA or other media, would provide a good 'selective medium' for our specific *Salmonella* serovar inoculum in various applications with raw meat if we could demonstrate enumeration equivalent to that on TSA. Additional platings with HE and XLD agars, both with and without antibiotics, showed no significant differences ($p > 0.05$) (data not shown).

3.4.1. Tryptic Soy Agar and Selenite Cystine Agar Containing Antibiotics

TSA containing spectinomycin (5 ug/mL), clindamycin (5 ug/mL), and novobiocin (50 ug/mL) was examined in a variety of situations where multi-strain (serovar) combinations of *Salmonella* were used. However, we obtained very high background counts on some samples of raw meat on TSA without antibiotics (Figure 4A, top row). When plated on TSA containing the three antibiotics (i.e., 3abc), the background levels were reduced by approximately 3 logs, but still had significant levels of colonies on the lowest dilution (Figure 4A, middle row). This background was further minimized when we used SCA in combination with our antibiotics for which our *Salmonella* serovars were resistant (Figure 4A, bottom row). Each of the *Salmonella* serovars gave slightly different colony sizes on TSA + 3abc for the same incubation time (data not shown) whereas they grew luxuriously on the SC agar (SCA) giving equivalent-sized and perfectly round colony morphologies (Figure 4B).

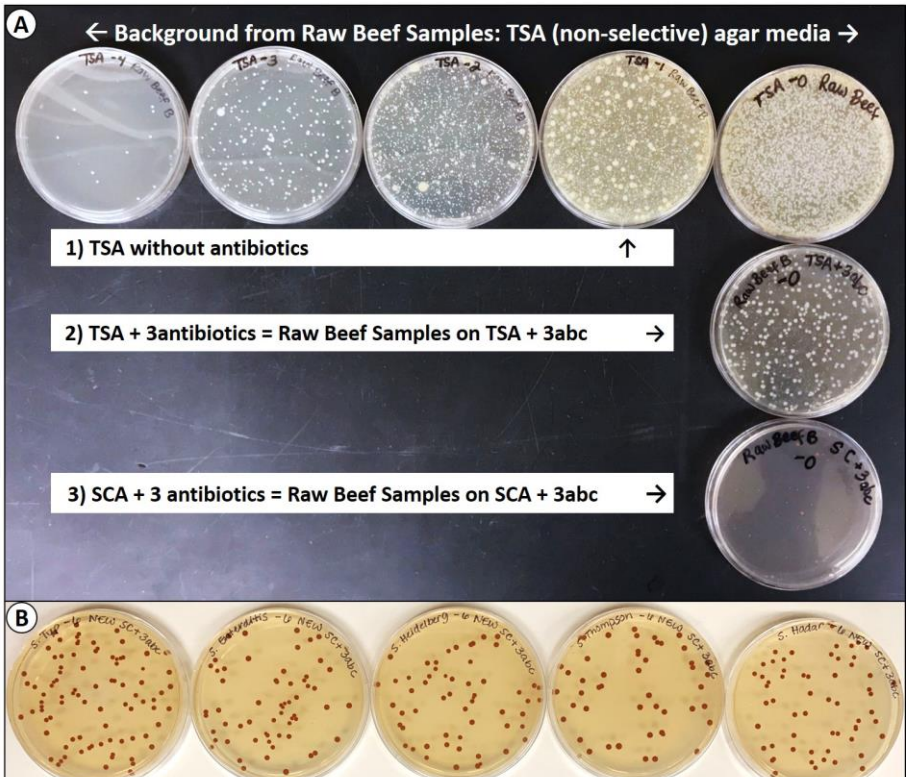

Figure 4. Comparison of TSA (without antibiotics) vs. TSA and selinite cystine agar (SCA) (with antibiotics). (A) Plating of samples taken from fresh raw meat onto TSA without antibiotics (top row), vs. TSA containing antibiotics (2nd row), vs. SCA containing antibiotics (3rd row). The "3abc" represents the 'three antibiotics' described previously: Spectinomycin (5 ug/mL), novobiocin (50 ug/mL), and clindamycin (5 ug/mL). (B) Individual *Salmonella* serovars surface plated (10^{-6} dilution) on SCA containing three antibiotics.

3.5. Comparitive Enumeration on Selective Agars with and without Sodium Pyruvate.

Selective agars were formulated that consisted of TSA, TSA-SP, SCA, SCA-SP, XLD, XLD-SP, HE, and HE-SP, all containing three antibiotics (spectinomycin, 5 ug/mL; novobiocin, 50 ug/mL; clindamycin, 5 ug/mL). Individual *Salmonella* serovars, or equal mixtures of them, were enumerated on these four media after various stress conditions.

3.5.1. Evaluation of Sodium Pyruvate for Recovery of Injured Cells

Sodium pyruvate was examined as a possible supplemental ingredient for the recovery of injured bacterial cells from acid-adapted and nutrient-starved Salmonella (Figure 5A) as well as acid-adapted and 2% salt/3% vinegar-marinaded *Salmonella* (Figure 5B). When nutrient-starved Salmonella were plated on the various selective media, no statistical difference ($p > 0.05$) was observed between TSA, TSA-SP, SCA, and SCA-SP, even though the data for SCA was slightly higher than TSA (Figure 5A). However, microbial counts on both HE and XLD (with and without sodium pyruvate), were approximately 1.5-log lower and significantly different ($p < 0.05$) than TSA and SCA. No significant differences ($p > 0.05$) were observed between HE and HE-SP, nor between XLD and XLD-SP (Figure 5A).

When inoculated beef pieces were subjected to a salt/vinegar spice marinade and vacuum-tumbled for 30 min, we observed lower counts as would be expected from salt/vinegar marination (inhibition) and subsequent sample stomaching of beef in 100 mL buffer (dilution). Results show that no significant difference ($p < 0.05$) was observed among TSA- or SCA-based media (with or without sodium pyruvate), nor between HE and HE-SP or XLD and XLD-SP (Figure 5B). Again, enumerations on HE, HE-SP, XLD, and XLD-SP were significantly different ($p < 0.05$) and lower than on TSA and SCA (Figure 5B).

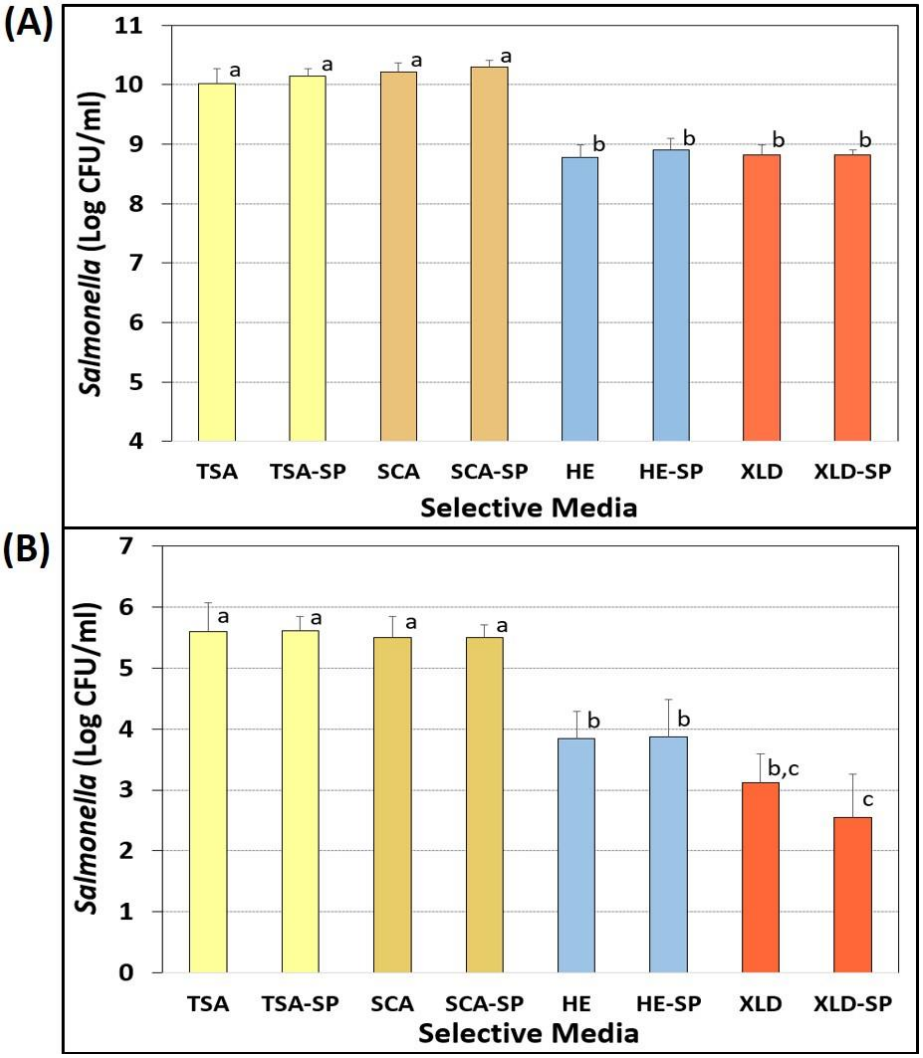

Figure 5. Evaluation of sodium pyruvate for recovery of injured *Salmonella* (**A**) after 10-day nutrient starvation or (**B**) after 30-min salt and vinegar (acid) marinade treatment on inoculated beef pieces. Enumeration of mixed-*Salmonella* serovars on TSA, SCA, Hektoen Enteric (HE), Xylose Lysine Desoxycholate (XLD), and on the same media containing 0.1% sodium pyruvate (TSA-SP, SCA-SP, HE-SP, XLD-SP). All media contained spectinomycin (5 ug/mL), novobiocin (50 ug/mL), and clindamycin (5 ug/mL). Cultures included *S*. Thompson 120, *S*. Heidelberg F5038BG1, *S*. Hadar MF60404, *S*. Enteritidis H3527, and *S*. Typhimurium H3380. Data are presented as the mean of triplicate replications, and error bars represent the standard deviation from the mean. Comparisons of means with different letters are significantly different ($p < 0.05$) as determined by one-way ANOVA using the Holm–Sidak test for pairwise multiple comparisons; means with the same letter are not significantly different ($p > 0.05$).

3.6. Salmonella Stress Conditions: Nutrient Depletion and Starvation

Individual *Salmonella* serovars (in triplicate) were plated on four selective media containing three antibiotics after fresh growth (18 hrs, 37 °C) in TSB (1% glucose) (Figure 6). No significant differences were observed between counts on TSA or SCA, whereas three of five serovars showed slightly lower, but no significant differences when enumerated on HE, and one of five serovars showed significantly lower counts on XLD of ~1.4-log lower levels (Figure 6A). When the same triplicate dilution tube series (in 0.1% BPW) was held at refrigeration temperature (4 °C) and plated again after 3- and 6-weeks, significant differences were observed for platings on HE and XLD which showed 2–3 log lower counts relative to platings on TSA and SCA (Figures 6B, 6C).

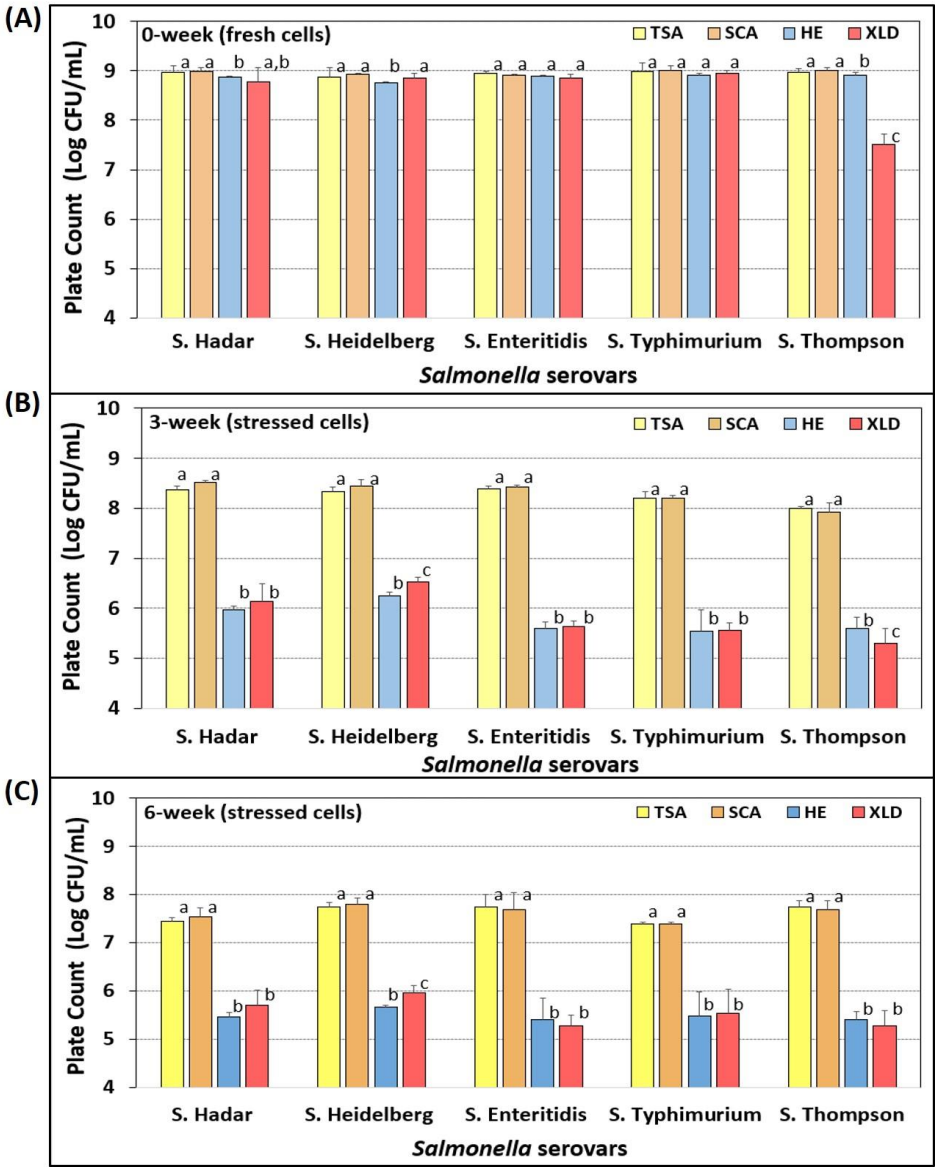

Figure 6. Comparison of four selective media for enumeration of individual *Salmonella* serovars after extended nutrient starvation for 0-weeks (**A**), 3-weeks (**B**), and 6-weeks (**C**) at 4 °C. Each serovar was plated on TSA, SCA, HE, and XLD containing spectinomycin (5 ug/mL), novobiocin (50 ug/mL), and clindamycin (5 ug/mL). Cultures included *S.* Thompson 120, *S.* Heidelberg F5038BG1, *S.* Hadar MF60404, *S.* Enteritidis H3527, and *S.* Typhimurium H3380. Data are presented as the mean of triplicate replications of cultures and their dilutions, and error bars represent the standard deviation

from the mean. Within the same serotype, comparisons of means with different letters are significantly different ($p < 0.05$) as determined by one-way ANOVA using the Holm–Sidak test for pairwise multiple comparisons; means with the same letter are not significantly different ($p > 0.05$).

3.7. Salmonella Stress Conditions: Exposure to Acidic Antimicrobials

Beef pieces inoculated with a five-serovar mixture of *Salmonella* were subjected to 30-sec dip treatment in vinegar (5% acetic acid), sodium acid sulfate (3%), or lactic acid (5%). The beef pieces were stomached in neutralizing BPW, further diluted in 0.1% BPW, and plated onto each of four different selective media described above: TSA, SCA, HE, and XLD, all containing three antibiotics (spectinomycin, 5 ug/mL; novobiocin, 50 ug/mL; clindamycin, 5 ug/mL). Nearly the same results were obtained within each antimicrobial dip treatment: No significant differences ($p > 0.05$) were observed between TSA and SCA, which showed a modest 0.3–0.6-log reduction from the controls (Figure 7). However, enumeration on HE and XLD agars showed approximately 1.5–2.0 log lower counts than that obtained using the same dilutions for samples plated on TSA and SCA.

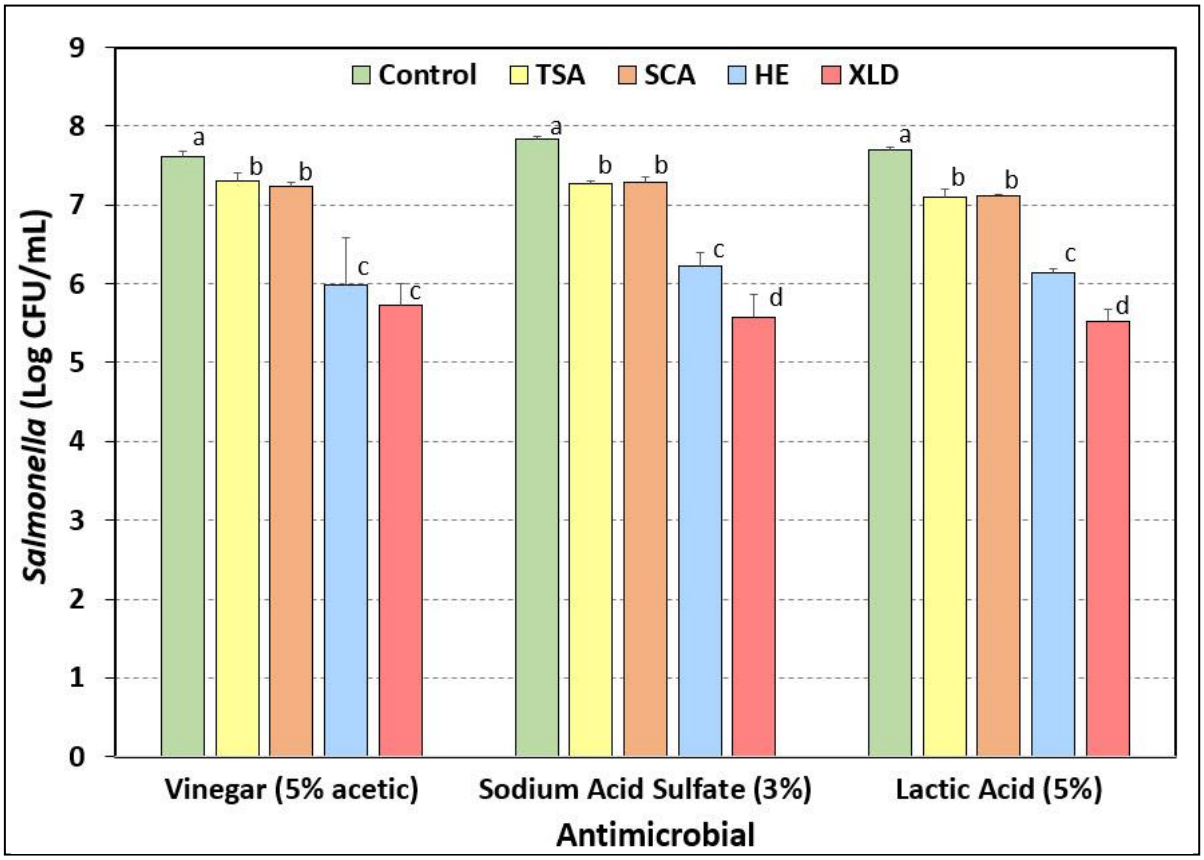

Figure 7. Acid-stress response of five-serovar mixtures of *Salmonella* inoculated on the surface of raw beef and subject to 30-sec dip treatment in vinegar (5% acetic acid), sodium acid sulfate (3%), and lactic acid (5%). Treated samples were then plated on TSA, SCA, HE, and XLD containing spectinomycin (5 ug/mL), novobiocin (50 ug/mL), and clindamycin (5 ug/mL). Cultures in the mixture included *S*. Thompson 120, *S*. Heidelberg F5038BG1, *S*. Hadar MF60404, *S*. Enteritidis H3527, and *S*. Typhimurium H3380. Data are presented as the mean of triplicate replications and error bars represent the standard deviation from the mean. Control represents inoculum level on beef pieces before antimicrobial treatment. Means with different letters are significantly different ($p < 0.05$) as determined by one-way ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences; means with the same letter are not significantly different ($p > 0.05$).

3.8. Salmonella Stress Conditions: Desiccation and Drying

Beef pieces inoculated with a five-serovar mixture of *Salmonella* were dry-marinaded with biltong spices and salt (no vinegar) before hanging in a humidity oven to be subjected to drying for up to 4 days at 75 °F and 55% RH. Samples retrieved at 0-, 2-, and 4-days of drying were plated on the four selective media described earlier (TSA, SCA, HE, and XLD, all containing three antibiotics). Again, the TSA and SCA based media had nearly identical counts, while HE and XLD based media both showed 1–1.5-log lower counts than the other selective media at each assay time (Figure 8).

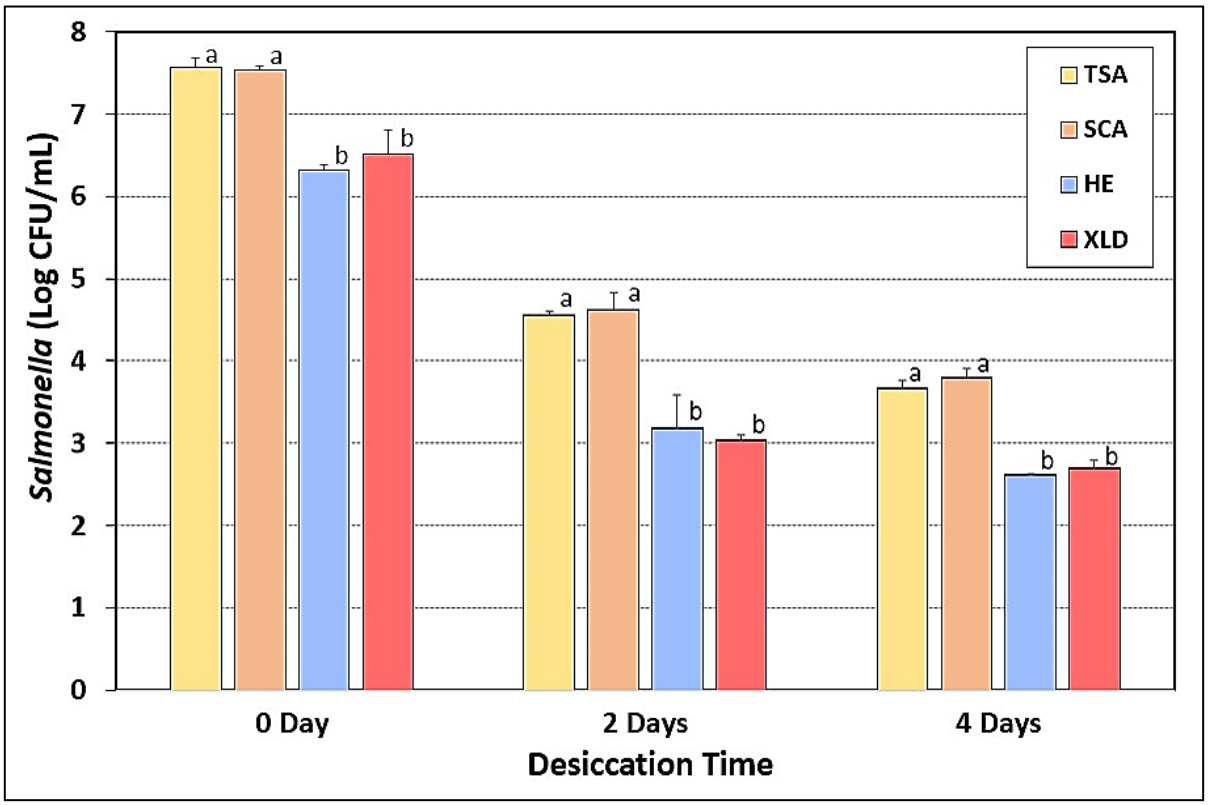

Figure 8. Desiccation-stress response of five-serovar mixtures of *Salmonella* inoculated on the surface of raw beef pieces and subjected up to 4-days drying in a humidity chamber at 23.9 °C (75 °F) and 55% RH. Treated samples were then plated on TSA, SCA, HE, and XLD containing spectinomycin (5 ug/mL), novobiocin (50 ug/mL), and clindamycin (5 ug/mL). Cultures in the mixture included *S*. Thompson 120, *S*. Heidelberg F5038BG1, *S*. Hadar MF60404, *S*. Enteritidis H3527, and *S*. Typhimurium H3380. Data are presented as the mean of triplicate replications and error bars represent the standard deviation from the mean. Means with different letters are significantly different ($p < 0.05$) as determined by one-way ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences; means with the same letter are not significantly different ($p > 0.05$).

3.9. Salmonella Stress Conditions: Thermal Stress

When 1-mL samples of the combined *Salmonella* serovars were subjected to heating at 145 °F (62.8 °C) for 75 sec in thin layer bags, equivalent counts were obtained on both TSA and SCA agar (~3.4–3.5-log reduction). Counts retrieved on HE and XLD agars showed microbial reductions of 4.75-log and 5.25-log, respectively (Figure 9).

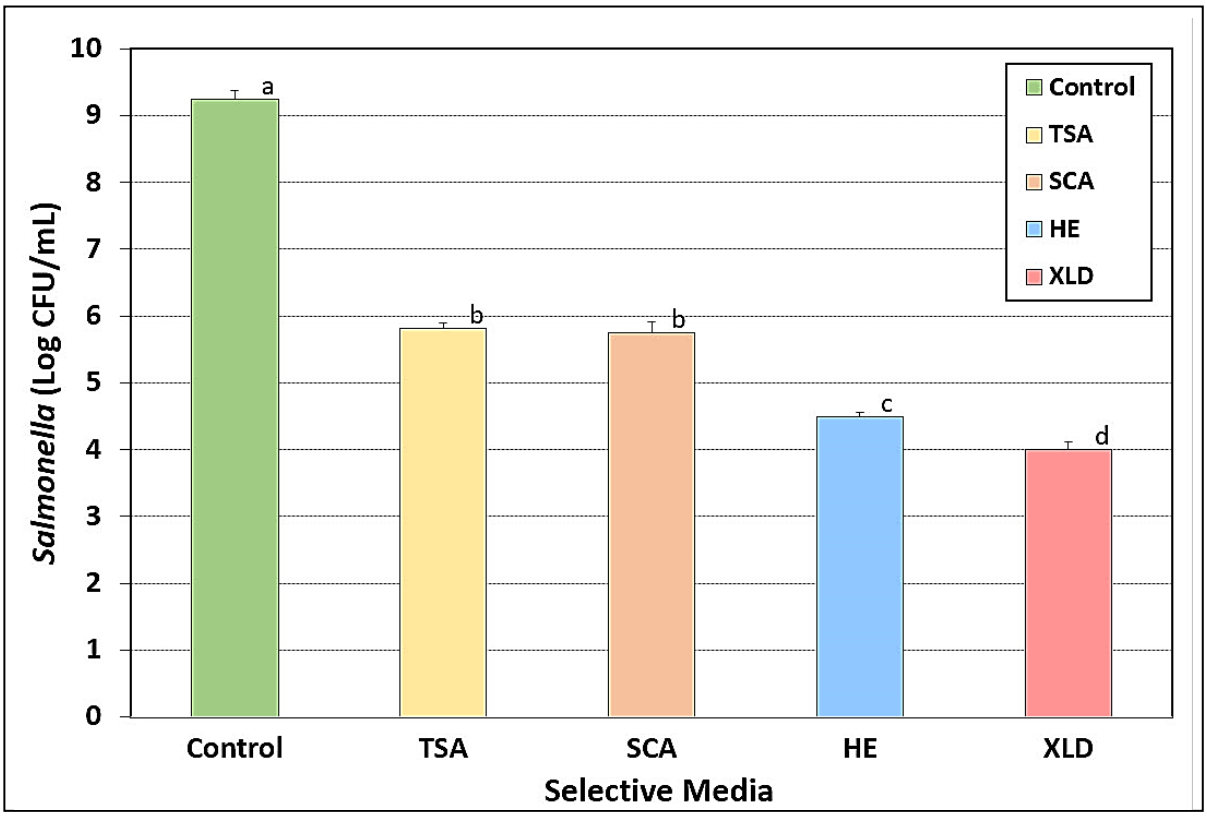

Figure 9. Thermal-stress response of five-serovar mixture of *Salmonella* heated at 62.8 °C (145 °F) for 75 sec. Treated samples were then plated on TSA, SCA, HE, and XLD; all contained spectinomycin (5 ug/mL), novobiocin (50 ug/mL), and clindamycin (5 ug/mL). Cultures in the mixture included *S.* Thompson 120, *S.* Heidelberg F5038BG1, *S.* Hadar MF60404, *S.* Enteritidis H3527, and *S.* Typhimurium H3380. The control is the level of the mixed culture before heating. Data are presented as triplicate replications and error bars represent standard deviation from the mean. Means with different letters are significantly different ($p < 0.05$) as determined by one-way ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences; means with the same letter are not significantly different ($p > 0.05$).

3.10. Bacterial Injury as Determined by Plating on Selective vs. Nonselective Media

When microbial counts of acid-adapted Salmonella were plated on TSA, and compared to platings on SCA, XLD, and HE, reduced levels of recovered counts were consistently obtained on XLD and HE (Table 2). This is consistent with the inhibition of sublethally injured cells by selective media components, as observed by Wesche et al. (Wesche et al., 2005). As determined with XLD and HE agars, injured cells were determined to comprise as much as 14%–19% of fresh acid-adapted cells and 73.0%–99.7% of the microbial populations of the remaining 11 trials after stressed conditions (Table 2). A similar comparison of acid-adapted *Salmonella* plated on SCA with those plated on TSA demonstrated a modest 4.4%–13.7% injury level in four of twelve trials, while showing 1.9%–46.7% enhancement of microbial recovery in eight of twelve trials (Table 2).

Table 2. Determination of % injury (-%) or enhancement (+%) of *Salmonella* by comparison of microbial counts on selective media (SCA, XLD, HE) to non-selective meda (TSA).

Process	% Injury (-) or %Enhancement (+)		
	SCA	**XLD**	**HE**
Sodium pyruvate trials			
• Nutrient depletion/starvation	+46.5	−94.0	−94.9
• Acid/vinegar marinade	−13.7	−99.7	−98.5
Extended nutrient depletion/starvation			
• Fresh cells	+1.93	−14.4	−19.0
• 3-weeks	+16.1	−99.4	−99.7
• 6-weeks	+6.6	−98.6	−99.2
Acidic stress (inoculated beef, 30-sec dip)			
• Vinegar (5% acetic acid)	−4.4	−95.1	−96.5
• Lactic acid (5%)	+4.8	−97.3	−89.1
• Sodium acid sulfate (3%)	+2.7	−97.9	−73.0
Desiccation stress (salt/spiced beef, dried)			
• 0-days	−6.5	−91.1	−94.4
• 2-days	+17.6	−96.9	−95.8
• 4-days	+31.8	−89.3	−91.1
Thermal stress (62.8 °C/145 °F for 75 sec)	−10.6	−99.2	−99.6

4. Discussion

In this study, various *Salmonella* serovars (*S.* Thompson 120, *S.* Heidelberg F5038BG1, *S.* Hadar MF60404, *S.* Enteritidis H3527, S. Typhimurium H3380) were characterized prior to their use as inocula in subsequent studies to evaluate antimicrobial interventions applied during the processing of dried beef (i.e., biltong, beef jerky). The *Salmonella* strains used in this study are widely distributed in academia and government research labs and have a long history of testing on the effects of antimicrobial interventions against *Salmonella* applied to meat and poultry products (Carpenter et al., 2011; Juneja et al., 2001; Juneja et al., 2010; Juneja et al., 2012; Mann & Brashears, 2007; Orta-Ramirez, Marks, Warsow, Booren, & Ryser, 2005; Tuntivanich, Orta-Ramirez, Marks, Ryser, & Booren, 2008; Velasquez et al., 2010; Warsow, Orta-Ramirez, Marks, Ryser, & Booren, 2008; Wesche et al., 2005).

Characterization of antibiotic resistance demonstrated that they are not only multi-drug resistant but are also resistant to the same antibiotics. This enables the use of the common antibiotics for the preparation of selective media to recover them from inoculated food studies. TSA containing novobiocin, spectinomycin, and clindamycin was considered a useful generic selective agar media for these *Salmonella* serovars. The approach to use antibiotics to which the inoculum bacteria are resistant has long been an effective method of selective enumeration of inoculum strains recovered from non-sterile foods that also contain other microorganisms (Flores, 2004; Luchansky et al., 2009; Mann & Brashears, 2006; Muriana & Klaenhammer, 1987; NACMCF, 2010; Price et al., 2000). However, the appearance of high levels of multi-drug resistant background bacteria from un-inoculated raw beef on TSA containing the three antibiotics was cause for concern and precipitated a search for a more selective medium.

The intended use of the *Salmonella* serovars was as inocula prior to antimicrobial (acid) interventions on raw beef, and therefore, the strains were acid-adapted prior to use throughout this

study. Acid adaptation of *Salmonella* serovars was reported in the early 1990s (Foster, 1991; Foster & Hall, 1990; G. J. Leyer & Johnson, 1992) whereby *Salmonella* pre-exposed to low pH were more resistant to acidic conditions than non-adapted cells. Foster and others (Foster, 1991; Foster & Hall, 1990) adjusted media with HCl to achieve low pH conditions. Subsequently, during investigations with enterohemorrhagic *Escherichia coli* and *Listeria monocytogenes*, Buchanan et al. (R. L. Buchanan & Edelson, 1996; Robert L. Buchanan, Golden, Whiting, Phillips, & Smith, 1994) augmented media with glucose to allow the organisms to lower pH during growth to induce the acid adaptation response. The ability of *Salmonella* and Shigatoxigenic *E. coli* to adapt to stressful environments has significant implications in the safety of processed foods (Álvarez-Ordóñez, Prieto, Bernardo, Hill, & López, 2012; Foster, 1991; Suehr, Chen, Anderson, & Keller, 2020). The *Salmonella* cultures used throughout this study were predisposed to acidic stress conditions by growth in media containing 1% glucose. This has been recommended by the NACMCF (NACMCF, 2010) for inoculated challenge studies and by USDA-FSIS when evaluating antimicrobial food processes involving acidic treatments.

Sodium pyruvate was initially considered as a possible media additive to allow recovery of injured cells since many intended applications would be stress-related. However, when examined in the context of nutrient depleted/starved cells, or when *Salmonella*-inoculated beef was exposed to the stress of salt (dehydration) and vinegar (acid) marination, no benefit was observed with sodium pyruvate. Similarly, neither Knabel and Thielen (Knabel & Thielen, 1995) nor Kirby and Davies (Kirby & Davies, 1990) observed improved recovery of heat-injured *Listeria* or *Salmonella*, respectively, when using sodium pyruvate in their recovery medium. Various investigators have shown that acid adaptation plays a role in enhanced survival of *Salmonella* and other bacteria, offering cross-protection not only against acid stress, but desiccation, salt, and thermal stress as well (Bacon et al., 2003; Greenacre & Brocklehurst, 2006; G. J. Leyer & Johnson, 1992; G J Leyer & Johnson, 1993). Although other injury recovery additives may have been more effective than sodium pyruvate (i.e., catalase, yeast extract), we continued without additional additives, since the SCA medium was demonstrating comparable results to TSA. In our study, enumeration of *Salmonella* was consistently and significantly lower on XLD and HE agars, as they likely inhibited the recovery of injured cells (Gorski, 2012; Pao et al., 2006; Strantz & Zottola, 1989). XLD or HE would have solved our background microorganism problem as the *Salmonella* would have appeared as black colonies. However, our data showed lower counts (corresponding to injury levels of 89%–99%) when *Salmonella* were plated on XLD or HE and compared to levels recovered on TSA. Strantz and Zottola (Strantz & Zottola, 1989) were only able to improve recovery with XLD and HE media by plating cells on TSA, allowing them to sit for 4 hrs of recuperation, and then overlaying with XLD or HE agar. Subsequent work by various investigators led to several modifications of the selective overlay technique, including the agar underlay (D. H. Kang & Siragusa, 1999) and thin agar layer (Chen et al., 2013; Ferreira, Horvath, & Tondo, 2013; Dong Hyun Kang & Fung, 2000; Wu et al., 2001) methods.

5. Conclusions

The *Salmonella* serovars examined in this study were intended for use in USDA-FSIS validation studies on antimicrobial interventions for dried beef processing to achieve 5-log reduction of *Salmonella*. Hence, it was important to accurately enumerate *Salmonella* survivors during processing in lieu of potential background bacteria from raw meat. Selenite cystine broth, a medium routinely used as a selective enrichment broth for luxuriant growth of *Salmonella* spp., provided more suppression of background microorganisms from raw beef when used as an agar medium than did TSA (with antibiotics). The ability to quantitatively enumerate these *Salmonella* serovars on SCA containing antibiotics facilitated experimental procedures and eliminated the need for cumbersome multiple layered plating schemes. Enumeration with SCA was not significantly different than that obtained with TSA, which demonstrates the adequacy of SCA as a selective media for these *Salmonella* serovars, and more so when supplemented with antibiotics to which the strains are resistant. The use of SCA should allow quantitative enumeration of these acid adapted *Salmonella* serovars when

recovered from inoculated food studies employing antimicrobial interventions in spite of the background organisms that might be present.

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Chapter IV

Processing of Biltong (Dried Beef) to Achieve USDA-FSIS 5-log Reduction of *Salmonella* without a Heat Lethality Step

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Abstract: In the US, dried beef products (beef jerky) are a popular snack product in which the manufacture often requires the use of a heat lethality step to provide adequate reduction of pathogens of concern (i.e., 5-log reduction of *Salmonella* as recommended by the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS)). Biltong, a South African-style dried beef product, is manufactured with low heat and humidity. Our objectives were to examine processes for the manufacture of biltong that achieves a 5-log reduction of *Salmonella* without a heat lethality step and with, or without, the use of additional antimicrobials. Beef pieces (1.9 cm × 5.1 cm × 7.6 cm) were inoculated with a 5-serovar mixture of *Salmonella* (*Salmonella* Thompson 120, *Salmonella* Heidelberg F5038BG1, *Salmonella* Hadar MF60404, *Salmonella* Enteritidis H3527, and *Salmonella* Typhimurium H3380), dipped in antimicrobial solutions (lactic acid, acidified calcium sulfate, sodium acid sulfate) or water (no additional antimicrobial), and marinated while vacuum tumbling and/or while held overnight at 5 °C. After marination, beef pieces were hung in an oven set at 22.2 °C (72 °F), 23.9 °C (75 °F), or 25 °C (77 °F) depending on the process, and maintained at 55% relative humidity. Beef samples were enumerated for *Salmonella* after inoculation, after dip treatment, after marination, and after 2, 4, 6, and 8 days of drying. Water activity was generally <0.85 by the end of 6–8 days of drying and weight loss was as high as 60%. Trials also examined salt concentration (1.7%, 2.2%, 2.7%) and marinade vinegar composition (2%, 3%, 4%) in the raw formulation. Nearly all approaches achieved 5-$\log_{10}$ reduction of *Salmonella* and was attributed to the manner of microbial enumeration eliminating the effects of microbial concentration on dried beef due to moisture loss. All trials were run as multiple replications and statistical analysis of treatments were determined by repeated measures analysis of variance (RM-ANOVA) to determine significant differences ($p < 0.05$). We believe this is the first published report of a biltong process achieving >5.0 $\log_{10}$ reduction of *Salmonella* which is a process validation requirement of USDA-FSIS for the sale of dried beef in the USA.

Keywords: *Salmonella sp.*; 5-log reduction; biltong; dried beef; antimicrobial; water activity; relative humidity; acid adaptation

1. Introduction

Dried meats are a popular food throughout the world due to shelf stability and nutrient content of the food product (Taormina & Sofos, 2014). Drying and curing of meats dates back centuries as an effective way to preserve foods, particularly meat (Wentworth, 1956). The drying of meats allows for reduction of moisture, creating a product with a low water activity (A_w) and therefore a microbially safe and shelf-stable product as less water is available for microbial growth (Taormina & Sofos, 2014). An example of a dried meat product is biltong, a ready-to-eat dried beef product native to South Africa. Biltong is usually made from lean strips of beef marinated in traditional spices (coriander, black pepper, salt) and vinegar (malt vinegar, red wine vinegar) and then dried at ambient temperature and humidity following marination (M. Jones, Arnaud, Gouws, & Hoffman, 2017). The microbial safety of biltong is due to the combination of vinegar (contributes to low pH), salt (binds water), and drying at low humidity (leads to low A_w) that inhibits the growth of microorganisms (Maxine Jones, Arnaud, Gouws, & Hoffman, 2019; K. Naidoo & D. Lindsay, 2010; Keshia Naidoo & Denise Lindsay, 2010; Petit, Caro, Petit, Santchurn, & Collignan, 2014). This style of dried beef is normally produced in a home setting. In the USA there has been a recent surge of interest from both entrepreneurs and commercial manufacturers to produce biltong using traditional methods.

Traditional South African biltong differs from American-style beef jerky mainly due to the absence of a heat lethality step. Beef jerky produced in the US generally includes a heat step to achieve the recommended 5-log reduction of the most likely foodborne pathogen, *Salmonella* (Buege, Searls, & Ingham, 2006; USDA-FSIS, 2014, 2017b). The lack of a heat lethality step and incubation at ambient temperature during the processing of biltong raises concern for the safety of biltong in regards to potential pathogenic survival and microbial growth such as *Salmonella* spp. and *Listeria monocytogenes* (K. Naidoo & D. Lindsay, 2010). Since biltong processing does not include a heat lethality step, but rather uses low temperature and humidity conditions to dry meat over an extended period, other ingredients must be used to achieve a microbially safe product. Biltong production utilizes salt, spices, and more importantly vinegar as one of the main additives to impart flavor and reduce microbial survival. Beef jerky in contrast, does not traditionally use vinegar but rather, uses heating, curing, smoking and drying techniques to achieve a microbially safe product (Carr et al., 1997). Ultimately, both processes must demonstrate their ability to produce a product that is safe for consumers.

In the US, beef jerky is manufactured under United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) compliance guidelines which state that relative humidity during the production of the beef product must be maintained at 90% or above and this humidity can be achieved by use of a sealed oven or continuously injected steam (USDA-FSIS, 2014). If these cooking/heating guidelines are not met, a microbial validation of the process must be provided to demonstrate sufficient reduction of the targeted foodborne pathogen. *Salmonella* has been historically associated with outbreaks related to beef (Laufer et al., 2015) or dried beef products (CDC, 1995; Eidson, Swell, Graves, & Olson, 2000). Although biltong processing does not maintain high humidity and heat, the manufacturing process must still demonstrate *Salmonella* lethality. Two options are available to achieve USDA-FSIS process validation for biltong. One option is to demonstrate a 2-log reduction of *Salmonella* and perform testing of every lot of edible ingredient to ensure the absence of *Salmonella* as was developed by the 'Blue Ribbon Task Force' circa 1996 for *E. coli* O157:H7 (Nickelson, Luchansky, Kaspar, & Johnson, 1996). For industry, this option is very difficult because if someone 'forgets' to carry out *Salmonella* testing on a given lot of product, or if the test result is positive, it creates a burden for the company, as well as the cost of all the *Salmonella* testing. The second option was that the process itself must provide a 5-log reduction of *Salmonella*, and even though this may be a tough target to achieve, once defined, it is the simplest to implement. The emphasis on achieving a 5-log reduction of *Salmonella* is paramount for the manufacture of biltong that is microbially safe. There are no published reports validating the biltong process in regard to pathogen reduction

according to USDA-FSIS guidelines. USA-based studies have not achieved the required 5-log reduction of *Salmonella* (Burnham, Hanson, Koshick, & Ingham, 2008) and manufacturers in other countries are not required to obtain a specified process reduction by their regulatory agencies (Maxine Jones et al., 2019; Keshia Naidoo & Denise Lindsay, 2010). Note: USDA-FSIS does not consider the 5-log reduction a 'requirement' because of the alternative 2-log process that could be used along with *Salmonella* testing of ingredients; however, if one were choosing the approach without *Salmonella* testing, then by default one would be required to demonstrate a 5-log process.

The purpose of this study was to examine various processes, antimicrobials, and conditions used for the manufacture of biltong to achieve a 5-log reduction of *Salmonella* without a heat lethality step to produce a microbially safe product that could satisfy USDA-FSIS process validation requirements.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Active cultures were grown in tryptic soy broth (TSB, BD Bacto BD211825, Franklin Lakes, NJ, USA) in 9 mL tubes at 37 °C. 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 enumeration was carried out on tryptic soy agar (TSA, BD Bacto; 1.5% agar) and plated in duplicate.

Salmonella serovars used in this study included: *Salmonella enterica* subsp. *enterica* serotype Thompson 120 (chicken isolate), *Salmonella enterica* subsp. *enterica* serotype Heidelberg F5038BG1 (ham isolate), *Salmonella enterica* subsp. *enterica* serotype Hadar MF60404 (turkey isolate), *Salmonella enterica* subsp. *enterica* serotype Enteritidis H3527 (phage type 13a, clinical isolate) and *Salmonella enterica* subsp. *enterica* serotype Typhimurium H3380 (DT 104 clinical isolate). These are well-characterized strains that have been used in numerous research publications involving antimicrobial interventions against *Salmonella* spp. (Carpenter, Smith, & Broadbent, 2011; Juneja, Eblen, & Marks, 2001; Juneja, Hwang, & Friedman, 2010; Juneja et al., 2012; Karolenko, Bhusal, Gautam, & Muriana, 2020).

Acid adaptation' of *Salmonella* serovars was reported in the 1990s by Foster (Foster, 1991) and Leyer and Johnson (Leyer & Johnson, 1992) whereby *Salmonella* pre-exposed to low pH for several doublings during growth were more resistant to low pH than non-adapted cells. Acid adaptation of our *Salmonella* serovars was carried out according to Wilde et al. (Wilde, Jørgensen, Campbell, Rowbury, & Humphrey, 2000) as modified by Karolenko et al. (Karolenko et al., 2020) in which these cultures were inoculated in TSB augmented with 1% glucose prior to use in various conditions of stress.

In preparation of acid-adapted cultures for use in biltong beef processing, individual cultures were propagated overnight at 37 °C in 9 mL TSB (BD Bacto BD286220). These cultures were then used to re-inoculate individual 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 for approximately 18 h. Individual cultures were harvested by centrifugation, and resuspended with 0.1% buffered peptone water (BPW, BD Difco), mixed in equal proportions, and held refrigerated (5 °C) until use shortly thereafter. USDA-FSIS 'highly recommends' the use of acid-adapted cultures when such inoculum strains would be used for stressed conditions to insure that they are not easily overcome by acidic processing conditions (NACMCF, 2010).

2.2. Beef Handling and Inoculation

Beef was processed in the Robert M. Kerr Food and Agricultural Products Center (FAPC; Oklahoma State University, Stillwater, OK, USA). Boneless beef round (i.e., outside round, flat), as per USDA Institutional Meat Purchase Specifications 171B (USDA-AMS, 2014) of either Select grade or ungraded were used. Beef was obtained for biltong trials from a local processor (Ralph's, Perkins, OK, USA) who obtained their beef through a broker that acquires beef from different source companies (i.e., Excel/Cargill, Dodge City, KS, USA; Kane Beef, Houston, TX, USA; High River Angus, Lake River, FL, USA; and others). Boxed, vacuum-packaged beef was held in our meat pilot plant coolers for 1–3 days when received (35 °F/1.7 °C), then trimmed to remove excess fat and sliced by our in-house meat-processing specialists in the FAPC meat pilot plant (Figure 1A).

Refrigerated, trimmed beef was subjected to a 'final trimming' prior to biltong processing to further approximate similar-sized pieces of ~1.9 cm thick × ~5.1 cm wide × ~7.6 cm long (~80–110 g)(Figures 1B,C). Inoculum cultures were grown as described earlier, acid adapted, centrifuged, and resuspended to a higher concentration (Figures 1D,E). Individual beef pieces on trays were inoculated with 150 μL (>2.0×10^9 cfu/mL) of the mixed-serovar *Salmonella* cocktail on each side. A 'gloved finger' was used to spread the inoculum over the side of the beef pieces, which were turned over and the same inoculation process was performed on the other side (Figures 1F–G). Inoculated beef pieces were then laid flat on foil-lined trays in a refrigerator (5 °C) to promote attachment for 30–60 min prior to use.

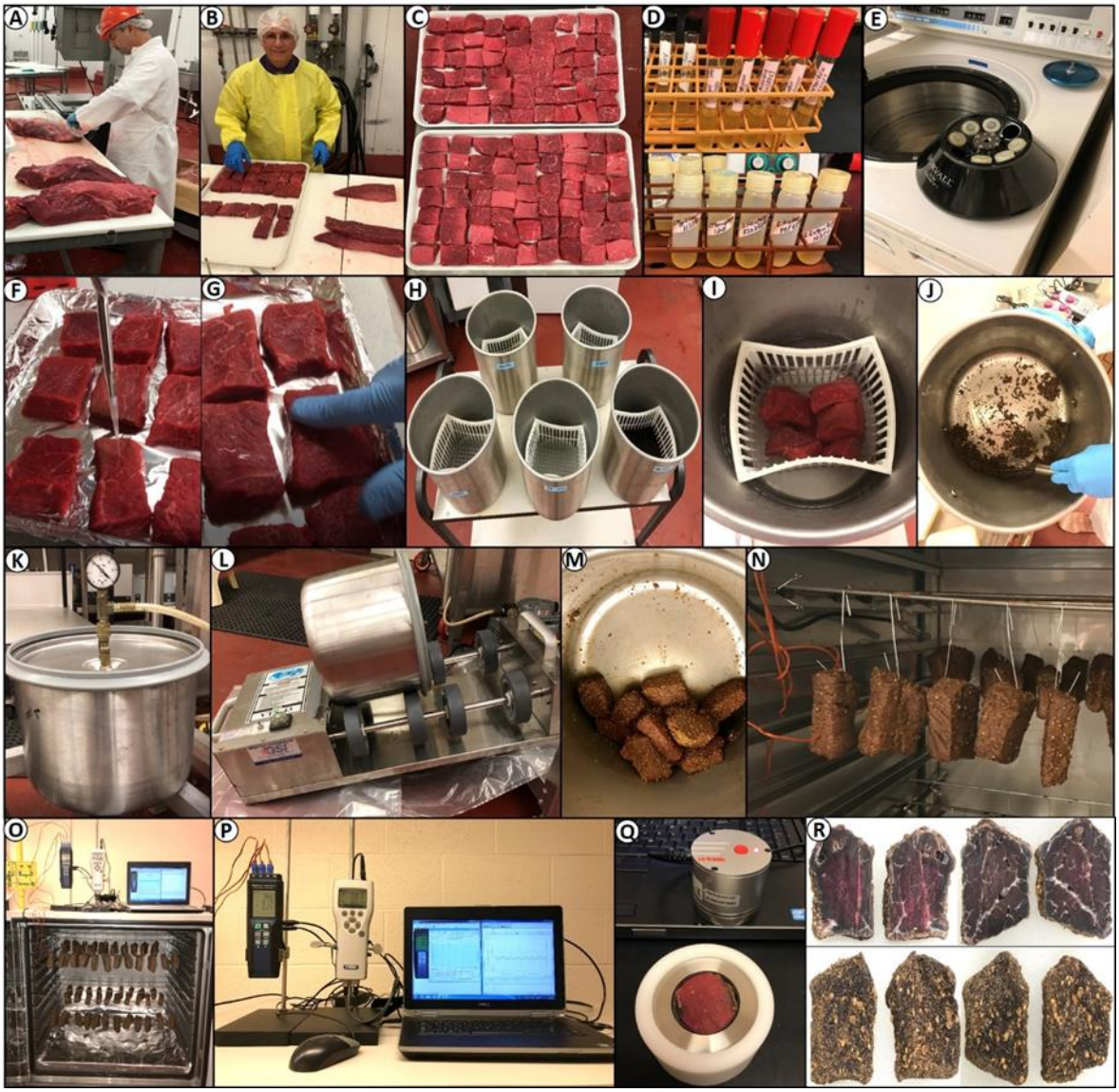

Figure 1. The biltong process: (**A–C**) Trimming beef bottom rounds into beef slices and final trimming into squares; (**D**,**E**) acid adapting cultures by growth in tryptic soy broth (TSB) with 1% glucose and centrifuging to concentrate them; (**F**,**G**) pipette inoculation of beef and 'gloved finger' spreading of inoculum; (**H**,**I**) baskets and containers for water/antimicrobial dip treatment of inoculated beef; (**J**) mixing vinegar and spices; (**K–M**) vacuum tumbling spices and beef; (**N**) hanging beef in humidity chamber; (**O**,**P**) humidity chamber with 4-channel temperature recorder and humidity probe); (**Q**) water activity meter; (**R**) biltong internal and external surface after 8 days of drying.

2.3. Biltong Processing: Antimicrobials

Beef processors are allowed to incorporate an antimicrobial treatment into their process to enhance *Salmonella* reduction. Some meat processors apply this treatment early on with intact beef bottom rounds where *Salmonella*, if present, would likely be on the surface of the beef. Treatment of intact bottom rounds prior to trimming would address *Salmonella* prior to further distribution onto beef surfaces by cutting/trimming, or onto equipment surfaces by additional contact contamination. Still, other processors trim intact bottom rounds into long strips first, and then proceed to take the strips into antimicrobials directly before continuing with marination, tumbling, and drying; this ensures every bit of resulting beef to have been dipped into antimicrobial. For the purposes of

experimental methodology, we follow the latter method as it would be impossible to inoculate intact bottom rounds, remove fat, and trim it to size, and follow the originally inoculated surfaces throughout the process. USDA-FSIS has indicated (personal communication) that the later method also mimics the inoculated surfaces of the former method, so our method accommodates both types of commercial applications. USDA-FSIS publishes a list of 'Safe and Suitable Ingredients for use on Meat and Poultry Products' that lists various allowable antimicrobials, use level, and product contact time to be considered as processing aids for various types of meat/poultry products (USDA-FSIS, 2018). The antimicrobials that were used in this study were chosen from this list (Table 1).

After inoculation and refrigerated hold time to promote bacterial attachment, inoculated beef pieces were dip treated (30 or 60 s) in various antimicrobial solutions (Table 1) using 15.25 × 15.25 × 15.25 cm perforated baskets and cylindrical stainless steel containers (Figures 1H,I); water dip treatment was used as a non-lethal dip control solution. An electronic timer was used to time the process while manually rotating the basket to ensure beef pieces would not be pinned up against each other. After the prescribed time, baskets were removed from the solutions and positioned above the container to allow excess liquid to drip into the container (~20–30 s); samples were then removed individually by sterile forceps into the stainless-steel marinade tumbler container for either vacuum- or non-vacuum tumbling (Figure 1J–M).

Antimicrobial (working stock) solutions included 5% lactic acid (FCC88, ADM Co., Decatur, IL, USA), 3% sodium acid sulfate (pHase, Jones-Hamilton Co., Walbridge, OH, USA), acidified calcium sulfate (diluted 2:1 with water: Mionix RTE-17 and Mionix RTE-01; Mionix LLC, Grain Valley, MO, USA) (Table 1).

Table 1. Antimicrobials used in this study as listed in the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) Safe and Suitable Ingredients List (7120.1).

Category	Substance	Intended Use of Product	Amount	Food Ingredient Label Key[a]
Antimicrobials • Lactic acid, 5%	Lactic acid (5%).	Beef and pork sub-primals and trimmings.	2% to 5 % solution of lactic acid, not to exceed 55 °C.	None under the accepted conditions of use (1)
Antimicrobials • Sodium acid sulfate, 3%	An aqueous solution of sulfuric acid and sodium sulfate.	In the form of a spray, wash, or dip on the surface of meat (beef and pork) and poultry products processing.	Solution of sulfuric acid and sodium sulfate at concentrations sufficient to achieve a targeted pH range of 1.0–2.2 on the surface of meat and poultry.	None under the accepted conditions of use (1)
Antimicrobials • Acidic calcium sulfate (Mionix RTE-17, 5% lactic acid)	An aqueous solution of acidic calcium sulfate and lactic acid.	Applied as a continuous spray or a dip on raw poultry carcasses, parts, giblets, and ground poultry.	Acidic calcium sulfate sufficient for purpose; lactic acid not to exceed 5.0 % and 55 °C.	None under the accepted conditions of use (1)

Antimicrobials • Acidic calcium sulfate (Mionix RTE-01, 10% lactic acid)	A solution of water, acidic calcium sulfate, lactic acid, and sodium phosphate (solution with a pH range of 1.45 to 1.55).	Raw whole muscle beef cuts and cooked roast beef and similar cooked beef products (e.g., corned beef, pastrami, etc.).	A solution of water, acidic calcium sulfate, lactic acid, and sodium phosphate (solution with a pH range of 1.45 to 1.55) spray applied for up to 30 s of continual application *sodium phosphate on finished product must not exceed 5000 ppm.	Listed by common name in ingredients statement of multi-ingredient products. Single ingredient raw whole muscle beef cuts must be descriptively labeled (2)

[a] **Food ingredient label key:** 1) The use of the substance(s) is consistent with the Food and Drug Administration's (FDA) labeling definition of a processing aid, 2) Generally Recognized as Safe (GRAS). The USDA-FSIS regulates which antimicrobials, dependent on use level and application time, must be included on food ingredient labels or can be excluded from such labels because their level and treatment time satisfies the definition of a 'processing aid'.

Antimicrobial (working stock) solutions included 5% lactic acid (FCC88, ADM Co., Decatur, IL, USA), 3% sodium acid sulfate (pHase, Jones-Hamilton Co., Walbridge, OH, USA), acidified calcium sulfate (diluted 2:1 with water: Mionix RTE-17 and Mionix RTE-01; Mionix LLC, Grain Valley, MO, USA) (Table 1).

2.4. Biltong Processing: Marination and Drying

Biltong beef marination, depending on the process, generally consisted of addition of a predicated amount of spices (coriander as the predominant spice, black pepper, and others), salt (generally ~2% total formulation), and vinegar (generally ~2% total formulation). An examination of 7 biltong recipes on the internet showed a variety of ingredient formulations that included: beef (82%–96% of total formulation), vinegar (2%–11%), salt (1.5%–8%), coriander (0.3%–2%), pepper (0.1%–1%), brown sugar (0.8%–6%), and bicarbonate (0.2%–1%); our use levels in this study fell within these common values. In addition to adding spices individually, some companies provide premixed biltong ingredients (i.e., Crown National, Freddy Hirsch, Tongmaster). Numerous biltong processes available on the internet also range from short marination periods (30 min to 4 h) to overnight marination (12–24 h); we used representative processes of both of these in our study.

2.4.1. Short-Term Biltong Marination Process

A short marination process was examined for biltong processing consisting of dipping inoculated beef into an antimicrobial solution (or water) for 30 or 60 s, and removing the basket and allowing excess liquid to drip for an additional 30–60 s. After dip treatment, beef pieces were transferred to a stainless-steel vacuum chamber containing a biltong spice blend of 1.7%–2.7% salt and 2–4% of 50- or 100-grain red wine (or white) vinegar (as a % of total weight of ingredients including beef). The chamber was evacuated to 38.1 cm (15 inches) Hg, and tumble-marinaded for 30 min on a rotating Biro VTS-43 tumbler (Biro, Marblehead, OH, USA) before hanging to dry. Beef pieces were hung in a temperature-controlled humidity oven (Hotpack, Warminster, PA, USA) at 25 °C (77 °F) and 55% relative humidity (RH) and allowed to dry for up to 8 days. Beef was sampled after inoculation (0 days), after antimicrobial (or water) dip treatment, after marination, and after 2, 4, 6, and 8 days of drying (or until >5-log reduction of *Salmonella* was obtained). The impact of both vinegar and salt was also examined via same-day processes comparing 2%, 3%, and 4% vinegar formulations (as % of total ingredient weight). Additional trials examined the effect of 1.7%, 2.2%, and 2.7% salt in the marinade formulation.

Trials included either 3 replicative trials with 2 samples taken at each testing period, or 2 replicate trials with 3 samples taken at each test period (n = 6/testing period) as per USDA-FSIS

(USDA-FSIS, 2015) and National Advisory Committee on the Microbiological Criteria for Foods (NACMCF) (NACMCF, 2010) guidelines.

2.4.2. Extended Overnight Biltong Marination Process

Several extended marination procedures were also examined. One included a 1 h hold at 5 °C after the antimicrobial dip followed by 40 min vacuum-tumbling in a complete meat-spice-salt-vinegar marinade mixture (formulation: 95–96% beef, 2.5% spices includes salt at 2% overall formulation, and 2% 50-grain red wine vinegar), and then the marinaded beef was held for an extended period (overnight, ~16–18 h) at 5 °C (41 °F). This process examined if further reduction of *Salmonella* could be achieved by an extended refrigerated hold period after antimicrobial/marination treatment as was demonstrated previously with *E. coli* O157:H7 (Muriana et al., 2019). After the extended marination period, the beef pieces were removed from the excess vinegar marinade and hung in the humidity chamber at 23.9 °C (75 °F) and 55% RH and sampled after 0 (post-marinade), 2, 4, 6, and 8 days of drying.

Another extended marination procedure involved non-vacuum tumbling (5 min) with just spices and salt (formulation: 95–96% beef, 4–5% spice which included salt at 2.1% of total dry formulation). Spiced beef pieces (dry) were then placed in stainless steel pans, and liquid marinade was slowly poured in for the beef to sit in while held at refrigeration temperature (5 °C/41 °F) overnight. Liquid marinade comprised 14% of total formulated weight (including beef); 50-grain white vinegar comprised 73% of the liquid marinade portion of the formulation and 10% of the total formulated weight. Marinaded beef pieces were then 'turned' after 30-min and again after 8–12 h and left to marinade for the remaining time in the refrigerator (total, 16–20 h). After marination, beef pieces were removed from the excess vinegar marinade and hung in the humidity chamber (22.8 °C/73 °F, 55% RH) as described previously and sampled at 0 (post-marinade), 2, 4, 6, and 8-days of drying.

2.4.3. Biltong Drying Process

After marination by either the short term or the longer extended processes, beef pieces were hung using large paper clips in a 10 cubic foot benchtop humidity oven (Hotpack Model #435315, SP Industries, Warminster, PA, USA) set at 22–25°C (72–77°F) depending on the process, and 55% RH. Relative humidity was established by a direct water line feed from an in-house deionized water supply to the built-in bottom water chamber with automatic water level and heating element to warm the water and generate humidity; an internal fan circulated the air within the chamber. Although air temperature and humidity level control was built-in, additional external monitors were used consisting of a handheld humidity monitor (Vaisala HM70, Helsinki, Finland) and a handheld thermocouple temperature recorder (Center 378, New Taipei City, Taiwan) with 4 temperature probes for chamber temperature (2 probes) and beef temperature (2 probes). A laptop computer was used to record temperatures and humidity during the entire process (Figures 1O–1P).

2.5. Water Activity and Moisture-Loss Determination

Water activity was measured using a HC2-AW-USB probe with direct PC interface and HW4-P-Quick software (Rotronic Corp., Hauppauge, NY, USA) (Figure 1Q). Samples for water activity and moisture loss were obtained using negative controls (i.e., non-inoculated beef, dipped, marinaded, and tumbled) during various stages of the process: the initial raw beef, beef after the spice/salt/vinegar marination step, and beef after 2, 4, 6, and 8–9 days of drying in the humidity chamber. Samples were cut in half and the interior portion of the biltong beef samples were positioned with the inside cut surface facing upward in the sampling cupules (Figure 1Q), towards the water activity meter sensor, as this was the region of highest water activity even with biltong at the end of drying (Figure 1R). This was determined by comparing water activity taken from the surface side facing the sensor, the inside facing the sensor, and chopped up pieces of entire biltong

pieces in the sample cupule (Figure 2). Samples from the refrigerator or drying oven were allowed to equilibrate to room temperature in the covered cupules before analysis.

Moisture loss was determined by weighing beef pieces (negative controls) prior to the start of drying and again after drying for 2, 4, 6, and 8 days. Comparison of latter weights to initial weights of the same pieces resulted in determination of % moisture loss as per Equation (1):

$$\%\ Moisture\ Loss = \frac{[(initial\ weight) - (latter\ weight)]}{(initial\ weight)} \times 100 \quad (1)$$

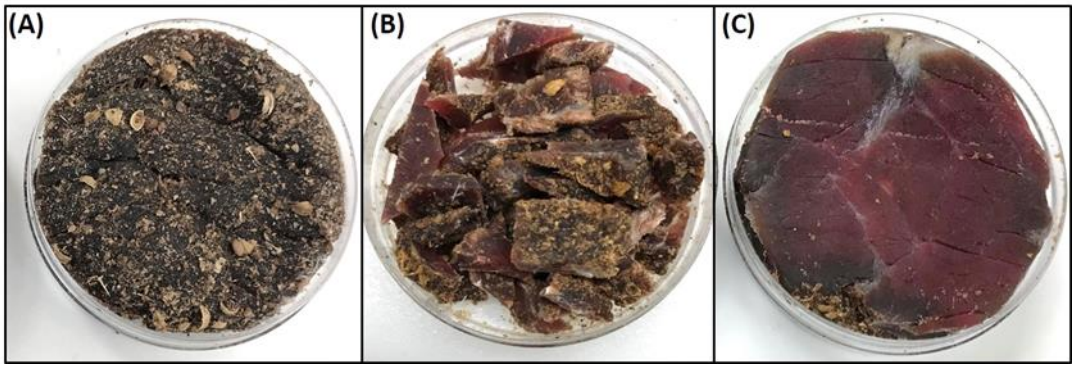

Figure 2. Water activity measurements. Examination of all possible samples from which water activity could be derived using the cupules for the water activity meter: (**A**) outer surface, (**B**) chopped pieces, and (**C**) inside surface. The sensor of the water activity meter would be positioned just above the retained sample in the cupule holder.

2.6. Microbial Sampling and Enumeration of Beef

Beef samples were randomly chosen and transferred to 4-mil sterile Whirl-pak filter-stomaching bags (Nasco, Fort Atkinson, WI, USA), followed by addition of 100 mL of chilled 1% neutralizing buffered peptone water (nBPW, Criterion, Hardy Diagnostics, Santa Maria, CA) and then stomached for 90 s in a masticator paddle-blender (IUL Instruments, Barcelona, Spain). The filter bag dilution (stomached sample) was considered the 10^0 dilution for all samplings, including the initially inoculated raw beef through the final samples at up to 8–10 days of drying so that microbial counts were directly comparable with each other at all stages of drying. After stomaching in nBPW, inoculated (experimental) and non-inoculated (negative control) samples were 10-fold serially-diluted with 0.1% BPW. Dilutions were then surface plated (0.1 mL) in duplicate on TSA or selenite cystine agar (SCA). Both medias contained spectinomycin (5 μg/mL), clindamycin (5 μg/mL), and novobiocin (50 μg/mL), and plates were incubated at 37 °C for 48 h before enumeration. In a prior study, SCA was shown to enumerate these same acid adapted *Salmonella* serovars comparably to TSA, even after exposure to different types of stress (Karolenko et al., 2020). When microbial counts were expected to be low, 0.2 mL was plated on each of 5 plates (1 mL total) to increase the sensitivity of plating (i.e., decrease the limit of detection).

2.7. Statistical Analysis

Each trial was performed in either triplicate replication with 2 samples tested per sampling period ($n = 6$) or duplicate replications with 3 samples tested per sampling period ($n = 6$) in accordance with validation testing criteria established by the NACMCF (NACMCF, 2010) and accepted by USDA-FSIS (USDA-FSIS, 2015). All replications were performed as autonomous and separate experiments using separately inoculated cultures, separately prepared plating media, 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 of timed series data was undertaken using repeated measures one-way analysis of variance (RM-ANOVA) and the Holm–Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$) of the treatments. Data treatments

with different letters are significantly different ($p < 0.05$); treatments with the same letter are not significantly different ($p > 0.05$).

3. Results

3.1. Short-Term Biltong Marination Process

A biltong process using a short marination process (i.e., a 30 min vacuum tumbling) facilitated process completion the same day it was initiated including hanging beef pieces in the humidity chamber. The data show that dip treatment in antimicrobials such as acidified calcium sulfate adjusted to 5% lactic acid (Mionix RTE-17) or 5% lactic acid (ADM FCM88), prior to marination, resulted in a ~5.0-log reduction of *Salmonella* in 6 days and ~5.5-log reduction by 8 days (Figure 3). The trials with acidified calcium sulfate (at 5% lactic acid) and 5% lactic acid were nearly identical and showed no significant difference ($p > 0.05$). Acidified calcium sulfate (Mionix RTE-01) adjusted to 10% lactic acid showed a steeper decline in reduction of *Salmonella* while drying and reached >5-log reduction in 4 days and >6-log reduction by 8 days and was significantly different ($p < 0.05$) than 5% lactic acid (ADM FCM88) or acidified calcium sulfate (Mionix RTE-17) adjusted to 5% lactic acid (Figure 3). Although the inoculated positive control trials without additional antimicrobial dip treatment did not reach the targeted 5-log reduction in 8 days and was significantly different to trials with antimicrobial treatment ($p < 0.05$), it could likely have reached 5-log reduction level if given a few more days of drying (Figure 3); some individual replicates did reach this level within 8 days, but the average of all replications was slightly less than 5-log reduction.

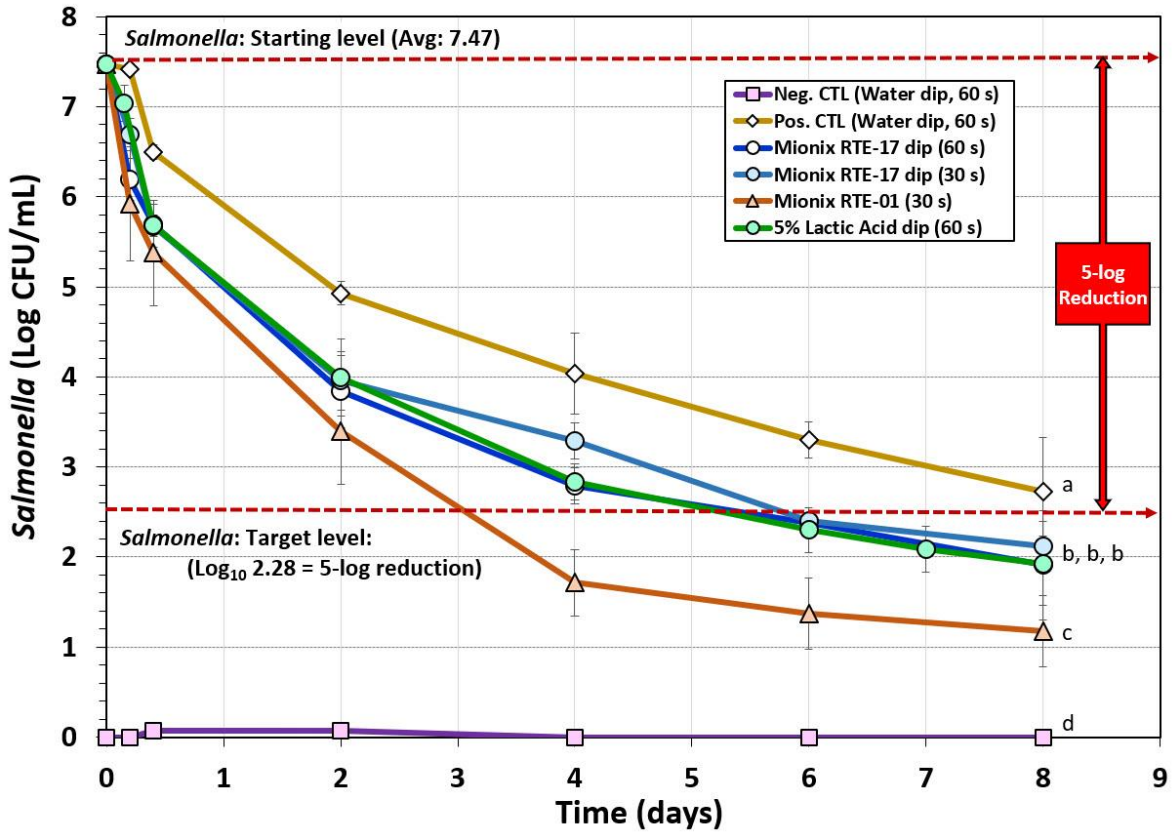

Figure 3. Short-term processing of biltong for reduction of *Salmonella*. Comparison of antimicrobials and pre-marination dip treatment time (30 or 60 s) with antimicrobials including lactic acid (5%) and acidified calcium sulfate (Mionix RTE-17 diluted to 5% lactic acid; Mionix RTE-01 diluted to 10% lactic acid). After treatment and marination, beef was held at 25 °C (77 °F) and 55% relative humidity (RH) for up to 8 days. Non-inoculated negative controls (Neg CTL) were used to

demonstrate the effectiveness of selective media against background organisms. Graphs of different trials were adjusted to a common starting level. Treatments were analyzed by repeated measures one-way analysis of variance (RM-ANOVA) using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences; treatments with different letters are significantly different ($p < 0.05$); treatments with the same letter are not significantly different ($p > 0.05$).

Biltong trials also examined the effect of vinegar level (2%, 3%, vs. 4% vinegar) and salt level (1.7%, 2.2%, or 2.7%) during marination on *Salmonella* lethality (Figure 4). The use of 2% vinegar in the marinade formulation again did not achieve 5-log reduction of *Salmonella* in 8 days of drying (Figure 4A). However, 3% and 4% vinegar demonstrated greater inhibition of *Salmonella*, dropping levels much earlier in the process yet showed no significant difference between them ($p < 0.05$) and both achieved >5-log reduction within 7–8 days (Figure 4A). Trials comparing the use of 1.7%, 2.2%, or 2.7% NaCl levels in the marinade formulation were not significantly different ($p < 0.05$) and all reached >5.5-log reduction in 6 days (Figure 4B). The data suggest that 1.7% NaCl may be used in order to reduce sodium levels in the final product.

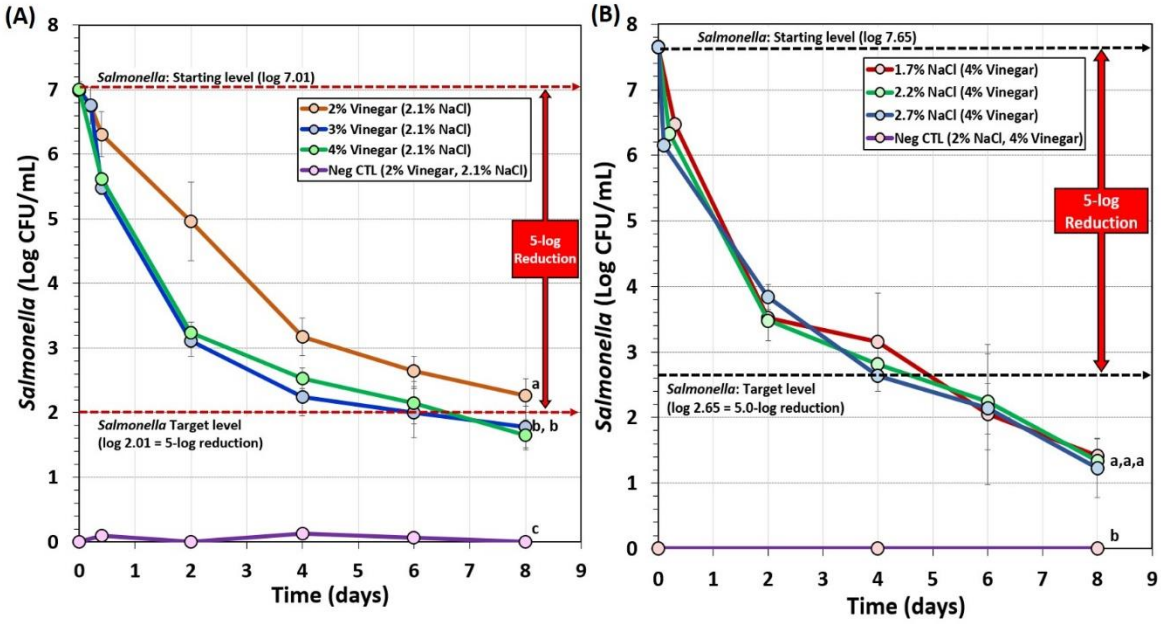

Figure 4. Short-term processing of biltong for reduction of *Salmonella*. (**A**) Comparison of 2%, 3%, or 4% vinegar in the marinade formulation. (**B**) Comparison of 1.7%, 2.2%, or 2.7% NaCl in the marinade formulation. After marination, beef was held at 25°C (77°F) and 55% RH for up to 8 days. Negative controls (Neg CTL) demonstrate the effectiveness of the selective media against background organisms from non-inoculated/processed beef. Graphs of various trials were adjusted to a common starting level. Treatments were analyzed by RM-ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences; treatments with different letters are significantly different ($p < 0.05$); treatments with the same letters are not significantly different ($p > 0.05$).

3.2. Long-Term Biltong Marination Process

Several additional overnight marination processes were examined. One involved vacuum-tumbling with the complete marinade mixture followed by overnight marination at refrigeration temperature (Figure 5A). This process included microbial testing after inoculation, after an antimicrobial dip treatment and an additional refrigerated hold period (1 h), after an overnight refrigerated marination step (0 days drying), and after 2, 4, and 6 days of drying (Figure 5A). A 5-log

reduction was achieved after 4 days of drying (Figure 5A). Background microorganisms that appeared on the 'negative control' plates (not inoculated with *Salmonella*) also declined during processing and were orders of magnitude lower than the levels of *Salmonella* on experimental samples inoculated with *Salmonella*. The data show that 5% lactic acid and 3% sodium acid sulfate both achieved 5-log reduction and were not significantly different ($p < 0.05$). Furthermore, positive control samples without additional antimicrobial dip treatment also achieved 5-log reduction, albeit later than the antimicrobial-treated samples (Figure 5A).

Another 'overnight marination' process included a traditional South African recipe whereby biltong beef samples were dry tumbled with spice and salt (without vacuum), placed in a pan, and vinegar was slowly added to prevent washing off the spices. This was marinaded overnight at 5 °C. The beef pieces sitting in the vinegar marinade (not completely covered) were turned over after 30–60 min, and then turned over again after 6–8 h until completion to allow equal marination of both sides prior to hanging in the humidity chamber. The extended overnight marinade provided a ~1.0–1.3 log reduction of *Salmonella* followed by an additional ~2.3-log reduction during the first 2-days of drying (Figure 5B). The targeted 5-log reduction of *Salmonella* was obtained after 6 days of drying (7th day on Figure 5B) and well beyond the 5-log reduction level by the 8th day. This method used the largest volume of vinegar (10% of total formulation) compared to the other methods.

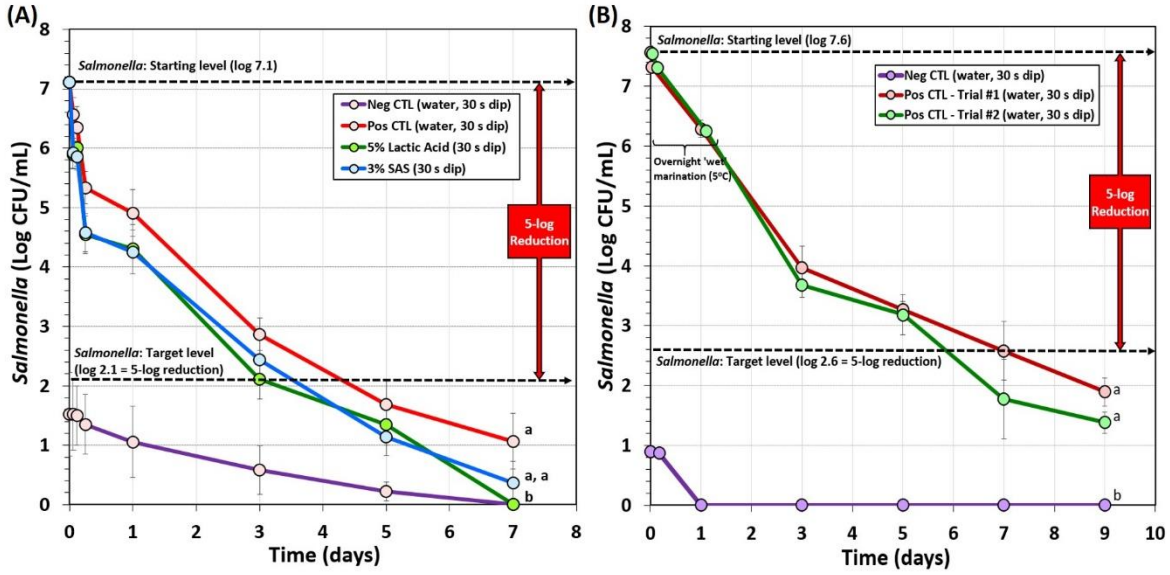

Figure 5. Processing of biltong for reduction of *Salmonella* involving overnight marination at 5 °C. (**A**) Overnight marination process including vacuum-tumbling of beef with spices, salt, and vinegar. Trials include pre-marination dip treatment with antimicrobials (5% lactic acid, 3% sodium acid sulfate) or water (positive and negative controls). After marination, beef was held at 23.9 °C (75 °F) and 55% RH for up to 6 days. (**B**) Overnight marination process, spiced-beef tumbled without vacuum and vinegar was added separately during static marination in the refrigerator. No separate antimicrobial dip treatment was used. After marination, beef was held at 22 °C (72 °F) and 55% RH for up to 8 days. Non-inoculated spice-processed beef (i.e., negative controls) was run in parallel with the other trials and plated on selective media. Graphs of different trials were adjusted to a common starting level. Treatments were analyzed by RM-ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences; treatments with different letters are significantly different ($p < 0.05$); treatments with the same letters are not significantly different ($p > 0.05$).

3.3. Temperature and Relative Humidity Measurements

Temperature measurements were the average of 2 probes placed in the humidity oven chamber (chamber temp) and 2 additional probes inserted into 2 pieces of beef (beef temp) at different positions in the chamber (Figure 6). The various temperature/humidity parameters targeted 25 °C/55% RH (Figure 6A), 23.9 °C/55% RH (Figure 6B), and 22 °C/55% RH (Figure 6C). The chamber temperature varied more than the beef because the unit would heat up the air when the temperature fell below the set point and then refrigerate and cool down when it ran above the set point, showing an oscillation over time. The solid beef pieces were buffered from these short temperature changes and probes inserted into the beef did not show the same type of incremental variation. Similarly, an external humidity probe was inserted into the chamber that was set at the same 55% RH for each of the 3 different temperature regimens (Figure 6A–C).

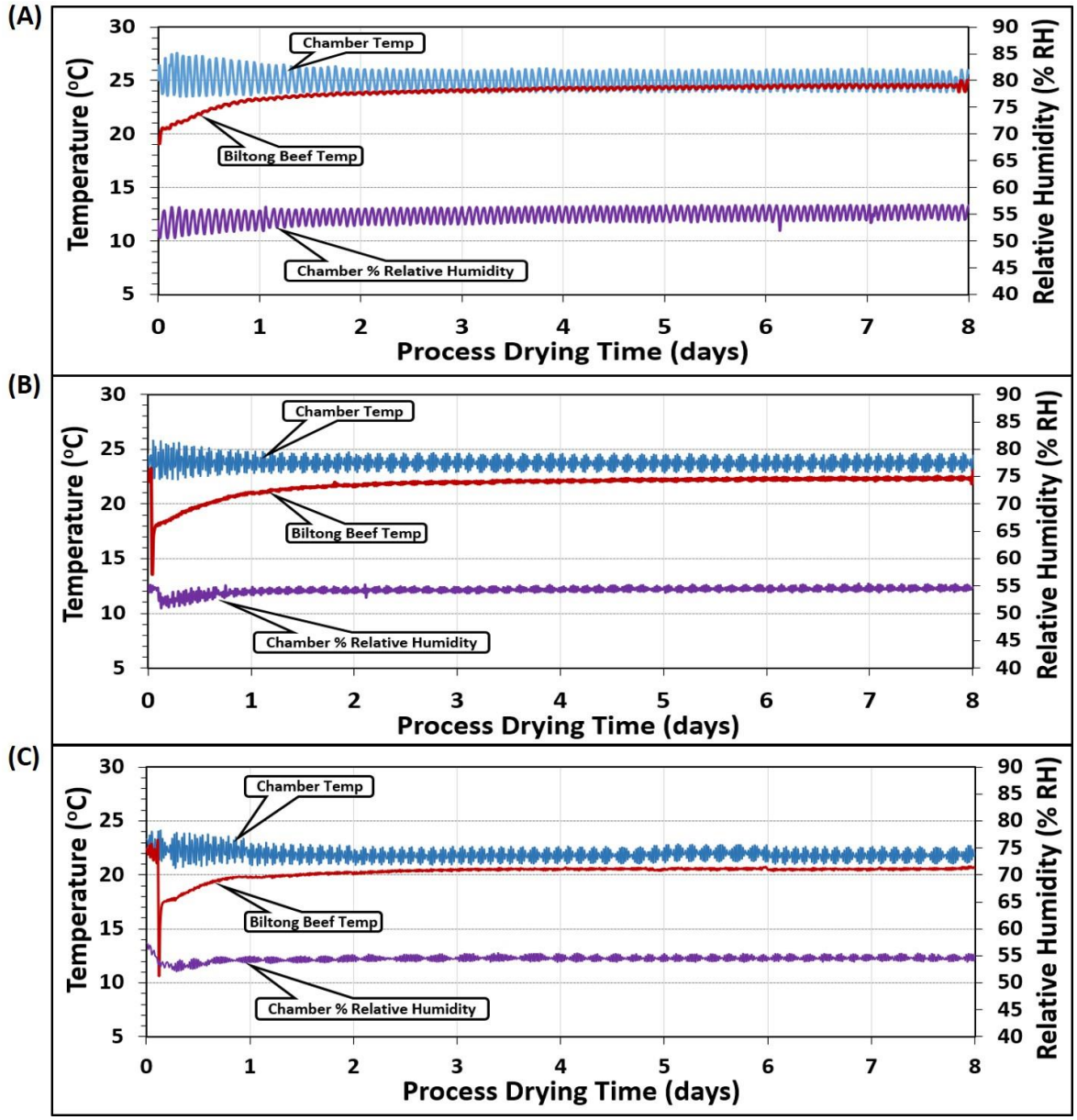

Figure 6. Temperature (°C) and relative humidity (% RH) during several different biltong processes covering three different ranges of temperature in the humidity oven: (**A**) 24–26 °C, (**B**) 23–24.5 °C, and (**C**) 21–23 °C, all maintained at 55% RH.

3.4. Water Activity and Moisture-Loss Measurements

Water activity (A_w) measurements were obtained using a Rotronic USB probe chamber (Figure 1Q), laptop, and software. Although many people suggested 'chopping' the biltong beef into pieces to get an overall 'average' of the product, it was determined that cutting the biltong beef so that the inside portion was tested for A_w provided the most conservative determination of water activity. Samples of the outer surface tested for water activity gave A_w ranges of 0.80–0.81 (Figure 2A). When biltong beef was positioned with the innermost portion directed upwards towards the sensor, A_w ranges of 0.89–0.90 were obtained (Figure 2C). Furthermore, when biltong beef was chopped up to have a combination of inner and the drier outside portions (Figure 2B), intermediate levels between these were observed (0.84–0.86).

Water activity measurements were obtained using non-inoculated 'negative control' samples to alleviate concerns of *Salmonella*, but were still processed with the same spice, salt, and vinegar marinade as the positive controls. Water activity and moisture loss measurements taken from 3 different processing temperature ranges (22 °C, 23.9 °C, and 25 °C) but the same relative humidity (55% RH) show incremental decrease in both A_w and moisture loss over time as processing temperature is increased (Figure 7).

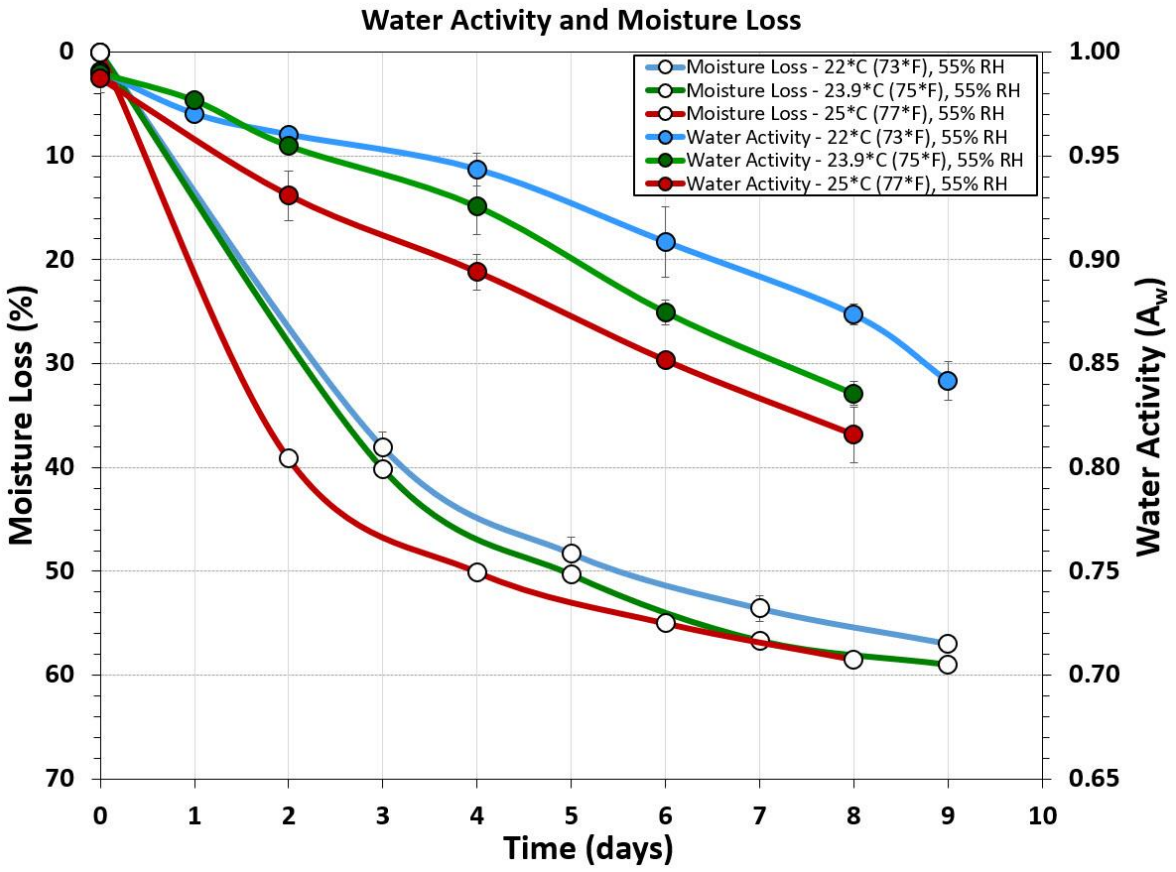

Figure 7. Water activity and moisture loss during several different biltong processes covering three different ranges of temperature at the same humidity level: 22 °C (73 °F), 23.9 °C (75 °F), and 25 °C (77 °F); all at 55% RH.

4. Discussion

Biltong is a new and expanding product line in the US dried beef market and is often marketed as a gourmet dried beef because it is not heated to high temperatures like beef jerky. Since it is an RTE shelf-stable dry beef, it must comply with USDA-FSIS guidelines (Revised Appendix A (USDA-FSIS, 2017a) and Jerky Guidelines (USDA-FSIS, 2014)) that address *Salmonella* reduction during the manufacture of such products. The fact that 'traditional' South African biltong does not use a heat lethality step was viewed as making it difficult to achieve the targeted reduction of *Salmonella* with this product.

One of the main reasons a 5-log reduction of acid adapted *Salmonella* was so readily achieved in this study was the manner in which *Salmonella* was enumerated. In a prior study on biltong Burnham et al. (2008) enumerated *Salmonella* on a 'colony forming unit per gram' (cfu/g) basis from start to finish in their process, and they never achieved a 5-log reduction (Burnham et al., 2008). Jones et al. (2019) also enumerated on a cfu/g basis in their biltong study, but they did not evaluate log reduction of *Salmonella*, but simply analyzed for log presence of various microorganisms on biltong at the end of the process (Maxine Jones et al., 2019). They were processing according to South African regulatory standards which does not require a specified log-reduction of targeted microorganisms. Similar enumeration on a cfu/g basis has also been reported for numerous studies with beef jerky (Allen, Cornforth, Whittier, Vasavada, & Nummer, 2007; Keshia Naidoo & Denise Lindsay, 2010; Petit et al., 2014; Porto-Fett, Call, & Luchansky, 2008). The comparison of microbial enumeration (i.e., cfu/g) between fresh raw beef (100% moisture) at the beginning of the process to the dried product at the end of the process (~60% moisture loss) is an inequitable microbial comparison. Drying of the underlying beef results in a concentration of residual microbial counts that undercuts the log reduction of the remaining *Salmonella* because the microorganisms are concentrated if enumerated on a 'per gram' basis. In this study, each similar-sized piece of beef was inoculated with a fixed quantity of inoculum (i.e., 150 μL/side; 300 μL/piece). Enumeration was then obtained by stomaching fresh (~100–110 g), partially-processed (~90–70 g), or dried beef (~50–40 g) in the same fixed volume of recovery diluent (i.e,., 100 mL), thereby overcoming problems due to concentration of the underlying beef tissue (and the overlying microbial population) by moisture loss. By maintaining a fixed volume of diluent, the microbial concentration factor is overcome and the final count is directly related to the earlier count without influence of beef tissue concentration due to drying.

Although traditional biltong processing does not include a thermal lethality step, it generally includes several ingredients (salt, vinegar) and processing conditions (drying) that either individually, or in combination, provides an antimicrobial effect. Salt is one of those ingredients that is not restricted, although the US FDA and Health and Human Services (HHS) have put in place voluntary programs to reduce the use of sodium because of overuse and excess consumption in the US food supply has resulted in high levels of hypertension and chronic heart disease (Department of Health and Human Services, 2016). In dried beef products, externally applied salt in the marinade helps to bind moisture and draw water out of meat (and bacteria) to facilitate drying. The use of different levels of sodium chloride (1.7%, 2.2%, and 2.7%) showed no significant difference ($p < 0.05$) in *Salmonella* lethality during biltong processing and, therefore, the use of 1.7% NaCl would do well to maintain *Salmonella* lethality while simultaneously reducing sodium levels in the finished product.

In the USA, vinegar and acetic acid are not considered equivalent ingredients, even at the same level of acetic acid concentration. On meat products, the application of acetic acid is regulated according to the USDA-FSIS 'Safe and Suitable Ingredients List' as a processing aid (USDA-FSIS, 2018). The USDA-FSIS does not regulate the use of vinegar because it is covered by US FDA regulations (United States Food and Drug Administration, 1995) as a Generally Recognized as Safe (GRAS) substance with no restriction on concentration limit (as 'grain' or % acetic acid) or treatment time. Thereby marination with vinegar can be short or as long as an overnight process. However, a process validated at a lower concentration of vinegar can be readily switched to a higher vinegar concentration, but the reverse is not true unless microbial (*Salmonella*) validation data is provided to

justify the decrease. In contrast, the use of 'acetic acid' as a processing aid may be limited to a particular concentration (i.e., ≤5%) and an 'appropriate treatment time' (i.e., 30–60 s is appropriate; 10 min is not appropriate). When vinegar is used, it must be listed on the ingredient label and the particular type of vinegar must be specified (i.e., white distilled vinegar, apple cider vinegar, balsamic vinegar, cane vinegar, coconut vinegar, malt vinegar, red wine vinegar, rice vinegar, sherry vinegar, white wine vinegar). Although vinegar is required to be listed on the ingredient label, it is generally considered an acceptable and innocuous ingredient by most consumers.

Additional antimicrobials may also be used to improve pathogen reduction prior to trimming or on the subsequent trimmed beef strips. Many of the preferred antimicrobials used on meat and poultry in the USA as per the USDA-FSIS Safe and Suitable list (USDA-FSIS, 2018) are those that are considered 'processing aids' (USDA-FSIS, 2008). Certain antimicrobials that can be designated as processing aids have restricted use levels and contact times, and if used as designated by USDA-FSIS, they are not required to be listed on the ingredient label. According to federal labeling requirements, processing aids are defined 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; US-FDA, 2016) and, therefore, are not required to be labeled. Of the antimicrobials used in this study, acidified calcium sulfate (RTE-01 at 10% lactic acid) would have to be listed on the label. However, lactic acid (5%), sodium acid sulfate (3%), and acidified calcium sulfate (RTE-17 at 5% lactic acid) are considered processing aids and need not be listed on the ingredient label in the USA. This type of 'clean/green label' ingredient is preferable to many companies that do not want excessive and complicated ingredient labels.

5. Conclusions

The absence of a heat lethality step during biltong processing and the inability of a prior biltong study to achieve 5-log reduction lead us to believe that a 5-log reduction of *Salmonella* in biltong would be difficult to obtain and initially we resorted to the use of additional antimicrobial treatments to achieve these reductions. The combination of vinegar/salt marinade by itself appears to give a significant reduction during processing and subsequent drying. Perhaps even more important was the manner of microbial enumeration that eliminated the effects of dried beef substrate that reduces the log reduction if performed on a cfu/g basis. We were able to demonstrate a 5-log reduction of *Salmonella* in all trials involving different processes and different ingredient formulations, even with the use of acid adapted cultures with reduced sensitivity to acidic conditions (i.e., vinegar) and without additional antimicrobials.

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Conflicts of Interest: The authors declare no conflict of interest.

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Chapter V

Quantification of Process Lethality (5-Log Reduction) of *Salmonella* and Salt Concentration During Sodium Replacement in Biltong Marinade

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Abstract: Salt (sodium chloride, NaCl) is commonly used in ready-to-eat (RTE) meat products such as biltong, a South African style dried beef product for flavor, enhanced moisture loss, and reduction of microbial growth. However, increased consumption of high sodium content foods is commonly associated with high blood pressure and heart disease. This study evaluated the use of alternative salts, potassium chloride (KCl) and calcium chloride ($CaCl_2$) in the biltong marinade to achieve a ≥ 5-log reduction of *Salmonella*, a pathogen of concern in beef products. Beef pieces (1.9 cm × 5.1 cm × 7.6 cm) were inoculated with a five-serovar mixture of *Salmonella* (*Salmonella* Thompson 120, *Salmonella* Enteritidis H3527, *Salmonella* Typhimurium H3380, *Salmonella* Heidelberg F5038BG1, and *Salmonella* Hadar MF60404), vacuum-tumbled in a traditional biltong marinade of salt, spices, and vinegar containing either NaCl, KCl or $CaCl_2$ (2.2% concentration) followed by an 8–10 day drying period at 23.9 °C (75 °F) and 55% relative humidity. Microbial enumeration of *Salmonella* was conducted following inoculation, after marination, and after 2, 4, 6, 8, and 10 days of drying in a humidity/temperature chamber. Biltong produced with $CaCl_2$, NaCl, or KCl achieved a > 5-log reduction of *Salmonella* after 6, 7, and 8 days, respectively. The *Salmonella* reduction trends with biltong made with NaCl or $CaCl_2$ were not significantly different ($p < 0.05$) while both were significantly different from that made with KCl ($p > 0.05$). Sodium, calcium, and potassium ion concentrations were measured using ion-specific electrode meters following biltong processing and drying. As expected, the biltong made with the corresponding salt had the most abundant ion in the sample. Regardless of the salt used in the marinade, the potassium ion levels were moderately elevated in all samples. This was determined to be from potassium levels naturally present in beef rather than from other ingredients. Sampling of several commercial brands of biltong for sodium content showed that some were significantly above the allowable level of claims made on package ingredient statements. The substitution of NaCl with KCl or $CaCl_2$ during biltong processing can also provide a 5-log reduction of *Salmonella* to produce a safe product that can be marketed as a more healthy low-sodium food alternative that may appeal to consumers who need to reduce their blood pressure and are conscientious of sodium levels in their diet.

Keywords: biltong; dried beef; salt replacement; *Salmonella*; 5-log reduction; marinade; potassium chloride; calcium chloride

1. Introduction

Salt has historically been used for hundreds of years in food for two main purposes: flavoring and preservation. However, sodium intake among consumers has dramatically increased with the consumption of processed foods (Mattes & Donnelly, 1991). High sodium intake is associated with many health issues including hypertension and cardiovascular disease (Cook et al., 2007; He et al., 1999; Zhao et al., 2011). The recommended dietary guidelines for intake of sodium should be less than 2300 milligrams (mg), however the average adult normally consumes more than 3000 mg per day (HHS, 2015). The World Health Organization and the American Heart Association recommend even lower levels, of less than 2000 and 1500 mg/day, respectively. Therefore, it is important for consumers to have options for food products that contain low levels of salt without compromising on the taste or texture of the food. Aside from the health consequences of an overabundance of sodium in a typical US diet, potassium and calcium deficiencies are a growing health concern for consumers. Various food items attempt to supplement these nutrients with claims of being 'fortified'. The replacement of NaCl during the production of biltong with other salts such as KCl or $CaCl_2$ could serve as an alternative source for these nutritive ions which are lacking in many consumers' diet.

High sodium content is of particular importance for processed, dried or cured meats that traditionally rely on the addition of sodium chloride (NaCl) during processing to help flavor and preserve the meat. Salt contributes to the decrease in water activity (a_w) by drawing water out of the meat, limiting the amount of free water available for microbial growth as well as influencing the overall flavor, appearance, and texture of the product (Taormina & Sofos, 2014). The addition of salts to dried meats can aid in interference with bacterial cellular mechanisms thus reducing the rate of bacterial growth (Shelef & Seiter, 2005). One strategy to reduce the sodium content in foods while preserving the microbiological inhibitory effects is to replace NaCl with an alternative salt such as KCl, $CaCl_2$, or $MgCl_2$ (Stringer & Pin, 2005; Taormina, 2010). Replacing sodium with alternative salts can also potentially help to achieve a healthier food product while still maintaining the same quality and safety. The concept of salt replacement in processed meat products is not new, as many studies have examined the replacement of NaCl with KCl, $CaCl_2$, or $MgCl_2$ in dried meat products (M. Aliño et al., 2009; Marta Aliño et al., 2010; Blesa et al., 2008). Partial replacement of NaCl with KCl and $CaCl_2$ in various dried meat products including dry-cured pork loins and Spanish dry-cured hams demonstrated similar organisms as traditional products made with NaCl and no significant differences in a_w (M. Aliño et al., 2009; Marta Aliño et al., 2010; Blesa et al., 2008). Although sensory attributes of salt and salt replacement are important in all meat products, they are more readily detected in large, intact muscle products (dry-cured hams) where the final product is associated with a recognized and expected meat flavor and where salt type can influence lipid oxidation over the extended drying period (Ripollés, Campagnol, Armenteros, Aristoy, & Toldrá, 2011; Vidal, Lorenzo, Munekata, & Pollonio, 2020). However, smaller and thin-shaved meat products (jerky, biltong) all have numerous product variations and a high degree of topical seasoning that may mask alternative salt sensory attributes. From a microbial safety standpoint, these studies have shown that it is possible to achieve a microbially safe, dry cured product with a partial or total alternative salt replacement similar to those that are produced with NaCl.

Biltong, is a South African style dried beef (jerky, kippered beef) product that uses lean cuts of beef that are marinaded in a traditional spice and vinegar mixture which includes salt and is then dried for an extended period of time at ambient temperature (Naidoo & Lindsay, 2010a, 2010b; Petit, Caro, Petit, Santchurn, & Collignan, 2014). In the United States (US), the US Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) requires manufacturers of biltong to provide microbial validation of reduction of *Salmonella* (a foodborne pathogen often associated with raw meat products) by one of two approaches (USDA-FSIS, 2017). One approach ("process reduction + *Salmonella* testing") is to demonstrate ≥ 2-log reduction of *Salmonella* by the process while simultaneously testing each lot of ingredients for *Salmonella* to insure they are *Salmonella*-free; the

other method ("process-only") is to ensure that the process itself (without *Salmonella*-testing of ingredients) demonstrates a ≥ 5-log reduction of *Salmonella* (USDA-FSIS, 2017). This latter process is the most sought after because continuous testing of ingredients for *Salmonella* in the former process is laborious and expensive, and if ingredients test positive, they cannot be used unless rendered free of *Salmonella*.

Currently, there is no known published research available that identifies whether the use of alternative salts in biltong manufacture has the same ability to contribute to the reduction of foodborne pathogens during biltong processing as does traditional salt (NaCl). In this study, we examined the efficacy of using alternative salts including KCl and $CaCl_2$ in place of traditionally used NaCl during biltong processing to achieve a USDA-FSIS required ≥ 5-log reduction of *Salmonella* if using the "process-only" method of biltong manufacture.

2. Materials and Methods

2.1. Bacterial Strains and Growth and Storage Conditions

Acid-adapted *Salmonella* serovars used in this study included: *Salmonella enterica* subsp. *enterica* serotype Thompson 120 (chicken isolate), *Salmonella enterica* subsp. *enterica* serotype Hadar MF60404 (turkey isolate), *Salmonella enterica* subsp. *enterica* serotype Heidelberg F5038BG1 (ham isolate), *Salmonella enterica* subsp. *enterica* serotype Typhimurium H3380 (DT 104 clinical isolate), and *Salmonella enterica* subsp. *enterica* serotype Enteritidis H3527 (phage type 13a, clinical isolate). These strains have been described in numerous research publications involving antimicrobial processing interventions against *Salmonella* spp (Carpenter, Smith, & Broadbent, 2011; Juneja, Eblen, & Marks, 2001; Juneja, Hwang, & Friedman, 2010; Juneja et al., 2012; Karolenko, Bhusal, Gautam, & Muriana, 2020; Karolenko, Bhusal, Nelson, & Muriana, 2020).

Salmonella cultures were inoculated into Tryptic Soy Broth (TSB, BD Bacto, Franklin Lakes, NJ, USA) and grown at 37 °C. Cultures were prepared for storage by centrifugation (6000× *g*, 5 °C) of 9–10 mL of fresh, overnight cultures and resuspending the resulting cell pellets with 2–3 mL of fresh sterile TSB containing 10% glycerol. The resuspended cells/freezing menstrum were placed in glass vials and stored in an ultra-low freezer (−80 °C). Frozen stocks were revived by transfer of 100 µL of thawed cell suspension into 9 mL of TSB, incubating overnight at 37 °C, and sub-culturing twice before use. Serial dilutions were made in 0.1% Buffered Peptone Water (BPW, BD Difco) and microbial enumeration was carried out on Tryptic Soy Agar (TSA, BD Bacto; 1.5% agar), plated in duplicate.

Acid adaptation of these *Salmonella* serovars was carried out with cultures inoculated into TSB augmented with 1% glucose according to Wilde et al. and others (Calicioglu, Sofos, Samelis, Kendall, & Smith, 2003; Karolenko, Bhusal, Gautam, et al., 2020; Karolenko, Bhusal, Nelson, et al., 2020; Wilde, Jørgensen, Campbell, Rowbury, & Humphrey, 2000). Individual cultures were harvested by centrifugation, resuspended with 0.1% BPW (BD Difco), and held at refrigerated temperature until use (5 °C). The centrifuged and resuspended individual cultures were then combined in equal proportions to obtain the mixed inoculum.

2.2. Beef Processing and Inoculation

Intact, select grade, beef bottom-round sub-primal cuts (Ralph's Packing Co., Perkins, OK, USA) were trimmed to approximately 5.1 cm wide × 1.9 cm thick × 7.6 cm long beef pieces at the R.M. Kerr Food and Agricultural Product Center (FAPC). Beef pieces used for this experiment were vacuum-packaged fresh, flash frozen (−80 °C), stored frozen (−20 °C), and thawed immediately before use. The beef pieces were inoculated by pipette with 150 uL of the 5-serovar *Salmonella* inoculum mixture on each side, and immediately spread with a 'gloved finger'. Inoculated beef pieces were then incubated for 30 min at 4–5 °C to allow for bacterial attachment prior to use.

2.3. Biltong Processing and Salt Replacement

Inoculated beef pieces were placed in plastic dip cages and dipped in sterilized water for 30 s (to mimic wetting by rinse treatment and/or alternative antimicrobial dips) and excess liquid was allowed to drain off. Meat pieces were then transferred to chilled steel tumbling vessels containing a biltong marinade. The biltong marinade was comprised of 2.2% salt, 0.8% black pepper, 1.1% coarse ground coriander and 4% red wine vinegar (100-grain; 10% acetic acid) as a percentage of total meat weight. Separate marinades were formulated to determine the efficacy of alternative salts in the biltong process whereby 2.2% NaCl (Fisher Chemical, Fisher Scientific, Atlanta, GA, USA) was replaced with 2.2% KCl (Fisher Chemical), or 2.2% $CaCl_2$ (Acros Organics, Fisher Scientific). Beef pieces were sealed in a steel drum with marinade, a vacuum was drawn to 15 inches Hg and then locked, and the drum was allowed to rotate for 30 min on a vacuum tumbler (Biro VTS-43, Marblehead, OH, USA). Meat pieces were then hung in a temperature-controlled humidity oven (Hotpack, Warminster, PA, USA) maintained at 55% relative humidity (RH) and 23.9 °C dried for up to 10 days. Sampling was conducted following marination (Day 0) and then again every 48 hrs.

For comparative purposes, the pH and a_w of raw beef starting product and 8-day product were compared. Analysis of a_w was performed as previously described and included both internal a_w of sliced biltong beef pieces as well as ground pieces (Karolenko, Bhusal, Nelson, et al., 2020). The pH of raw beef was performed directly after grinding the raw beef, or with dried beef by adding 2 parts deionized water to 1 part dried beef (ground) for samples that had been dried for 8 days.

2.4. Microbial Anaylsis

At each sampling time point, meat pieces were randomly selected from each salt marination batch and transferred to sterile Whirl-pak filter-stomaching bags (4-mil; Nasco, Fort Atkinson, WI, USA). After addition of 100 mL of 1% neutralizing Buffered Peptone Water (nBPW, Criterion, Hardy Diagnostics, Santa Maria, CA, USA), samples were stomached for 90 sec in a Masticator paddle-blender (IUL Instruments, Barcelona, Spain). Serial dilutions were surface plated on Selenite Cystine Agar (SCA) containing spectinomycin (5 μg/mL), clindamycin (5 μg/mL) and novobiocin (50 μg/mL) as described previously by Karolenko et al. (Karolenko, Bhusal, Gautam, et al., 2020). The filter bag dilution was considered the 10^0 dilution. Plates were incubated for 48 h at 37 °C and enumerated as log CFU/mL relative to the 10^0 filter bag dilution. Treatments were performed in duplicate replication and sampled in triplicate at each sampling time.

2.5. Determination of Salt Ion Concentration in Experimental Biltong and Comparison to That in Commercially Available Biltong

Following drying, biltong pieces (Figure 1A) were cut into smaller pieces and finely ground using a laboratory blender (Waring Commerical, New Harford, CT) until a homogenized mixture was formed (Figure 1B). Five (5) g of the finely ground dried beef was weighed out and brought up to 100 g with distilled water in a stomacher bag and macerated in a paddle mixer to homogenize the sample thoroughly.

Individual Horiba LAQUA Twin Pocket Ion Meters (Horiba Intruments, Irvine, CA, USA) were used for Na^+, Ca^{2+}, and K^+ ion quantitation. Although the instruments came with an ion standard solution, we also prepared a series of standards that would reflect the various levels experienced in our testing to ensure the linearity of the response throughout this range. As per the manufacturer's instructions, 300 μL of the diluted/mixed sample was placed into the sensor chamber of the appropriate salt meter and stable readings (in ppm) were recorded. Readings were taken in duplicate from three different batch trials. Additionally, each sample was also tested in the remaining two ion meters to confirm the presence or lack of any additional salt ions. To determine the ion concentration content per serving size, the following equation was used:

$$\text{Calculated Ion ppm (mg/100g)} = \text{Meter reading } \left(\frac{\text{mg}}{\text{L}}\right) \times \frac{\text{Volume after Dilution (L)}}{\text{Biltong Weight (g)}} \times 100 \quad (1)$$

$$\text{Calculated Ion per Serving Size } \left(\frac{\text{mg}}{28\text{ g}}\right) = \text{Calculated Ion } \left(\frac{\text{mg}}{100\text{ g}}\right)/3.57 \quad (2)$$

The serving size was determined by using the commonly-used serving sizes listed on commercial biltong products that list a serving size of 28 g (1 oz).

Ion concentrations (Na^+, Ca^{2+}, K^+) of commercially produced biltong were sampled using the protocol listed in Section 2.5 with slight modifications. Two different formulations of biltong products were tested from each of two different biltong manufacturers (i.e., Company A, Company B) for a total of four products analyzed in total. Samples were ground until a fine uniform consistency was achieved using the laboratory blender (Figure 1B). Samples were processed as described above and ion concentrations measured with each of the ion meters. Three different ground mixtures were made from each type of biltong product and then readings were averaged. Readings for each sample taken with the ion meters were made from duplicate samplings. Percent recovery of sodium was calculated by:

$$\text{Percent Recovery (Sodium)} = \frac{\text{Calculated Na } \left(\frac{\text{mg}}{28\text{ g}}\right)}{\text{Listed Sodium on Label } \left(\frac{\text{mg}}{28\text{ g}}\right)} \quad (3)$$

Calcium and potassium ion concentrations of each biltong product were also determined using the equations in Section 2.5.

Figure 1. Sampling biltong for salt ion analyses: (**A**) representative in-house manufactured biltong after 8 days of drying showing the inside of dried beef muscle tissue (left) and retention of surface seasoning (right); (**B**) finely ground biltong samples to be mixed in water for ion analysis.

2.6. Statistical Anaylsis

Replicate process validation trials were performed in duplicate, with 3 samples tested per sampling period within each replication (n = 6), in accordance with inoculated validation criteria established by the National Advisory Committee on Microbial Criteria for Foods (NACMCF) (NACMCF, 2010) and accepted by USDA-FSIS (USDA-FSIS, 2015). Replications were performed as autonomous and separate experiments using separately-inoculated cultures and prepared plating media. All other tests were performed in triplicate replication. All data are presented as the mean of multiple replications with standard deviation of the mean represented by error bars. Statistical analysis of timed series data was performed using repeated measures one-way analysis of variance (RM-ANOVA); statistical analysis of all other data was done using one-way analysis of variance (ANOVA). Pairwise multiple comparisons were done using the Holm–Sidak test to determine significant differences. Data treatments with the same letter are not significantly different ($p > 0.05$); treatments with different letters are significantly different ($p < 0.05$).

3. Results

3.1. Microbial Lethality Validation of Salt Replacement

A 2.2% salt concentration was applied in the ingredient formulation for all three salts (NaCl, KCl, or $CaCl_2$) used in separate biltong marinades for comparison of their effect on reduction of *Salmonella* during biltong processing. All inoculated beef pieces were subjected to a 30-s rinse treatment in sterile water to mimic commercial rinse practices, resulting in ~0.2-log reduction of the inoculated *Salmonella* (not shown on graph; combined with post-marinade reduction). Subsequent marination of beef pieces with NaCl had a post-marinade reduction of 1.38-log while treatments using KCl and $CaCl_2$ had a post-marinade reduction of 1.11- and 1.43-log, respectively (Figure 2). During the drying of biltong over the next 4 days, *Salmonella* levels continued to decline at a similar rate (Figure 2). Biltong formulated with $CaCl_2$ was able to achieve ≥ 5-log reduction by day 6, biltong formulated with NaCl achieved this same benchmark by day 7 (by extrapolation), and that made with KCl by day 8. By the end of 10 days of drying, biltong made with either $CaCl_2$ or NaCl achieved an overall reduction of *Salmonella* of 6.37-log and 6.22-log, respectively, while that made with KCl achieved a 5.57-log reduction over the same time period. Statistical analysis of these three biltong processes demonstrated that the NaCl and $CaCl_2$ formulations were not significantly different from each other ($p > 0.05$), but they were significantly different than biltong formulated with KCl ($p < 0.05$). In spite of these differences, all three formulations achieved > 5-log reduction of *Salmonella* within 8-days.

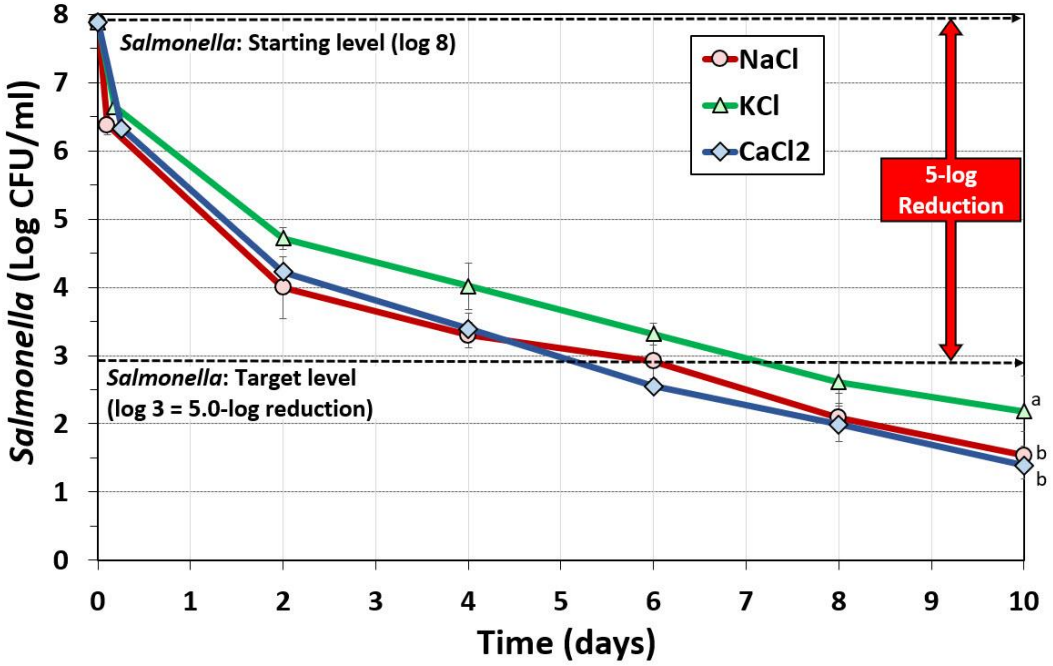

Figure 2. Biltong processing using alternative salts for reduction of *Salmonella.* Comparison of NaCl, KCl, and $CaCl_2$ at a concentration of 2.2% to attempt a 5-log reduction of *Salmonella* population over a period of ten days at 23.9 °C (75 °F) and 55% relative humidity (RH). Statistical analysis of entire time course of graphs: treatments with the same letter are not significantly different ($p > 0.05$); treatments with different letters are significantly different ($p < 0.05$).

3.2. Determindation of Final Salt Ion Concentration, pH, and A_w in Biltong

Specific ion selective electrode (ISE) meters were used for analysis of Na^+, Ca^{2+}, and K^+ ion levels. The testing of standardized salt solutions throughout the range that might be tested in our biltong samples gave excellent results (R^2 values of 0.9999) for each range of standards used for the various individual ISE meters (Figure 3).

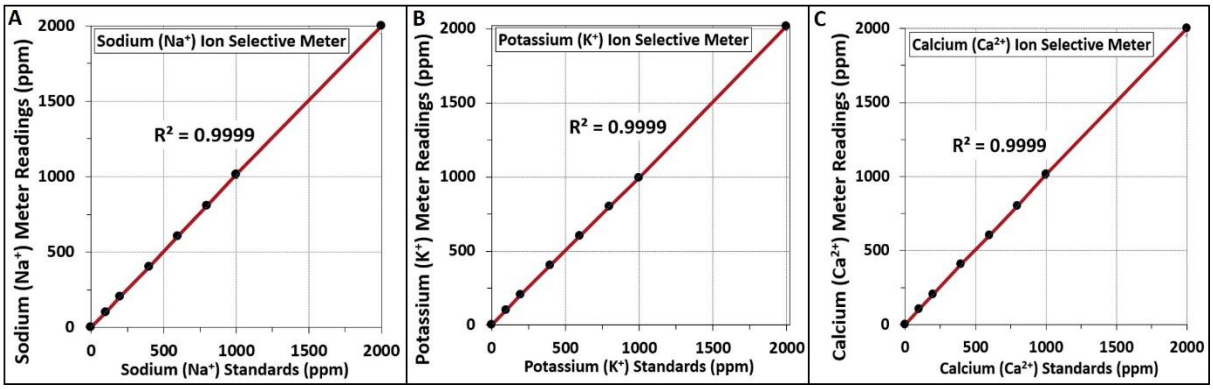

Figure 3. Ion analyses on standardized salt solutions using ion selective electrode (ISE) meters used in this study. Panel (**A**), ion analysis of Na^+ using NaCl standards; (**B**), ion analysis of K^+ using KCl standards; (**C**), ion analysis of Ca^{2+} using $CaCl_2$ standards. Individual standards were prepared in triplicate and data points represent the mean of triplicate samplings with error bars representing the standard deviation of the mean. The coefficient of determination is a measure of the linearity of the data points to the trendline is indicated by R^2 value.

The ion corresponding to the appropriate salt that the marinade was formulated with was the most abundant ion in the sample (Figure 4). Biltong made with NaCl resulted in a Na^+ concentration of 620 ppm, followed by K^+ (408 ppm) and Ca^{2+} (7 ppm). Biltong made with KCl resulted in a K^+ concentration of 1475 ppm followed by Na^+ (57 ppm) and Ca^{2+} (5.8 ppm). Similarly, biltong made with $CaCl_2$ resulted in a Ca^{2+} concentration of 525 ppm followed by K^+ (457 ppm) and Na^+ (54 ppm) (Figure 4).

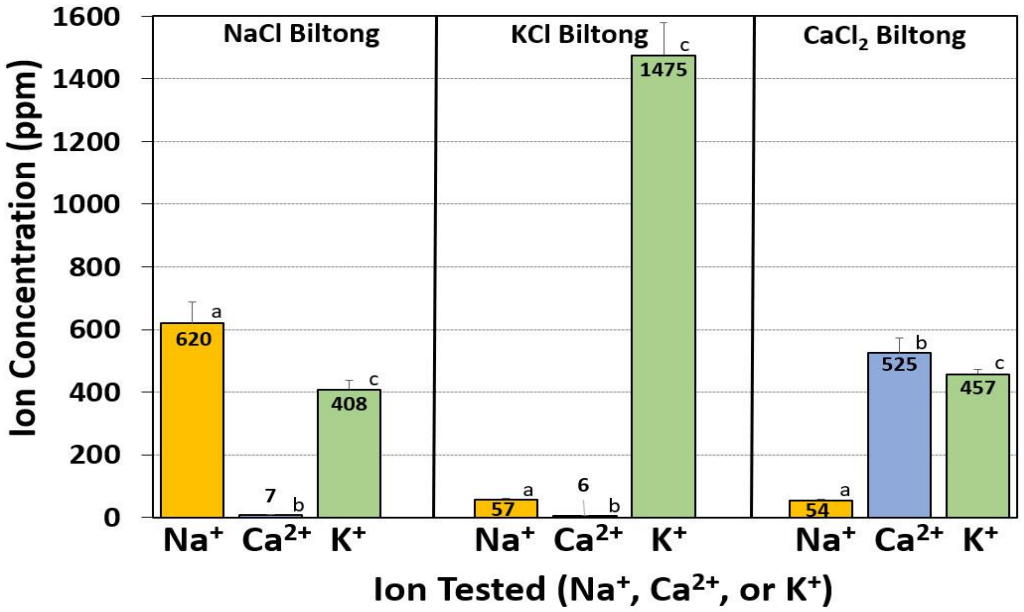

Figure 4. Analysis of Na^+, Ca^{2+} and K^+ ions in biltong made with NaCl, KCl, or $CaCl_2$. Comparison of all 3 ion concentrations in in each batch of biltong made with a single added salt. Data are presented

as the mean of triplicate replications and error bars represent the standard deviation from the mean. Statistical analysis was only performed on ion analyses within the same biltong salt formulation. Means with the same letter are not significantly different ($p > 0.05$) whereas means with different letters are significantly different ($p < 0.05$).

In order to determine the source of additional potassium, dried biltong beef was produced using unseasoned meat pieces without spices, vinegar, or salt, as well as meat pieces seasoned only with spices and vinegar (without salt). Both trials were then dried for 8 days, finely ground, and sampled for ion analysis as described previously. The resulting ion levels for both the unseasoned meat pieces as well as the spice/vinegar biltong pieces were low in both Na^+ (43–45 ppm) and Ca^{2+} (1–5 ppm) ions, but high in K^+ ions at 377 ppm and 370 ppm, respectively (Figure 5).

During biltong manufacture, several processing parameters converge to inhibit the *Salmonella* inoculum: acidic antimicrobials (antimicrobials/vinegar), salt, a_w, and dessication. To further assess the contribution of acidic solution vs. drying on reduction of *Salmonella*, we examined pH and a_w at the beginning and end of the process. The pH of the initial raw beef was pH 5.5 and after marinade processing followed by 8-days of drying, the beef was still pH 5.0 ($CaCl_2$ biltong), 5.26 (NaCl biltong), and 5.38 (KCl biltong) (Table 1). Similarly, the a_w of the initial raw beef was 0.9865 and after 8 days of processing/drying, the internal a_w of sliced biltong pieces was reduced to 0.8206 (NaCl), 0.8276 ($CaCl_2$), and 0.8380 (KCl) (Table 1). The a_w of finely ground biltong beef pieces was significantly lower: 0.6690 (KCl), 0.6860 (CaCls), and 0.6879 (NaCl) (Table 1).

Table 1. Initial and final pH and water activity (a_w) of biltong beef (before processing and after 8 days of drying) made with NaCl, KCl, or $CaCl_2$. All analyses were performed on a triplicate series of samples.

	pH of Biltong		**A_w of Biltong**		
Type of Biltong	**Initial pH (Raw Beef)**	**Final pH (8 Days)**	**A_w Initial (Raw Beef)**	**A_w Final (8 Days) Internal**	**A_w Final (8 Days) Ground**
Made using NaCl	5.50 ± 0.04	5.26 ± 0.06	0.9865 ± 0.0054	0.8206 ± 0.0150	0.6879 ± 0.0196
Made using $CaCl_2$		5.38 ± 0.01		0.8380 ± 0.0032	0.6690 ± 0.0334
Made using KCl		5.00 ± 0.01		0.8276 ± 0.0141	0.6860 ± 0.0230

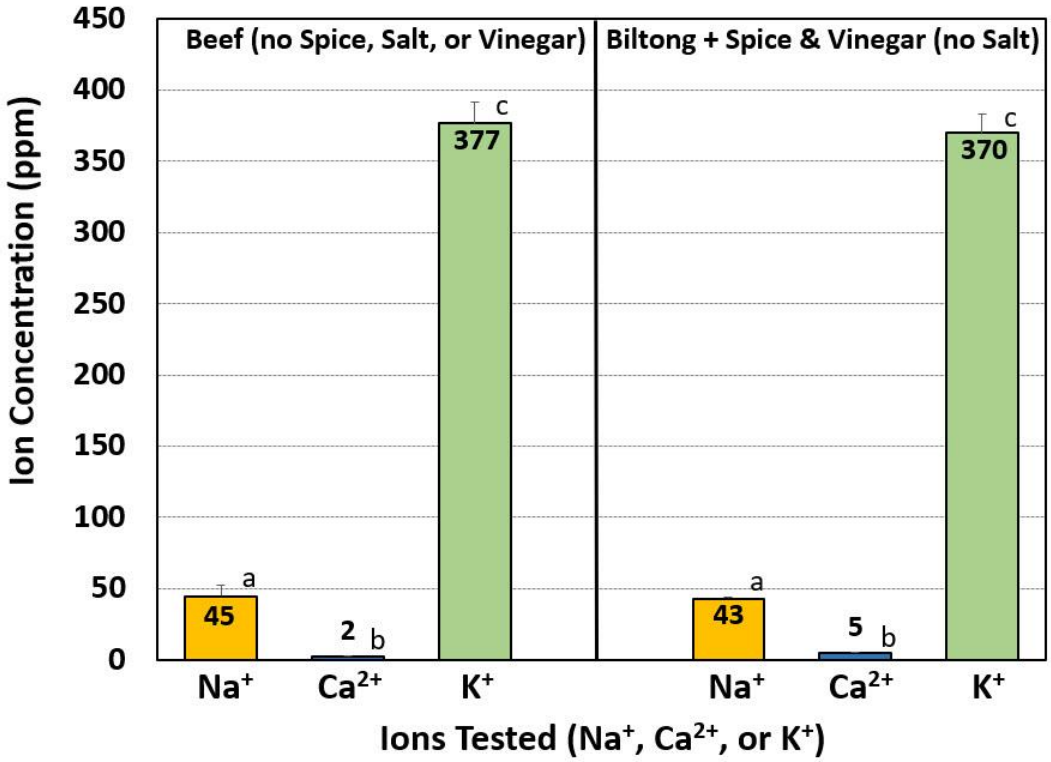

Figure 5. Comparison of ion concentrations of biltong made with dried beef without spice, salt, or vinegar and biltong made with only spice and vinegar marinade (without salt). Data are presented as the mean of triplicate replications, and error bars represent the standard deviation from the mean. Comparisons of means with different letters are significantly different ($p < 0.05$) as determined by one-way ANOVA using the Holm–Sidak test for pairwise multiple comparisons; means with the same letter are not significantly different ($p > 0.05$).

3.3. Salt Ion Analysis from Commercial (Retail) Biltong

Samples of commercially available biltong from retail supermarkets were analyzed for various salt ion contents (Na^+, Ca^{2+}, K^+; Figure 6) to compare with levels determined from our in-lab manufactured biltong (Figure 5). As with our own biltong made without $CaCl_2$, all of the retail biltong samples had very low levels of Ca^{2+} (Figure 6). Additionally, similar elevated levels of K^+ were observed in the commercial biltong (336–591 ppm) and compared favorably to our laboratory biltong which ranged from 370–456 ppm K^+ (excluding the levels of K^+ observed from biltong produced with KCl). The biltong made in this study using NaCl (Figure 4) demonstrated comparable Na^+ concentrations (620 ppm) when compared to that produced by Company A (650, 673 ppm Na^+), but had significantly lower levels than produced by Company B (702, 775 ppm Na^+) (Figure 6).

Additionally, the Na^+ content per serving size (28 g) of each of the commercial biltong samples was calculated using the experimental Equation (3) and was compared to the reported sodium content on the dietary label. Both products from Company A over-stated the Na^+ content on their label compared to the calculated (lower) Na^+ content that was determined from their product using the ISE meters (Figure 7). Conversely, the products made by Company B significantly under-stated the Na^+ content on their labels compared to the significantly higher Na^+ content calculated by analyses of their products (Figure 6). Ion analyses done for experimental biltong produced in this study obtained similar values as Company A (i.e., 374 mg Na^+/28 g biltong). Substitution of different salts for NaCl demonstrated significantly lower levels of Na^+ per serving size of biltong: i.e., 30 mg Na^+/serving when $CaCl_2$ was used, or 32 mg Na^+/serving when KCl was used (Table 2) while still achieving ≥ 5-log reduction of *Salmonella* (Figure 2).

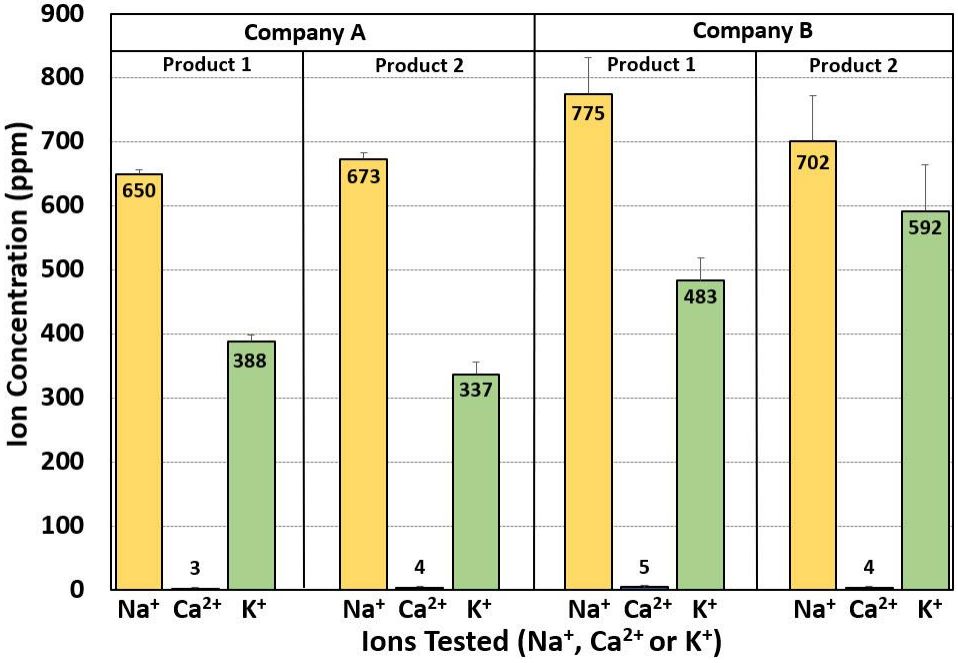

Figure 6. Salt ion analyses of two commercially-available biltong products (Product 1; Product 2) from each of two different companies (Company A; Company B). Analyses were done in triplicate on each product with errors bars indicating the standard deviation of the mean.

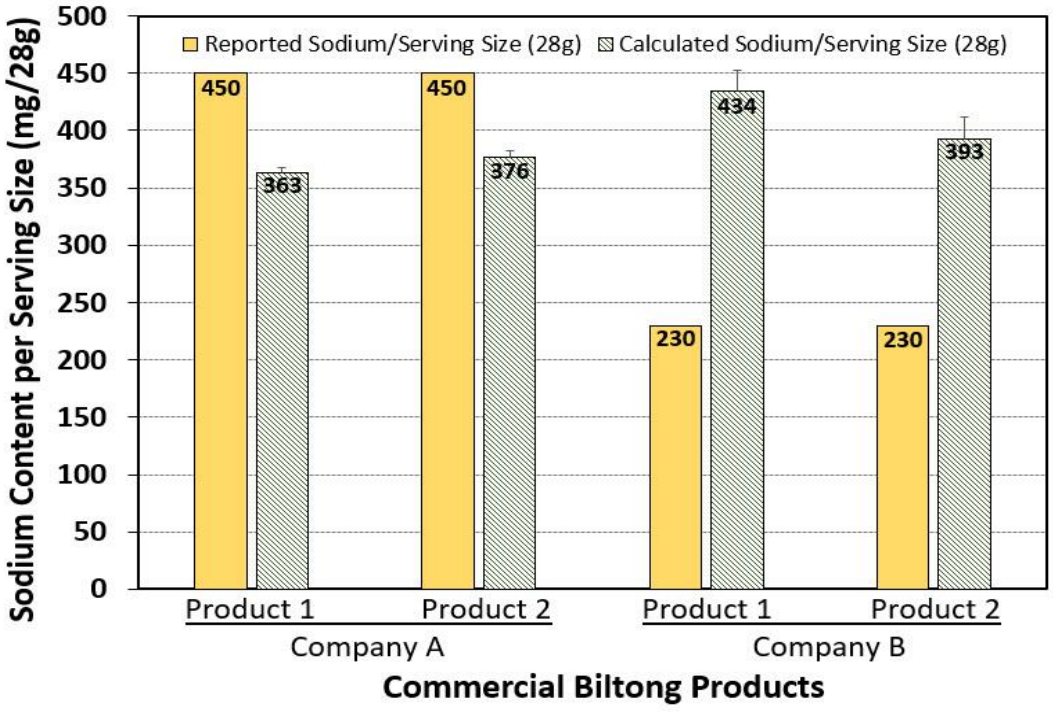

Figure 7. Comparison of reported Na^+ content (per serving size) to the calculated Na^+ content of two commercial biltong products (Product 1, 2) for each of two companies (Company A, B). Reported values were obtained from product labels; calculated values were based on triplicate replication of Na^+ analyses using the method described herein. Products were obtained locally from supermarkets.

Table 2. Salt ion concentration of biltong beef produced during this study based on a comparable 28 g (1 oz) serving size.

	Avg/Serving (mg/28 g Biltong)		
Type of Biltong	**Na^+**	**Ca^{2+}**	**K^+**
Made using NaCl	374.3	3.9	228.3
Made using $CaCl_2$	30.3	293.5	255.3
Made using KCl	31.9	3.3	823.0
Made with unseasoned beef	25.0	0.4	211.0
Beef + spice, vinegar (no salt added)	23.9	2.5	206.9

4. Discussion

Biltong is a ready-to-eat (RTE) dried beef product that is not processed to as high a temperature as beef jerky. Rather, it is dried at moderate temperatures (i.e., 23.9 °C/75 °F) and relative humidity (~55% RH) and relies on salt, spices, and vinegar, along with extended drying (4–10 days) relative to jerky, to achieve USDA-FSIS recommended ≥ 5-log reduction of *Salmonella*. The use of acid-adapted *Salmonella* cultures ensures that the *Salmonella* inoculum is not easily inhibited by acid treatment with vinegar and/or other antimicrobials that are allowed during biltong processing (Karolenko, Bhusal, Gautam, et al., 2020). The vinegar/acid treatment provided by a short term antimicrobial dip treatment (30–60 s) or meat marinade (i.e., 30 min) is effective as a microbial inhibitor during the short time the surface pH is lowered by vinegar or acid treatment. During the vinegar/marinade treatment, we observed approximately 1.1–1.43-log reduction of *Salmonella* that when combined with a pre-marinade rinse treatment (~0.2-log reduction), results in a post-marinade total reduction of ~1.31-1.63 log reduction (Figure 2). Upon removal from the marinade, residual moisture is absorbed, the vinegar is diluted by diffusion into the beef, and the acidic pH is buffered by the underlying mass of beef protein which equilibrates back to the normal pH of beef. Acid inhibition of surface bacteria is highest when there is sufficient acid to render a low surface pH level. Weak organic acids are more effective when the pH is below the pK_a of the acid (acetic acid $pK_a = 4.76$) at which they are more capable of diffusing into bacterial cells where they dissociate into the toxic anion adduct of the weak acid at the neutral pH of the bacterial cytoplasm that is well above the pK_a of the acid (Mani-López, García, & López-Malo, 2012; Smittle, 2000). The acidic effect of marinade may be prolonged for those processes that have an extended/overnight marination (Karolenko, Bhusal, Nelson, et al., 2020). In the current study, the marination was performed for 30 min and the beef was removed and hung to dry for up to 10 days at 23.9 °C (75 °F) and 55% RH. Biltong beef was examined for pH after 8-days of drying (to be consistent with our prior studies that stopped at 8 days) and, regardless of the salt used in the marinade, the pH was observed to be pH 5.00–5.30 (Table 1). This pH range is close to the isoelectric point of meat protein (pH 5.1–5.2) at which it has net zero charge and less water binding capacity than beef of higher or lower pH (Cheng & Sun, 2008; Haque, Timilsena, & Adhikari, 2016).

External salt on the beef provided by the marinade, and that which is absorbed into the beef periphery via vacuum tumbling, theoretically binds available water, and initiates the lowering of a_w as observed in prior post-marination a_w analyses (Karolenko, Bhusal, Nelson, et al., 2020). This, together with drying conditions (temperature, RH, time), are present for the entire drying time during which biltong is usually harvested (6–10 days), and work in concert to lower the a_w of the product to limit growth and inhibit microorganisms (Karolenko, Bhusal, Nelson, et al., 2020). Under these drying conditions, biltong results in ~60% moisture loss (Karolenko, Bhusal, Nelson, et al., 2020) and the relative concentration of salt likely increases to higher levels. In our study, the a_w of raw beef (0.9985) was reduced to 0.8206–0.8380 (internal) or 0.6690–0.6879 (ground) after 8 days of drying. Chopped samples are often used to obtain average a_w values. However, the internal a_w is a much more important parameter when vacuum tumbling is involved because of the potential to draw bacteria

internally during this process (Bosse, Thiermann, Gibis, Schmidt, & Weiss, 2017; Pokharel, Brooks, Martin, & Brashears, 2016; Warsow, Orta-Ramirez, Marks, Ryser, & Booren, 2008). Therefore, the USDA-FSIS considers vacuum tumbled beef as 'non-intact' beef product (similar to ground beef and blade/needle-tenderized beef) and the internal a_w must be < 0.85 to prevent growth and enterotoxin production by *Staphylococcus aureus* should it be internalized during vacuum tumbling. In combination, these factors help to achieve the necessary ≥ 5-log reduction of *Salmonella* that is sought for a USDA-FSIS validated dried beef process (Karolenko, Bhusal, Nelson, et al., 2020).

Given the concerns for high sodium in our diets, questions have developed on whether alternative salts such as KCl or $CaCl_2$ can provide an equivalent 5-log reduction of *Salmonella* while reducing the amount of sodium in the product. Using acid-adapted cultures to reduce the acid sensitivity of *Salmonella* (Karolenko, Bhusal, Gautam, et al., 2020), a > 5-log reduction of *Salmonella* was obtained in 7-days using 2.2% NaCl and 4% vinegar as observed in this paper (Figure 2) and as described previously (Karolenko, Bhusal, Nelson, et al., 2020). By replacing NaCl with either $CaCl_2$ or KCl in the marinade formulation, we still achieved a > 5-log reduction of *Salmonella* in 6 and 8 days, respectively (Figure 2). Although beef pieces were cut to similar sizes by hand, we have not determined whether slight differences in size could influence the rate of drying and affect the timeline of log reduction.

Salt ion concentrations (Na^+, Ca^{2+} and K^+) of biltong made with different salts were determined using ion selective electrode (ISE) meters. These meters have steadily improved during the last 30 years and compare favorably with more sophisticated instruments (Garcia, Vanelli, Pereira Junior, & Corrêa, 2018; Lai, Gardner, & Geddes, 2018; Megahed, Hiew, Grünberg, Trefz, & Constable, 2019). Although the meters provide direct readings as 'ppm', we further examined the accuracy of readings using standardized solutions throughout the entire range examined in this work. The meters demonstrated excellent accuracy and repeatability with R^2 values of ≥ 0.9999 (Figure 3). As expected, the ion present in the highest concentration was the same ion from the salt added to the marinade. Surprisingly, K^+ was not only high in biltong made with KCl, but it was also moderately high in biltong produced with the other salts (Figure 3). The unexpectedly high values of K^+ present in all of the biltong samples, even when KCl was not added to the marinade, caused us to search for additional sources of K^+ in the process. Testing of beef marinaded with just spices and vinegar (no salt), or just the dried beef alone, helped determine that the beef itself was a dominate source of K^+ (Figure 4). There are numerous studies documenting moderately high levels of K^+ within beef similar to the levels observed in the present study (Service, 2019).

Salt ion levels were also examined for two flavored formulations produced by each of two manufacturers available at local supermarkets. We noticed high Na^+ levels which was expected as "salt" is listed on the ingredient label and negligible Ca^{2+} levels. The retail brands were moderately high in K^+ levels and is likely contributed by the beef source (Figure 5) as per our results with unseasoned dried beef (Figure 4). The ion levels in the retail brands were further re-calculated based on serving size (Figure 6) and then compared to our own biltong (Table 2). On a "per serving" basis (28 g, 1 oz), the products produced by Company A demonstrated lower Na+ levels (360 and 373 mg/serving) that resulted in 80% and 83% of the on-package ingredient label claims (450 mg/serving), respectively. Analyses of biltong made in our study (2.2% NaCl, 4% vinegar, spices) demonstrated levels comparable with Company A (374 mg/serving) (Table 2). Conversely, the products produced by Company B were 67% and 85% higher (393 and 434 mg/serving) than their ingredient labels claims for sodium (235 mg/serving) (Figure 6). The level of salt ion on the final product could readily be affected by a change in processing conditions such as injected marinade vs. dip marinade, fine salt vs. coarse granulated salt, vacuum-tumbling vs. no vacuum-tumbling, and short time marination vs. overnight margination, such that the final product no longer lives up to claims listed on the ingredient label. This can more readily occur when another company (i.e., a co-packer) manufactures for the seller. US Food and Drug Administration (FDA) regulations suggest that listed ingredients that occur

at more than 20% less than, or more than 20% greater than, declared level claims (beyond the limits of analytical variability) the product could be deemed 'misbranded' (US-FDA, 2016).

Additional studies could examine the effects these salts have on the sensory characteristics of biltong including taste, texture and tenderness. Studies of salt substitution in other types of beef have described off-tastes such as "bitter" and "metallic" and an increase in hardness associated with $CaCl_2$ and KCl at high concentrations (Marta Aliño et al., 2010; Scanga et al., 2000b). However, some studies have indicated a preference of KCl over other alternative salts and/or no issues when limited to partial replacement of traditional NaCl (Miller, David, Seideman, & Ramsey, 1986; Vidal et al., 2020) or masked when flavorings are added in marinades (Scanga et al., 2000a). Although some of these meat products are significantly different than biltong, this may not be an issue given the strong spice profile of surface seasonings from the marinade on these types of products. As we strive to examine additional processing modifications that may also affect flavor, future studies could evaluate that the product is not only microbially safe for consumers but also tastes acceptable as there is not much work done in this area with biltong.

5. Conclusions

The replacement of NaCl with other ingredients such as KCl or $CaCl_2$ in the biltong marinade resulted in a ≥ 5-log reduction of *Salmonella* to achieve the USDA-FSIS validation of biltong with either NaCl, $CaCl_2$, or KCl, providing manufacturers a choice of alternative salt to reduce sodium content if desired. Although the objective of our work was to demonstrate that KCl or $CaCl_2$ could also obtain a sufficient reduction of *Salmonella* to meet USDA-FSIS biltong validation criteria, there are also human health benefits to use of these different salts. Such alternative salts allow biltong to be produced and marketed as a more healthy low-sodium food alternative while simultaneously being considered to be "fortified" with either K^+ or Ca^{2+} as consumers seek to supplement deficiencies of these minerals in their diet.

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Chapter VI

Evaluation of Various Lactic Acid Bacteria and Generic *E. coli* as Potential Non-Pathogenic Surrogates for In-Plant Validation of Biltong Dried Beef Processing

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Abstract: Validation studies conducted within a food processing facility using surrogate organisms could better represent the manufacturing process than controlled laboratory studies with pathogenic bacteria on precision equipment in a BSL-2 lab. The objectives of this project were to examine potential surrogate bacteria during biltong processing, conduct biltong surrogate validation lethality studies, and measure critical factors and intrinsic parameters during processing. Beef pieces (1.9-cm x 5.1-cm x 7.6-cm) were inoculated with 4-strain mixtures of *Carnobacterium divergens*/*C. gallinarum*, *Pediococcus acidilactici*/*P. pentosaceous*, Biotype 1 *E. coli* ATCC BAA (-1427, -1428, -1429, -1430), 2-strain mixture of *Latilactobacillus sakei,* and other commercially available individual bacterial cultures (*P. acidilactici* Saga200/Kerry Foods; *Enterococcus faecium* 201224-016/Vivolac Cultures). Inoculated beef was vacuum-tumbled in marinade and dried in a humidity-controlled oven for 8-10 days (24.9 °C; 55% relative humidity). Microbial enumeration of surviving surrogate bacteria and evaluation of intrinsic factors (water activity, pH, salt concentration) were performed post-inoculation, post-marination, and after 2, 4, 6, 8, 10-days of drying. Trials were performed in duplicate replication with triplicate samples per sampling time and analyzed by one-way RM-ANOVA. Trials conducted with *E. faecium, Pediococcus* spp., and *L. sakei* never demonstrated more than 2-log reduction during the biltong process. However, *Carnobacterium* achieved a >5-log (5.85-log) reduction over a drying period of 8 days and aligned with the reductions observed in previous trials with pathogenic bacteria (*Salmonella, E. coli* O157:H7, *L. monocytogenes, S. aureus*) in biltong validation studies. Studies comparing resuspended freeze-dried/frozen cells vs freshly grown cells for beef inoculation showed no significant differences during biltong processing. *Carnobacterium* spp. would be an effective non-pathogenic in-plant surrogate to monitor microbial safety that mimics the response of pathogenic bacteria to validate biltong processing within a manufacturer's own facility.

Keywords: biltong; surrogate; lactic acid bacteria; dried beef; validation; *Carnobacterium*.

1. Introduction

Biltong is a South African style dried beef product that is growing in popularity in the United States. This dried meat product is traditionally made using lean strips of beef that are marinated in a mixture of traditional spices (coriander, pepper), salt, and vinegar and then dried at low or ambient temperature and humidity. Dried beef processing guidelines, as issued by the United States Department of Agriculture Food and Safety Inspection Service (USDA-FSIS) require dried beef products to be heated to an internal temperature of 160°F (71.1°C) in a sealed oven or steam injector with a relative humidity greater than 90% during the cooking/heating process (USDA-FSIS, 2014). Since biltong does not have a heat lethality step during processing and deviates from these guidelines, biltong manufacturers must conduct a validation or challenge study to evaluate the ability of their process to sufficiently inactive bacterial pathogens such *Salmonella* spp. which have been historically linked to outbreaks and recalls of dried meat and poultry products (USDA-FSIS, 2017). USDA-FSIS does give processors two different options to safely produce these alternative dried meat products. The first option requires *Salmonella* testing of every lot of edible ingredients used during processing and an overall process reduction of a 'pathogen of concern' of at least 2-log. Alternatively, processors can forego ingredient testing if they can demonstrate that their process can achieve ≥ 5-log reduction of *Salmonella* by the end of processing (Nickelson, Luchansky, Kaspar, & Johnson, 1996).

USDA-FSIS regulatory guidance for manufacture and sale of biltong requires processors to demonstrate product safety by process validation against a 'pathogen of concern'. In recent BSL-2 in-lab studies, this has been done with *Salmonella* serovars (Caitlin E. Karolenko, Arjun Bhusal, Jacob L. Nelson, & Peter M. Muriana, 2020), *E. coli* O157:H7 and *L. monocytogenes* (Gavai, 2021), and *S. aureus* as well (Muriana, unpublished data). These experiments, while successful in achieving a > 5-log reduction of foodborne pathogens and the data is currently used by processors in support of their in-plant food safety (HACCP) processes, are often conducted in highly controlled BSL-2 laboratory environments with research-grade equipment. The food processing environment is extremely variable between small vs large processors, and both likely have greater variability of process parameters than that found in BSL-2 lab equipment. USDA-FSIS has recognized this difference and has allowed consideration of 'in-plant' validation studies using surrogate organisms if the surrogate can mimic a pathogen's response to a process (USDA-FSIS, 1995, 2003, 2021b). The intention is that in-plant data would more likely reflect the actual process variability and conditions than scientific equipment from a BSL-2 lab. Conducting a validation study within a processor's own facility would allow for a more accurate representation of a commercial process' impact on pathogenic bacteria. Due to food safety concerns, it is unsafe to introduce pathogenic bacteria into a manufacturing facility to test whether the process achieves sufficient microbial reduction. Therefore, non-pathogenic surrogate bacteria would be better suited to mimic the response of pathogens to actual processing conditions (USDA-FSIS, 2021b). This presents the question, what surrogate organism should be used for the biltong process?

A surrogate organism for a challenge study is typically a non-pathogenic organism that has similar survival capabilities and susceptibility to injury as the target pathogen, and closely mimics how the pathogen would react under similar processing conditions (Hu & Gurtler, 2017; National Advisory Committee on the Microbiological Criteria for Foods, 2010). A variety of organisms have been used as surrogates in place of pathogens to mimic pathogenic responses in commercial food processes, predominantly *E. faecium, Pediococcus* spp., and Biotype 1 *E. coli*. *Enterococcus faecium* ATCC 8459 (NRRL B-2354) has been used as a surrogate for *Salmonella* Enteritidis PT 30 in the thermal processing of wheat flour (Liu, Rojas, Gray, Zhu, & Tang, 2018), as a *S. enterica* surrogate for storage time and temperature of milk powders (Wei, Agarwal, & Subbiah, 2021), in thermal extrusion of low moisture foods (Bianchini et al., 2014), and in plant level validation of thermal processes for peanuts and pecans (Brar & Danyluk, 2019). Investigators also found that *Pediococcus* strains had similar heat tolerances as *Salmonella* spp. and would be suitable surrogates for validation studies of jerky style dried meat products (A. G. Borowski, S. C. Ingham, & B. H. Ingham, 2009a, 2009b; Dierschke, Ingham,

& Ingham, 2010). *Pediococcus acidilactici* ATCC 8042 was examined as a *Salmonella* surrogate for thermal processing of toasted oats for cereal and peanuts for peanut butter (Deen & Diez-Gonzalez, 2019b), and for processing of low-moisture pet food (Ceylan & Bautista, 2015; Deen & Diez-Gonzalez, 2019a). Biotype 1 *E. coli* ATCC BAA-1427, BAA-1428, BAA-1429, and BAA-1430 have been used as thermal surrogates for *E. coli* O157:H7 in meat processes (Keeling, Niebuhr, Acuff, & Dickson, 2009), as *Salmonella* surrogates for thermal processing of ground beef (Redemann et al., 2018), and thermal treatment of almonds and pistachios (California, 2007; Ma, Kornacki, Zhang, Lin, & Doyle, 2007). These strains have been recommended by USDA-FSIS as surrogate indicator organisms for food process validation studies (USDA-FSIS, 2021b).

Despite the prevalence of studies performed with surrogate bacteria for various food processes, there have not been any surrogate organism that has been proven to suitably represent the response of pathogens during biltong processing. The objective of this study was to examine potential non-pathogenic lactic acid bacteria and generic *E. coli* strains that could be used for in-plant studies to mimic pathogen lethality during biltong processing.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Bacterial cultures used in this study were obtained from various sources including our laboratory culture collection, commercial starter cultures, and bacteria isolated from biltong trials as listed in Table 1.

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

Organism	Strain Designation	Culture Collection Designation	Antibiotic Resistance (ug/ml)*	Source
Pediococcus acidilactici	ATCC 8042	PMM 128	GM, 10; RF, 5	Muriana Culture Collection
Pediococcus acidilactici	PO2K5	PMM 331	GM, 10; RF, 5	Muriana Culture Collection
Pediococcus pentosaceous	ATCC 43200	PMM 104	GM, 10; RF, 5	Muriana Culture Collection
Pediococcus pentosaceous	FBB61-2	PMM 105	GM, 10; RF, 5	Muriana Culture Collection
Pediococcus acidilactici	Saga200	PMM 444	NA, 10; CL, 10	Kerry Foods, Beloit, WI, USA
Enterococcus faecium	201224-016	PMM 445	NA, 10; CL, 10	Vivolac Cultures, Indianapolis, IN, USA
Escherichia coli	ATCC BAA-1427	PMM 876	OX, 1; NB, 2.5	ATCC, Muriana Culture Collection
Escherichia coli	ATCC BAA-1428	PMM 877	OX, 1; NB, 2.5	ATCC, Muriana Culture Collection
Escherichia coli	ATCC BAA-1429	PMM 878	OX, 1; NB, 2.5	ATCC, Muriana Culture Collection
Escherichia coli	ATCC BAA-1430	PMM 879	OX, 1; NB, 2.5	ATCC, Muriana Culture Collection
Latilactobacillus sakei	GO-R2C	PMM 446	GM, 2.5; RF, 2.5	Isolated from biltong
Latilactobacillus sakei	GO-R2D	PMM 447	GM, 2.5; RF, 2.5	Isolated from biltong
Carnobacterium divergens	GO-R2E-B	PMM 448	GM, 2.5; RF, 2.5	Isolated from biltong
Carnobacterium divergens	GO-R1B	PMM 449	GM, 2.5; RF, 2.5	Isolated from biltong
Carnobacterium gallinarum	NB-R2A	PMM 450	GM, 2.5; RF, 2.5	Isolated from biltong
Carnobacterium gallinarum	NB-R2B	PMM 451	GM, 2.5; RF, 2.5	Isolated from biltong

*Antibiotic designations: Gentamicin, GM; Rifamycin, RF; Nalidixic acid, NA; Colistin, CL; Oxacillin, OX; Novobiocin, NB

Bacterial isolates obtained from previous biltong beef trials after marination and drying for eight days at 24.9 °C (75 °F) and 55% relative humidity (RH) were identified by 16S rRNA PCR/sequencing

(Shah & Muriana, 2021) as *Carnobacterium gallinarum*, *Carnobacterium divergens*, and *Latilactobacillus sakei* for examination as biltong process surrogates (Table 1).

Other lactic acid bacteria used in this study included *Pedicoccus acidilatcici* ATCC 8042, *P. acidilactici* P02K5, *P. pentosaceus* FBB61-2, and *P. pentosaceus* ATCC 43200 that have been maintained in our laboratory culture collection. Some of these strains have been evaluated in other surrogate studies (Ceylan & Bautista, 2015; de Souza de Azevedo et al., 2019). Non-pathogenic *E. coli* ATCC BAA-1427, BAA-1428, BAA-1429, and BAA-1430 have been used as Biotype 1 surrogate strains in various process validation studies and recommended for such by USDA-FSIS (Keeling et al., 2009; Niebuhr, Laury, Acuff, & Dickson, 2008; USDA-FSIS, 2021b). *P. acidilactici* Saga200, used as a protective starter cultures, was obtained as a frozen slurry from Kerry Foods (Beloit, WI, USA). *Enterococcus faecium* 201224-016 was obtained as a freeze-dried powder from Vivolac Cultures (Indianapolis, IN, USA) and is sold as a probiotic.

Carnobacterium spp., *E. faecium, and E. coli* cultures were inoculated into typtic soy broth (TSB, BD Bacto, Franklin Laes, NJ, USA) and grown at 30 °C for 24 hrs. *L. sakei* and *Pedicoccus* spp. were inoculated into De Man, Rogosa and Sharpe broth (MRS, BD Bacto) and grown at 30 °C for 24 hrs. Cultures were prepared for storage by centrifugation (7200 x*g*, 5 °C) of 9 mL of fresh, overnight culture and the resulting pellet was resuspended with 2-3 mL of fresh, sterile TSB or MRS broth containing 10% glycerol. The cells in freezing media were then placed in 8-mL sterile glass vials and stored in an ultra-low freezer (-80 °C) until use. Prior to use, frozen stocks are revived by transferring 100 µL of partially thawed culture into 9 mL of either TSB or MRS broth and incubated overnight at 30 °C.

Several cultures were used directly after suspension from the freeze-dried or frozen state for comparison of biltong process performance with metabolically-active forms grown in liquid media. Prior to use, *P. acidilactici* Saga200 (frozen) was resuspended by adding 0.5 g of the frozen culture to 9 mL of 0.1% buffered peptone water (BPW, BD Difco) and vortexting until completely incorporated. *E. faecium* 201224-016 was resuspended by adding 0.1 g of the freeze-dried culture to 9 mL of 0.1% BPW and vortexing until completely dissolved.

2.2. Acid Adpation of Cultures

Acid adaptation of active 4-strain mixtures of *Carnobacterium* spp., *Pediococcus* spp., and *E. coli* BAA-strains were conducted as first described by Wilde et al. (Wilde, Jørgensen, Campbell, Rowbury, & Humphrey, 2000) and as used in previous biltong studies (Karolenko, Bhusal, Gautam, & Muriana, 2020; Caitlin E. Karolenko et al., 2020). In brief, individual cultures were inoculated into TSB or MRS containing 1% glucose, incubated overnight at 30 °C, harvested by centrifugation, and cell pellets were then resuspended with 0.1% BPW. For mixed culture biltong inocula, individual strains were combined in equal proportions to create a mixed inoculum cocktail. The commerical starter cultures (*P. acidilactici* Saga200 and *E. faecium* 201224-016) were not acid-adapted and used as a single strain inoculum.

2.3. Beef Sample Preparation and Inoculation

USDA select-grade boneless beef rounds were obtained from a local meat processor (Ralph's Perkins, OK, USA) who obtains beef from a wholesale beef broker. Beef rounds were trimmed of fat and cut into approximately 5.1-cm wide x 1.9-cm thick x 7.6-cm long beef squares and held overnight at 5 °C on foil-lined trays wrapped in plastic bags. Beef pieces were inoculated the following morning with the respective inoculum depending on the trials being performed that day. Beef pieces were inoculated with either the *Carnobacterium* spp. mixture (*C. divergens* GO-R2E-B, GO-R1B; *C. gallinarum NB-R2A, NB-R2B*), the *L. sakei* mixture (*L. sakei* GO-R2C, GO-R2D), the *Pediococcus* spp. mixture (*P. acidilactici* ATCC 8042, PO2K5; *P. pentosaceous* ATCC 43200, FBB61-2), *P. acidilactici* Saga200, or *E. faecium* 201224-016. The inoculum suspension (150 uL) was applied to each side of the beef pieces and

immediately spread with a gloved finger. Inoculated beef pieces were then allowed to incubate for 30 min at 5 °C to allow for bacterial attachment prior to use.

2.4. Biltong Processing, Marination and Drying

Biltong processing was conducted as described whereby trials were performed in duplicate and triplicate samples were harvested at each time point (n = 6) (C. Karolenko & P. Muriana, 2020; C. E. Karolenko, A. Bhusal, J. L. Nelson, & P. M. Muriana, 2020). Following inoculation and attachment, the beef pieces were then dipped in sterile water to mimic rinse treatments that processors often apply using antimicrobials or water during processing. The inoculated pieces were placed in a plastic basket, dipped in sterile water in a stainless-steel tub for 30 sec, and drained for 60 sec to release excess liquid. The beef pieces were then placed into a chilled metal tumbling vessel containing a biltong marinade. The biltong marinade consisted of 2.2% salt, 0.8% black pepper, 1.1% coarse ground coriander, and 4% red wine vinegar (100-grain; 10% acetic acid) in relation to the total meat weight. Beef pieces were vacuum tumbled (15 inches Hg) in a Biro VTS-43 vacuum-tumbler (Marblehead, OH, USA) for 30 min and then hung to dry in a humidity-controlled oven (Hotpack, Model 435315, Warminster, PA, USA) at 55% relative humidity and 24.9 °C (75 °F) for 8-10 days.

2.5. Selective Recovery of Inoculum Bacteria from Biltong-Inoculated Beef

The bacteria assessed in this study as potential biltong proccessing surrogates were inoculated onto raw beef and initial and residual inoculum enumeration had to preclude other natural contaminants also found on raw beef, those contributed during trimming of beef, or from the marinade spice mix. Prior studies indicated that such processing conditions induces stresses and injured cells may not be recovered on harsh selective media and thereby giving a falsely lower count (C. E. Karolenko, A. Bhusal, D. Gautam, et al., 2020). To eliminate the possibility of inhibiting injured-but-viable cells, we used generic growth media (TSA, MRS agar) supplemented with antibiotics to which the strains are resistant as a selective media to enumerate our inoculated organisms from samples taken during biltong processing (C. E. Karolenko, A. Bhusal, D. Gautam, et al., 2020; Caitlin E. Karolenko et al., 2020). Antibiotic resistance was determined using antibiotic susceptibility discs (BD BBL Sensi-Discs, BD Labs, Franklin Lakes, NJ, USA) to determine innate antibiotic resistance (Table 1). After identification of antibiotic resistances, cultures were then enumerated on media with, and without, antibiotics to ensure the absence of inhibition from the use of antibiotics in the media as described previously (Bhusal, Nelson, Pletcher, & Muriana, 2021; C. E. Karolenko, A. Bhusal, D. Gautam, et al., 2020; Caitlin E. Karolenko et al., 2020). For some strains used as inoculum cocktails that did not have consensus of the same antibiotic resistances, antibiotic resistance was acquired by plating on low level antibiotics known to generate spontaneous antibiotic resistance (i.e., gentamycin, rifamycin).

2.6. Comparison of Commerically Available Starter Cultures as Biltong Inoculants in their Lyophilized and Metabolically Active Forms

2.6.1. Culture Preparation

Lactic acid bacteria obtained as freeze-dried cultures from starter culture companies for use in validation studies may present a facile method of use as validation inocula by simply resuspending the cells in buffer and directly inoculating beef samples (A. G. Borowski et al., 2009a, 2009b). Freeze-drying or lyophilization of bacteria exposes them to stressful conditions that can have an effect on subsequent cell viability or activty [21,22]. Therefore, the activity of lyophilized (*E. faecium* 201224-016) and frozen (*P. acidilactici* Saga200) starter cultures and their metabolically active forms (i.e., after growth in media) were compared in their response to biltong processing.

For the lyophilized culture (*E. faecium* 201224-016), 0.1 g freeze dried powder was added to 9 mL of sterile 0.1% BPW and vortexed until completed suspended. The resuspended mixture was then used to inoculate each beef piece (300 uL; 150 uL/side) prior to marination.

For the frozen starter culture (*P. acidilactici* Saga200), a sterile hollow hole puncher was used to core ~0.8 g of frozen Saga200 from the manufacture's container which was added to 9 mL of sterile 0.1% BPW and vortexed until homogonized. The culture suspension was kept chilled on ice and used shortly thereafter to inoculate beef pieces.

Metabolically active versions of these cultures were obtained by growth in 150 mL of the appropriate media (TSB, MRS) for 24 hrs at 30 °C, centrifugation, and resuspension of the recovered cell pellet with 5 mL of sterile 0.1% BPW. The resuspended culture was then used to inoculate beef pieces prior to use in the validation study. The lyophilized and metabolically active forms of each *E. faecium* 201224-016 and *P. acidilactici* Saga200 were used in parallel and simultaneous biltong trials to reduce any variables that might influence the observed effect of the marinade and drying process.

2.6.1. Lyophilization of *Carnobacterium gallinarum* NB-R2A

To the author's knowledge, there is no commerically available *Carnobacterium* strain available in the United States. Therefore, *C. gallinarum* NB-R2A, isolated from biltong, was lyophilized via freeze-drying to examine a lyophilized version for comparison with the actively grown culture. *Carnobacterium gallinarum* NB-R2A was inoculated into 9 mL of TSB from frozen stock and incubated for 18 hrs at 30 °C. Following incubation, the 9 mL culture was transferred to 190 mL of TSB and incubated again for 18 hrs at 30 °C. The culture was then centrifuged at 7200 xg for 20 min. The supernatant was removed and the cell pellet was resuspended with 5 mL of sterile BPW and repeated. The supernatant was removed following centrifugation and the final cell pellet was resuspended with 10 mL of autoclaved milk-based freeze drying medium consisting of 11 g skim milk powder, 1 g dextrose, 1 g trehalose, and 0.2 g yeast extract per 100 mL. The milk/cell suspension was added to Oak Ridge tubes (5 mL each) and freeze-dried using a Heto vacuum centrifuge (Model VR-maxi) connected to a Heto freezing condensor (Model CT 60E) and a Leybold Trivac vacuum pump (Model D2.5F) setup for 24 hours under vacuum. The freeze-dried powder was then stored at -80 °C until use in our biltong study. Just before use, 0.25 g of powder was added to 9 mL of sterile 0.1% BPW, vortexed until mixed, and used to inoculate beef pieces for biltong processing.

2.7. Evaluation of Critical Parameters and Intrinsic Factors in the Biltong Process

2.7.1. Water Activity

Uninoculated beef pieces were sampled for water activity (A_w) measurements at various stages throughout processing (in triplicate) including the initial raw beef, beef after marination, and then beef after drying for 2, 4, 6, 8 and 10 days. To obtain measurements, beef pieces were cut in half and placed in a sampling cup with the interior portion of the sample facing upwards (towards the sensor). Samples were then covered with sampling cup cover containing the sensor and allowed to equilibrate to the temperature of the room. Water activity was measured using a HC2-AW-USB probe with direct PC interface and HW4-P-Quick software (Rotronic Corp., Hauppauge, NY, USA). Measurements were taken in triplicate for each sample at each timepoint.

2.7.2. Moisture Loss

Following marination each beef piece was individually weighed and labelled prior to being hung in the humidity-controlled oven. Three pieces were selected and weighed prior to processing, and then sampled every two days while drying. The weight at the time of sampling was compared to the intial weight of the same piece recorded prior to drying. The determination of percent mositure loss was calculated as per Equation (1):

$$\%\ Moisure\ Loss = \frac{[(inital\ weight) - (final\ weight)]}{(inital\ weight)} \times 100 \quad (1)$$

2.6.3. Measurement of Biltong Beef pH

Measurements of beef pH were obtained at various points in the biltong process including: raw beef, beef following marination, and beef after 2, 4, 6, 8, and 10 days of drying. At each timepoint, three pieces of uninoculated beef were collected, weighed and then added to a laboratory blender with steel blades (Waring Commerical, New Harford, CT, USA) with sterile water of equal weight to the weight of the beef pieces. The water and beef mixtures were blended until a finely ground mixture was formed. The pH of the homogenized meat mixture was measured in triplicate using an H-series pH meter and probe (Hach, Loveland, CO, USA).

2.7.4. Salt Concentration

The homogenized meat mixture used to measure pH was also used to obtain salt concentrations of each sample. Horiba LAQUA Twin Pocket Meter (Horiba Instruments, Irvine, CA, USA) was used to quantify sodium ion concentraion. Appropomately 300 µL of the homogenized sample was placed in the sample chamber and allowed to stablize before recording. Readings (in ppm) were taken in triplicate for each sample. To determine the salt (NaCl) concetration from the sodium ion concentration, the following equations were used:

$$Na\ \left(\frac{mg}{100g}\right) = Meter\ reading\ (ppm) \times \frac{Weight\ after\ Dilution\ (g)}{Sample\ Weight} \times 100 \quad (2)$$

$$NaCl\ Salt\ \left(\frac{g}{100g}\right) = Na\ \left(\frac{mg}{100g}\right) \times \frac{NaCl\ molar\ mass}{Na\ molar\ mass} \times \frac{1}{1000} \quad (3)$$

2.8. Microbial Sampling and Inoculum Enumeration of Biltong Beef

At each sampling timepoint biltong beef processing (raw beef, after marinade, after every 2, 4, 6, 8 and 10 days of drying), 3 beef pieces were selected at random and placed in a sterile Whirl-pak filter stomaching bag (Nasco, Fort Atkinson, WI, USA) in combination with 100 mL of 1% neutralizing buffered peptone water (nBPW, Criterion, Hardy Diagnostics, Santa Maria, CA, USA). Samples were stomached for 60 sec in a paddle-blender masticator (IUL Instruments, Barcelona, Spain). Serial dilutions were made with 1% BPW and plated on either TSA containing gentamicin and rifamycin (2.5 µg/mL each) for *Carnobacterium*, or on MRSA containing gentamicin and rifamycin (2.5 µg/mL each) for *L. sakei*, or MRSA containing gentamicin (10 µg/mL) and rifamycin (5µg/mL) for *Pediococcus spp.*, TSA containing naldixic acid and colistin (10 µg/mL each) for *E. faecium* 201224-016, and MRS containing nalidixic acid and colistin (10 µg/mL each) for *P. acidilactici* Saga200; the filter bag dilution was considered the 10^0 dilution. Plates were incubated at 30 °C for 48 hrs and enumerated as log CFU/mL. Samples were collected in triplicate replication and plated in duplicate at each sampling timepoint.

2.9; Statistical Analysis

Validation trials were conducted in duplicate with tripicate sampling at each timepoint (n = 6) per validation criteria established by the National Advisory Committee on Microbial Criteria for Foods (NACMCF) (National Advisory Committee on the Microbiological Criteria for Foods, 2010) and supported by the USDA-FSIS (USDA-FSIS, 2015). Data is presented as the mean of multiple replications with standard deviation of the mean represented by error bars. Statistical analysis of data collected over time was done using one-way repeated measures analysis of variance (RM-ANOVA). Pairwise multiple comparisons were done using the Holm-Sidak test to determine significant

differences. Data treatments with the same letter are not significantly different ($p > 0.05$); treatments with different letters are significantly different ($p < 0.05$).

3. Results and Discussion

3.1. Critical Parameters and Intrinsic Factors

3.1.1. Water Activity, Moisture Loss and Salt Concentrations

To complement the surrogate validation trials, we measured and recorded critical operational parameters and intrinsic factors at each key stage of processing (raw beef, inoculation, marination and every two days of drying) as recommended by USDA-FSIS (USDA-FSIS, 2014). Water activity (A_w) is a measure of free, unbound water available for bacterial growth. USDA-FSIS considers vacuum-tumbled beef as 'non-intact beef' whereby A_w is a primary safety factor as there is no heat lethality step in biltong processing and biltong is processed as thick beef samples (Pokharel, Brooks, Martin, & Brashears, 2016; USDA-FSIS, 2021a). Therefore, A_w is a critical safety factor for control of bacteria that might be internalized due to vacuum tumbling. *S. aureus* that can tolerate lower A_w and high salt levels would be a concern for possible production of staphylococcal enterotoxin. The targeted A_w for shelf-stable beef jerky is < 0.85 which was achieved after day 7 of drying (Figure 1) (USDA-FSIS, 2014, 2017). Water activity after 8 and 10 days of drying ranged from 0.82 to 0.79 respectively. Similarly, beef samples showed incremental moisture loss with 59% and 62.5% loss at 8 and 10 days, respectively (Figure 1).

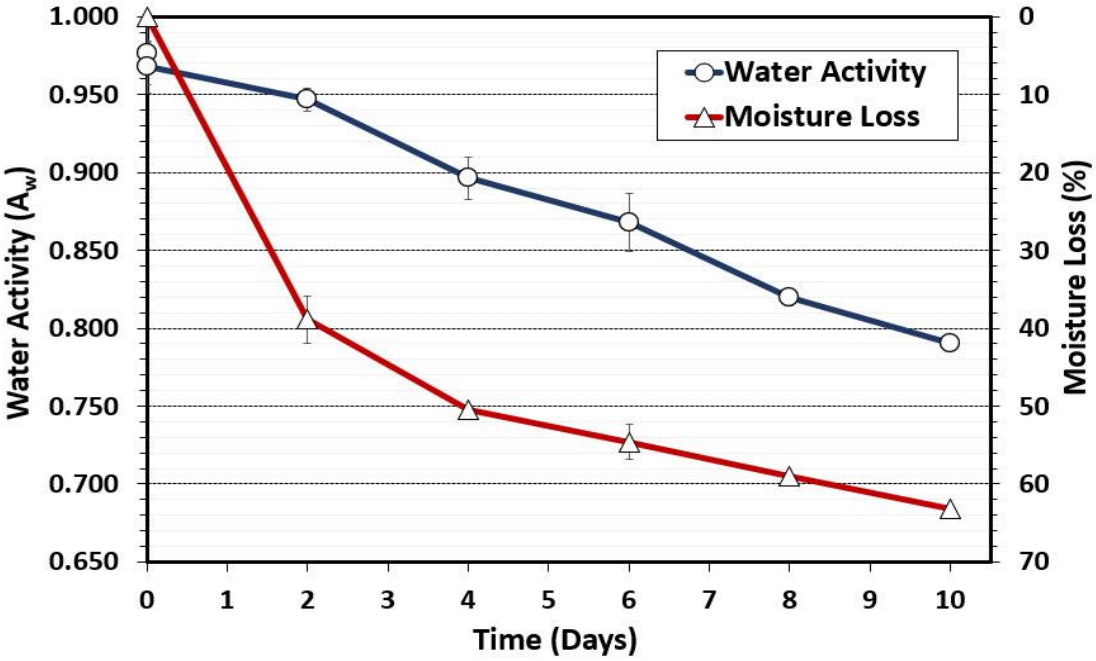

Figure 1. Water activity (A_w) and moisture loss during biltong processing at 24.9 °C (75 °F) and 55% RH. The data represents the average of measurements taken during duplicate trials with triplicate samples taken at each time interval (n = 6).

Salt concentration was also determined during the biltong process. Salt concentration was calculated from sodium readings obtained with the LAQUAtwin NA-11 sodium ion meter (Horiba Inc, Irvine, CA, USA). The initial calculated salt concentration determined on raw beef was 0.12% NaCl and then following the marination step the beef salt concentration shot up to 2.17% (2.17 g NaCl/100 g beef). The initial salt level falls in line with expectations given that the biltong marinade is formulated at 2.2% salt (w/w). The salt concentration increased over time and was indirectly proportional to moisture loss during the drying process (Figure 2). As

expected, as moisture loss occurs, A_w is also reduced to below 0.85 A_w (Figure 1) and the salt concentration increases to approximately 4% (Figure 2), both conditions are inhibitory to most bacteria, helps to ensure a safe product for consumers (Gurtler et al., 2019). Biltong safety involves an interplay between moisture, salt concentration, and A_w since moisture loss increases salt concentration and salt binds water and helps to draw it out of the interior of the beef, reducing A_w. For consumer issues regarding high sodium levels, the use of alternative salts ($CaCl_2$, KCl) instead of NaCl can help lower sodium levels in finished biltong while still maintaining a 5-log reduction of pathogen (*Salmonella*) (Caitlin Karolenko & Peter Muriana, 2020).

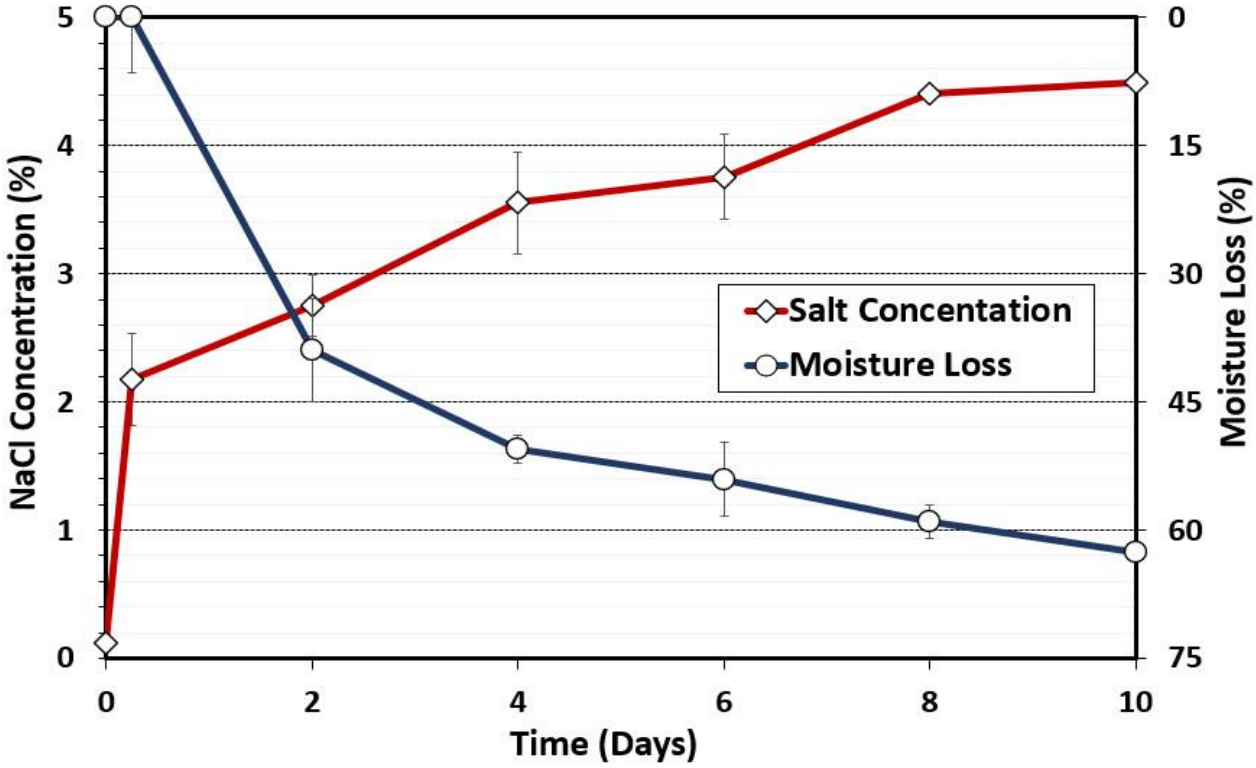

Figure 2. Moisture loss (%) and salt concentration (%) during biltong processing. Measurements were taken with initial beef samples, after marination, and after 2, 4, 6, 8, and 10-days of drying at 24.9 °C (75 °F) and 55% RH. Data points represent the mean of duplicate trials with triplicate samples taken at each time interval (n = 6).

3.1.2. The pH of Beef During Biltong Processing

The initial pH of the raw meat pieces was on approximately pH 5.43 (Figure 3) which was determined by blending beef samples in sterile water in a laboratory blender. The pH of the samples then decreased following the marination step down to 5.02 which can be attributed to the presence of residual 100-grain red wine vinegar in the marinade. After removal from the marinade, the pH of biltong beef samples then equilibrated slightly higher to ~5.18-5.20 for the remainder of the drying process in the humidity-controlled oven (Figure 3). The pH of the marination solution is much lower (pH 2.5-2.7) and during 30 min vacuum tumbling, the surface bacteria are immersed in the low pH marinade solution which can lead to cell death and inactivation of pathogenic bacteria (Jin & Kirk, 2018; Lund et al., 2020) as observed in the current study and prior biltong trials where levels of inoculated pathogens were reduced after marination (Caitlin E. Karolenko et al., 2020). After removal from the vacuum tumbler, the residual marinade on the surface is absorbed and the pH of biltong beef samples equilibrates to ~5.18-5.20 for the remainder of the drying process in the humidity oven (Figure 3).

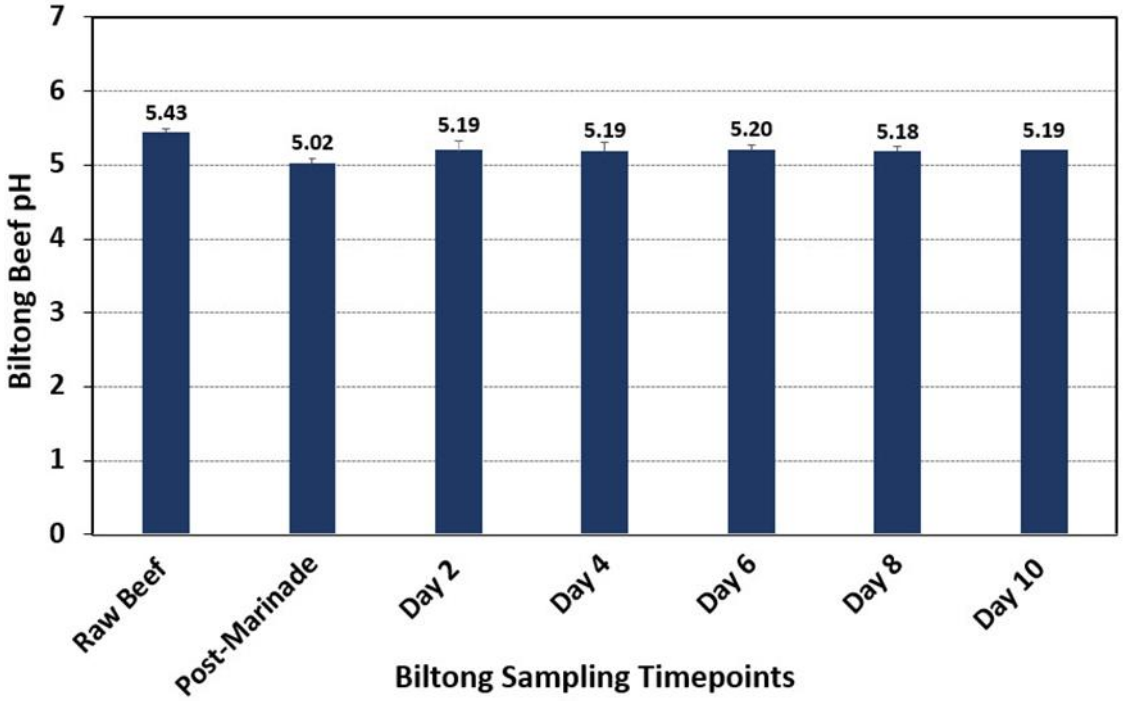

Figure 3. The pH of meat at each sampling timepoint during biltong processing. Samples were taken in triplicate at each timepoint following blending with sterile water in a laboratory blender (n = 6).

3.1.3. Temperature and Relative Humidity during Biltong Processing

Temperature and RH measurements were recorded by computer software connected to the handheld temperature and humidity recorders to which the probes in the oven chamber were connected (Figure 4). Two temperature probes were inserted separately into two beef pieces to measure the internal beef temperature during processing while humidity probe was place midway within the chamber. Air temperature and humidity were set to 23.9 °C (75 °F) and 55% throughout the duration of each trial but cycled above and below the set points. The internal temperature of the beef was more consistent and steadily increased from their initial temperature to match the temperature of the chamber. Long term storage at low RH helps to evaporate moisture from the beef.

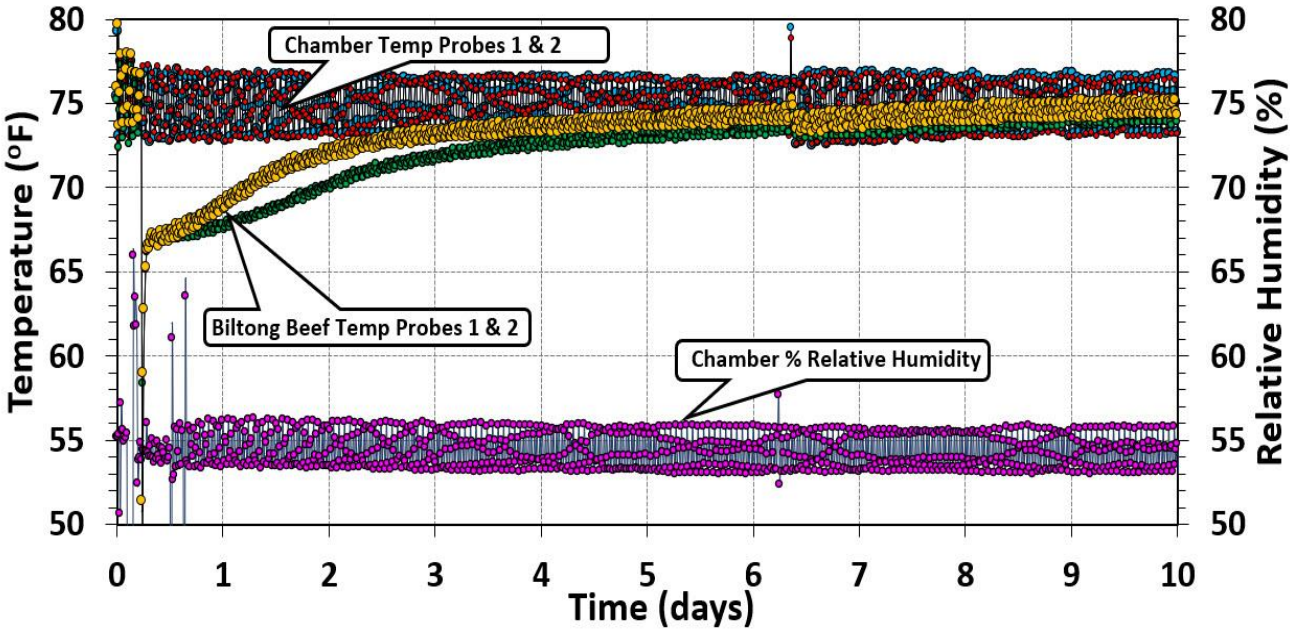

Figure 4. Humidity oven temperature and relative humidity measurements. The temperature was set to 24.9°C (75 °F) and relative humidity setpoint was 55% RH during the drying process over a period of 10 days. Graphical data shows the typical cycling of oven control above/below setpoint. Two temperature probes were placed in various places in the chamber and two additional probes were inserted into separate pieces of beef to track the internal temperature of the biltong product over the same drying period.

3.2. Surrogate Log-Reductions During Biltong Processing

Various bacteria were considered for examination as possible non-pathogenic surrogates, including strains recovered from biltong after processing. These included a 2-strain mixture of *L. sakei* GO-R2C and GO-R2D and a 4-strain mixture of *C. divergens* GO-R2E-B and GO-R1B and *C. gallinarum* NB-R2A and NB-R2B (Figure 5). We also examined a 4-strain mixture of *P. acidilactici* and *P. pentosaceous* strains (*P. acidilactici* ATCC 8042 and PO2K5; *P. pentosaceous* ATCC 43200 and FBB61-2) vs. starter cultures that were available through culture companies (*E. faecium* 201224-016, *P. acidilactici* Saga200) as surrogate organisms (Figure 5).

Only a slight reduction from inoculated levels was observed following vinegar/spice/salt marination (0.65, 0.58, 0.75, and 0.61-log reduction) with all cultures used, except for the 4-strain mixtures of *Carnobacterium* spp. and *E. coli* ATCC BAA series, (Figure 5). A larger log reduction was observed after marination of the 4-strain mixtures of *Carnobacterium spp.* (1.23-log) and *E. coli* ATCC BAA-strains (0.86-log) (Figure 5). Trials using *E. coli* ATCC BAA (4-strain mix), *L. sakei* (2-strain mix), *Pediococcus* spp. (4-strain mix), *E. faecium* 201224-016, and *P. acidilactici* Saga200 failed to achieve a 5-log reduction during biltong processing with overall reductions of 4.86-log, 2.03-log, 1.87-log, 1.68-log, and 1.83-log respectively. Of all the non-pathogenic strains examined, only the 4-strain mixture of *Carnobacterium* spp. achieved an overall reduction of greater than 5-log (5.85-log) during the 8-day drying period (Figure 5). Based on these results, *Carnobacterium* spp. were the only organisms that achieved a 5-log reduction (within 6-8 days) comparable to that observed for the pathogenic strains and present the best case for use as a *Salmonella, L. monocytogenes, E. coli* O157:H7, or *S. aureus* surrogate for biltong processing (Figure 5).

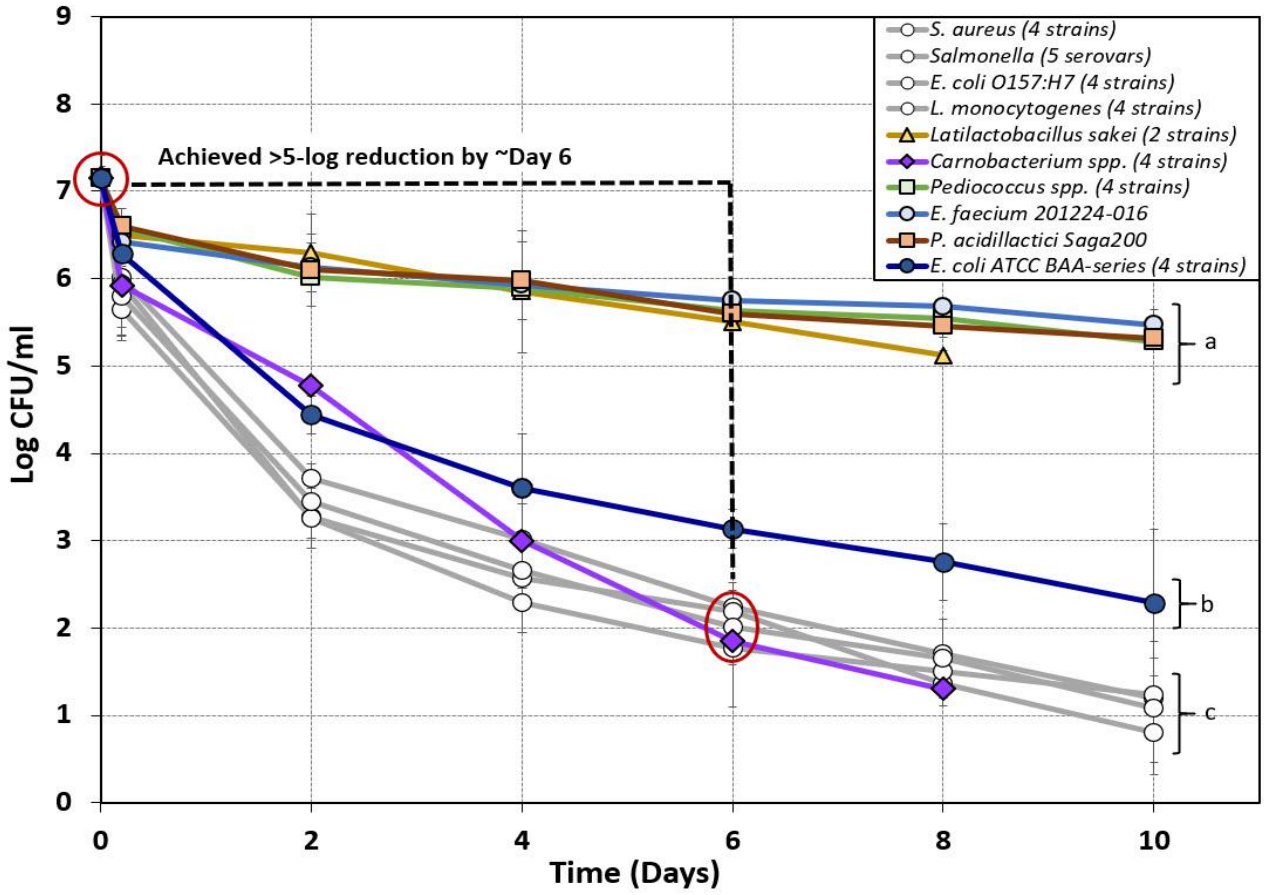

Figure 5. Composite graph of biltong processing data of non-pathogenic bacteria attempting to mimic the biltong process log reduction of pathogenic bacteria (light grey lines) to be considered a possible 'biltong processing surrogate' organism for in-plant validation. Log reduction curves of various lactic acid bacteria (*Carnobacterium* spp., *Pediococcus spp., L. sakei, E. faecium*), and Biotype I *E. coli* strains tested as potential surrogate organisms for biltong processing over a period of 8-10 days. Strains were compared to the log reduction curves observed during previous biltong validation studies using pathogenic bacteria including *Salmonella* serovars (Caitlin E. Karolenko et al., 2020), *S. aureus,* and *E. coli* O157:H7 and *L. monocytogenes* (Gavai, 2021). Data points are the mean of duplicate trials sampled in triplicate (n = 6). Statistical analysis was performed using one-way repeated measures analysis of variance (RM-ANOVA) of the entire time course of data; curves with the same letter are not significantly different ($p > 0.05$); isolates with different letters are significantly different ($p < 0.05$).

3.3. Comparison of Lyophilized/Frozen Starter Cultures with Metabolically Active (Grown) Versions in Biltong Processing Trials

Several reports in the literature have used freeze-dried or frozen cultures, resuspended directly in buffer, to inoculate food samples in process trials for direct comparison to pathogens grown in microbiological media (that we describe as 'active cultures') (Bianchini et al., 2014; Alena G. Borowski, Steven C. Ingham, & Barbara H. Ingham, 2009). The ease of availability of freeze-dried/frozen cultures from culture companies would facilitate the use of such cultures for in-plant validation studies, however we were interested to see if they provide the same response in a biltong process as the actively grown cultures (Figure 6). The comparisons were between two commercially available starter cultures, *E. faecium* 201224-016 (Vivolac Cultures; freeze-dried) and *P. acidilactici* Saga200 (Kerry Foods; frozen), and a lyophilized *C. divergens* NB R2A which was chosen from among the *Carnobacterium* mixed strains demonstrating >5-log reduction in Figure 5.

Neither the lyophilized version of *E. faecium* 201224-016 (1.43-log reduction) nor the frozen version of *P. acidilactici* Saga200 (1.54-log reduction) achieved the 5-log reduction target; survival curves of the lyophilized/frozen forms were also not significantly different when compared to their metabolically active forms, 1.68- and 1.83-log reduction, respectively (Figure 6). The lyophilized single strain *C. divergens* NB R2A also showed no significant difference from the metabolically active culture and again achieved 5-log reduction during the biltong process (Figure 6). The data shows lyophilized or frozen versions of *E. faecium*, *P. acidilactici*, or *C. gallinarum* do not respond differently than actively grown cultures to biltong processing conditions and when possible, use of them might do well to facilitate inoculated studies.

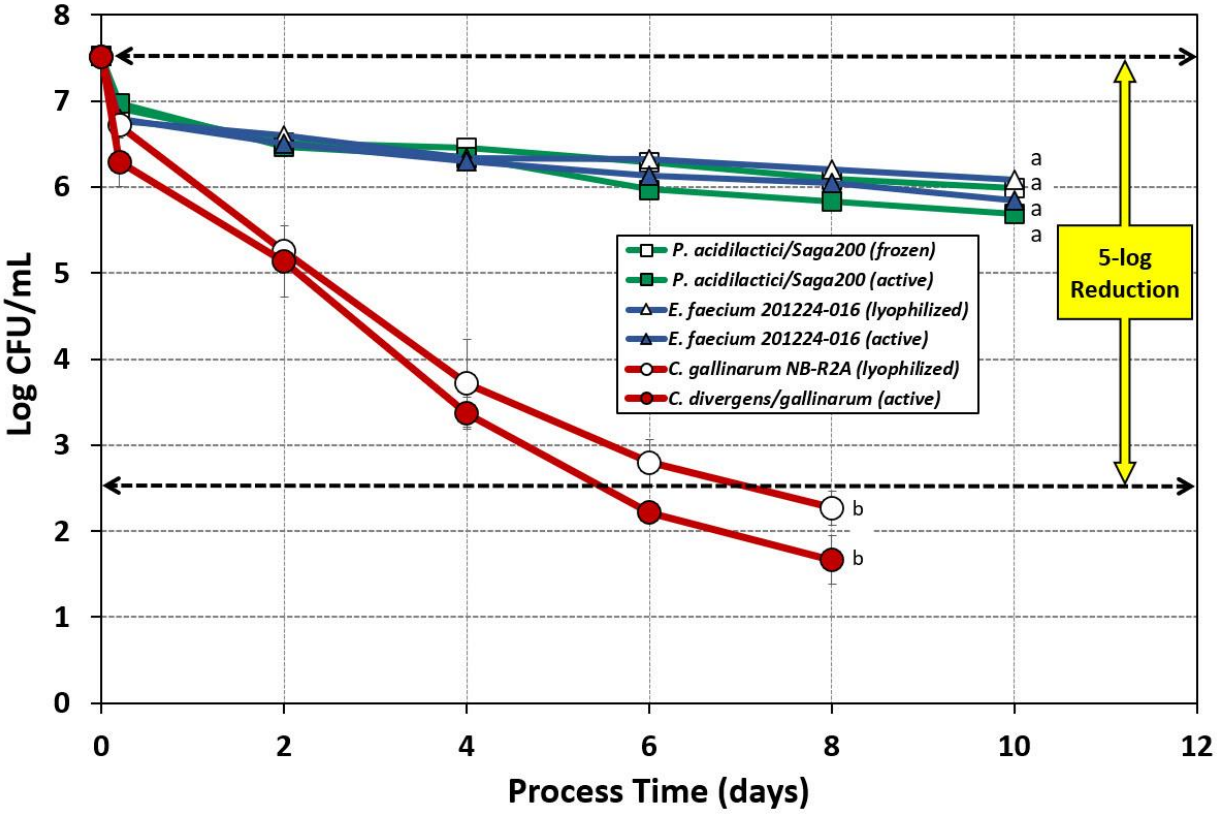

Figure 6. Biltong processing of beef inoculated with lyophilized/frozen cells vs metabolically active cells (freshly grown) of *E. faecium* 201224-016, *P. acidilactici*, and *C. gallinarum* NB-R2A. Lyophilized *C. gallinarum* NB-R2A was compared to a four-strain cocktail of metabolically active *C. divergens*/*gallinarum*. Graph curves of frozen or lyophilized cultures have hollow symbols. Statistical analysis was performed by one-way repeated measures analysis of variance (RM-ANOVA) over the entire time course of the data sets; graphs with the same letter are not significantly different ($p > 0.05$); isolates with different letters are significantly different ($p < 0.05$).

4. Conclusion

The lethality observed in the biltong process with *Carnobacterium* spp. aligned with that observed with 4 major pathogenic organisms indicating that *Carnobacterium* spp. could be an effective in-plant surrogate organism to monitor the effectiveness of biltong processing within a manufacturer's facility. *Enterococcus faecium, L. sakei, and Pediococcus spp.* are not reduced much (<2-logs) and are resilient towards the acid, salt, and low A_w experienced during 10 days of biltong processing. The use of lyophilized/frozen cells as inoculum for biltong processing was not significantly different than using actively grown cells. This work helps to fill USDA-FSIS knowledge gaps in air-dried shelf-stable dried beef (biltong) processing with regards to potential surrogate organisms and critical factors involved in the biltong process.

Supplementary Materials: Not applicable.

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Chapter VII

Microbial Profiling of Biltong Processing Using Culture-Dependent and Culture-Independent Microbiome Analysis

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Abstract: Biltong is a South African air-dried beef product that does not have a heat lethality step, but rather relies on marinade chemistry (low pH from vinegar, ~2% salt, spices/pepper) in combination with drying at ambient temperature and low humidity to achieve microbial reduction during processing. Culture-dependent and culture-independent microbiome methodologies were used to determine the changes in the microbial community at each step during biltong processing through 8 days of drying. Culture-dependent analysis was conducted using agar-based methods to recover viable bacteria from each step in the biltong process that were identified with 16S rRNA PCR, sequencing, and BLAST searching of the NCBI nucleotide database. DNA was extracted from samples taken from the laboratory meat processing environment, biltong marinade, and beef samples at 3 stages of processing (post-marinade, Day 4, and Day 8). In all, 87 samples collected from 2 biltong trials with meat obtained from each of 3 separate meat processors (n = 6 trials) were amplified, sequenced with Illumina HiSeq, and evaluated with bioinformatic analysis for a culture-independent approach. Both culture dependent and independent methodologies show a more diverse population of bacteria present on the vacuum-package chilled beef that reduces in diversity during biltong processing. The main genera present after processing were identified as *Latilactobacillus* sp., *Lactococcus* sp., and *Carnobacterium* sp. The high prevalence of these organisms is consistent with extended cold-storage of vacuum-packaged beef (from packers, to wholesalers, to end users), growth of psychrotrophic bacteria at refrigeration temperatures (*Latilactobacillus* sp., *Carnobacterium* sp.), and survival during biltong processing (*Latilactobacillus* sakei). The presence of these organisms on raw beef and conditions of beef storage appears to 'front-load' the raw beef with non-pathogenic organisms that are present at high levels during and after processing. This may result in a natural microbial suppression of mesophilic foodborne pathogens that are also reduced during the biltong process.

Keywords: biltong; microbiome; dried beef

1. Introduction

Biltong is a South African style dried beef product made using lean beef rounds that are sliced, marinated in a mixture of salt, vinegar, and spices, and then dried at ambient temperature and humidity. Since biltong is produced without a heat lethality step, the safety of the product relies on the addition of vinegar and salt in the marinade step and an extended drying period to achieve a low water activity (A_w) to make the product safe for consumers (C. E. Karolenko, Bhusal, Nelson, & Muriana, 2020). Biltong manufacturers obtain beef rounds from a variety of meat processors that have been chilled and vacuum-packaged prior being procured for use in the biltong production process. This 'wet-aging' stage takes place during the 4-14 days between slaughter and sale when the meat has been vacuum-packaged in an oxygen-barrier film and stored above freezing temperatures (Sitz, Calkins, Feuz, Umberger, & Eskridge, 2006).

Chilled storage and packaging helps to prevent the growth of common foodborne pathogens and slow the rate of meat spoilage (Barros-Velázquez et al., 2003; Venter, Shale, Lues, & Buys, 2006). However, it also leads to prevalence of psychrophiles and psychrotrophs in the microbiota of the raw meat. Additionally, the type of packaging can also select for different types of bacterial species and strains to colonize the meat surface depending on the intrinsic and extrinsic environmental factors of the meat system including oxygen availability, temperature, meat chemistry, and the presence of other bacteria (Dainty & Mackey, 1992; Ercolini, Russo, Torrieri, Masi, & Villani, 2006; Gram et al., 2002).

Bacteria found in the processing environment (slaughter, fabrication, further processing) also adds to the total microbiota on the meat. This in combination with any contributing bacteria found in added processing ingredients (i.e., marinade ingredients) results in the initial microbiota of the food product (Johansson et al., 2020; Nieminen et al., 2012). The initial microbiota can then change as processing conditions change (i.e., temperature, drying). Other studies have investigated the contribution of the environment on chilled, vacuum-packaged beef as well and the changes in microbial communities on dried beef products during processing (Chaillou, Chaulot-Talmon, Caekebeke, Cardinal, Christieans, Denis, Desmonts, et al., 2015; De Filippis, La Storia, Villani, & Ercolini, 2013; Pini, Aquilani, Giovannetti, Viti, & Pugliese, 2020). Little research has been done with the changes in the microbiome of air-dried beef products like biltong.

To evaluate changes in microbial communities, microbial profiling of the biltong product can be done to determine the differences in the microbial population at each step during processing and whether a process will select for similar organisms even though differences exist in the makeup of the initial microbiota. This can be done in one of two ways: culture-dependent methodology or culture-independent methodology. The culture-dependent methodology relies on agar-based methods to isolate, identify and characterize bacteria from the food matrix in question. While culture-based methodologies are standard in many laboratories and industry, culture-dependent techniques can only detect 0.1% of a complex community thus overestimating bacterial species that are culturable and underestimating the species that are unculturable (Cao, Fanning, Proos, Jordan, & Srikumar, 2017; Jarvis et al., 2018). Therefore, to understand the extent of all bacteria present, nucleic acid sequencing-based techniques (i.e., culture-independent methods) can be used to understand the complex microbiomes on foods. Previous studies have utilized culture-independent microbiome analysis to investigate the bacterial community of processed meats including modified atmosphere packaged beef, beef steaks and dry-aged beef from manufacturing facility to final product including the influence of the facility environment on the microflora of the final product (Clark, Clark, Bass, Capouya, & Mitchell, 2020; Säde, Penttinen, Björkroth, & Hultman, 2017; Yang et al., 2016) .

The objectives of this study were to identify bacterial populations present at different stages of biltong processing and to characterize how they change during processing through culture-independent and culture-dependent microbiome analyses. This change in bacterial population was also assessed using duplicate trials of beef obtained from each of three different beef processors to determine the influence different facilities would have on the native vs. final microbiota of biltong beef.

2. Materials and Methods

2.1. Beef Sources

Beef was obtained from various 3 different meat producers and used in biltong trials with dual objectives of both culture dependent (colony isolation, 16S rRNA identification) and culture independent microbiome analysis (DNA extraction, 16S gene sequencing, and microbial community analysis). USDA Select grade bottom round beef was sourced from Nebraska Beef (Omaha, NE, USA), Greater Omaha Packing Co, Inc. (Omaha, NE, USA), and High River Angus (JBS USA Food Company, Greeley, CO, USA). Beef from each of these processors was purchased from a local meat processor (Ralph's Packing Co., Perkins, OK, USA) who obtained the beef from a regional beef broker. The beef rounds were then transported to a cold room at the Robert M. Kerr Food and Agricultural Products Center at Oklahoma State University (FAPC, Stillwater, OK, USA) and stored for 2-3 days at 5 °C. From the time the beef was initially processed, the rounds were stored at refrigeration temperatures in vacuum-packaged polyethylene bags for 14-18 days from time of manufacture to time of use in biltong processing to represent how most biltong processors receive their beef.

2.2. Beef Preparation and Sampling

2.2.1. Preparation of Beef for Biltong Process and Microbiome Analyses

The same beef and biltong process would serve as a source of samples for both culture dependent and culture independent microbiome analysis. Initial trials of DNA extraction tests with raw untrimmed beef were of low quality due to high lipid content that interfered with DNA extraction buffers (Ojo-Okunola et al., 2020). We therefore followed the procedures of Hanlon *et al.* (Hanlon et al., 2021) to massage the vacuum-packaged bags and recover purge to minimize fat recovery. Vacuum-packaged beef bottom rounds were hand-massaged for 60 sec to encourage detachment of bacteria from meat pieces with minimal disruption to the fat (Hanlon et al., 2021).

Bags were carefully sanitized prior to opening using a sanitized knife, new gloves and sanitized trays and care was taken to minimize introduction of external contamination. After removal of the bottom rounds, 30 mL of purge liquid was taken to represent bacteria on the surface of the beef, distributed into two separate 15 mL sterile conical centrifuge tubes, and placed in a refrigerator for 30 min to allow the lipid content at the surface to solidify. An additional 2 mL of purge sample was collected for enumeration purposes. Following refrigeration, the liquid portion from each tube was removed from the solidified lipid layer and placed in new sterile 15 mL conical tubes (two 15 mL tubes per sample). The tubes were centrifuged at 4,280 xg for 20 min at 4 °C. The supernatant fractions were discarded, and 1 mL of cold sterile molecular-grade water was added to resuspend the pellet. The entire volume was then transferred to the second tube and used to resuspend the second pellet, thus combining the two tubes into one sample. The combined resuspended pellets were then transferred to a 2 mL DNA-free sterile microcentrifuge tube to start DNA extraction for culture-independent analysis.

2.2.3 Biltong Beef Processing, Marination, and Drying

Each beef round was trimmed of fat, sliced lengthwise, and cut to approximately 5.1 cm wide x 1.9 cm thick x 7.6 cm long beef rectangles, and held on covered trays overnight at 5° C. One round (~15 lbs) from each of the commercial packing plants was sufficient for one biltong trial. Beef used in these trials were never frozen and used within 2-3 days of receipt.

Biltong processing was conducted as described previously by Karolenko et al. (C. Karolenko & Muriana, 2020; C. E. Karolenko, Bhusal, Nelson, et al., 2020), but without bacterial inoculation. Individual beef pieces were placed in plastic baskets and dipped in sterile water in stainless steel vessels to replicate water rinse treatments or antimicrobial dips to enhance microbial reduction that is often used by biltong processors. The beef pieces were placed in the water for 30 sec after which the basket was removed and excess liquid allowed to drain for 60 sec. The beef pieces were then

placed into chilled stainless steel tumbling vessels containing a biltong marinade consisting of 2.2% salt, 0.8% black pepper, 1.1% coarse ground coriander, and 4% red wine vinegar (100-grain, 10% acetic acid) in relation to the total meat weight. Beef pieces were vacuum-tumbled (15 inches Hg; Biro VTS-43 Marblehead, OH, USA) for 30 min and then hung to dry in a humidity-controlled oven (Hotpack, Model #435315, Warminster, PA, USA) at 55% relative humidity and 24.9 °C (75 °F) for 8 days.

During each duplicate trial run from each of the 3 beef suppliers (n = 6 per sampling period per supplier), samples were collected at each sampling time point (raw beef/purge, beef after marinade, and beef after 4 and 8 days of drying). Beef samples were placed in a sterile Whirl-Pak filter stomaching bag (Nasco, Fort Atkinson, WI, USA) with 50 mL of sterile water and stomached to resuspend attached bacteria. The only samples that gave problems with DNA extractions were raw beef samples, which is why we used purge from the package as representative of what microbiota was present on the beef from the supplier as per Hanlon *et al.* (Hanlon et al., 2021).

All samplings were performed in triplicate at each stage of duplicate biltong trials from each of the 3 beef processors (n = 6 /sampling point/beef supplier).

2.2.2. Environmental and Marinade Sample Preparation for Microbiome Analysis

Environmental samples were also collected from surfaces that the meat would have contacted during fabrication and processing. Sterile premoistened sponge swabs (Sponge Sticks, 3M, St. Paul, MN, USA) were used to sample the cutting knife (both sides of the blade), cutting tray (plastic, 360 cm^2) and gloves of the person trimming the beef. The same sponge was used to swab all three surfaces. Swabbing of the environmental surfaces was done in triplicate for each trial. A clean tray, knife, and fresh gloves were used for trimming and cutting separate rounds from each processor if multiple beef bottom rounds were processed. Following swabbing, 25 mL of sterile water was added to the sample bag and hand massaged for 60 sec. The resulting liquid was collected in sterile 15 mL conical tubes. Tubes were centrifuged at 4280 xg for 20 min at 4 °C. The supernatant was discarded, and 1 mL of cold sterile water was added to one of the two tubes per sample to resuspend the pellet. The entire volume was then transferred to the second tube and used to resuspend the second pellet, thus combining the two tubes into one sample. The combined resuspended pellet was then transferred to a 2 mL DNA-free sterile microcentrifuge tube to start DNA extraction for culture-independent analysis.

Three separate marinade samples were made for each of 2 trials performed for each of the 3 beef processors tested. Each marinade was formulated based on 100 g of meat (the average amount of beef per individual biltong sample). Sterile water (8.3 mL) was added to the marinade, and samples were hand stomached for 60 sec at high setting and transferred to a sterile 15 mL conical tube. Tubes were centrifuged at 4280 xg for 20 min at 4°C. The supernatant was discarded and 1 mL of cold sterile water was added to resuspend the pellet. The combined resuspended pellet was then transferred to a 2 mL DNA-free sterile microcentrifuge tube to start DNA extraction for culture-independent analysis.

2.3. Culture Dependent Analysis

2.3.1. Bacterial Enumeration

Microbial enumeration was evaluated by total viable aerobic bacterial counts. Serial 10-fold dilutions were made by transferring 1 mL of each sample from the stomacher bags into 9 mL sterile 0.1% buffered peptone water (BPW, BD Bacto) tubes. Dilutions were then surface plated (0.1 mL) on tryptic soy agar (TSA) plates (BD Bacto; 1.5% agar) in duplicate and incubated for 48 hours at 30 °C before being counted.

2.3.2. Microbial Profiling (16S PCR, Sequencing, and Identification of Isolates)

Following incubation and enumeration, five bacterial isolates were collected from petri plates of the last dilution from each timepoint for each duplicate trial for each of the 3 processors (raw beef,

post-marination, day 4 and 8 of drying); an effort was made to select phenotypically different colonies if present. Isolates were streaked onto TSA for further purification and then single colony isolates were inoculated into tryptic soy broth (TSB) and allowed to grow for 24 hrs at 30 °C. Bacterial cells were harvested by centrifugation and washed three times in 0.1M Tris Buffer (pH 8.0) and lysed using the bead beating method using sterile, acid-washed glass beads (425-600 uM; Sigma; (Coton & Coton, 2005)) to extract the DNA. The resulting template was then used for 16S rRNA polymerase chain reaction (PCR) to amplify the DNA. Amplification for each isolate was done with two separate reactions each with a forward and reverse primer. The first reaction utilized the primers 7F (5′-RAGAGTTTGATCHTGGCTCAG-3′) and 928R (5′-CCCCGTCAATTCHTTTGA-3′) and the second reaction was done using the primers 759F (5′-CAGGATTAGATACCCTGGTAGTCC-3′) and 1541R (5′-AAGGAGGTGATCCARCCGC-3′). Following amplification, the resulting PCR amplimers were cleaned using the GenCatch Advanced PCR Extraction Kit (Epoch Life Sciences; Missouri City, TX, USA) per the manufacturer's procedures. The resulting products were sent to the Core Sequencing Facility at Oklahoma State University (Stillwater, OK, USA) for sequencing in both the forward and reverse direction for each template. Sequences were aligned using Molecular Evolutionary Genetic Analysis (MEGA; Version 10; (Kumar, Stecher, Li, Knyaz, & Tamura, 2018)) and then identified by using Standard Nucleotide BLAST (blast.ncbi.nlm.nih.gov) to compare our sequences with those in the NCBI 16S rRNA gene sequence database.

2.3.3. Phylogenetic Relationship

The relationship between the obtained and identified isolates were derived using phylogenetic tree constructed for the data using the Maximum Likelihood method with MEGA X software (Kumar et al., 2018). The evolutionary analyses were conducted in MEGA X software. Initial trees were automatically generated using the Neighbor-Joining method and pairwise distances were computed for the variant trees with the Maximum Composite Likelihood approach. The topology with the superior likelihood value was selected.

2.4. Culture Independent Analysis

2.4.1. DNA Extraction

The resuspended pellets from Sections 2.2.3. and 2.2.4. were then extracted with the DNeasy PowerFood Microbial DNA extraction kit and protocol (QIAGEN, Germantown, MD, USA). All samples were eluted using 100 µL elution buffer and then quantified using a NanoDrop-1000 spectrophotometer (ThermoFisher Scientific, Walthram, MA, USA; (Desjardins & Conklin, 2010)). Extracted DNA was then stored at -20 °C until shipped for further amplification, sequencing, and analysis. For shipment, samples were stored in 1.5 mL Eppendorf tubes wrapped in Parafilm, placed inside a sample box wrapped in a plastic bag, placed in a styrofoam shipping box with 10 pounds of dry ice, and shipped by overnight carrier to Novogene Co. Sample Receiving (Sacramento, CA, USA).

2.4.2. 16S rRNA Sequencing

From the extractions, 96 samples (30 from Greater Omaha; 36 from Nebraska Beef; 30 from High River-JBS) were submitted to Novogene (for 16S rRNA sequencing and analysis. A mock community made up equal proportions of *Escherichia coli* ATCC BAA 1427, *Enterococcus faecium* 201224-016, and *Pediococcus acidilactici* ATCC 8042 was used as a positive control for comparison. Per the company's protocols, the V3-V4 regions of the 16S bacterial rRNA gene were amplified using primers 341F (5′-CCTAYGGGRBGGASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′). Amplicons were sequenced on Illumina NovaSeq 6000 paired-end platform to generate 250 base pair paired-end reads. Paired-end reads were then merged using FLASH (V1.2.7) creating raw tags (30K). Raw tags were quality filtered to obtain clean tags via QIIME (V.1.7.0) software. Tags were then compared with a reference database (SILVA138 database) using UCHIME to detect chimera sequences. Any chimera sequences found were removed, obtaining effective tags used for

bioinformatic analysis. Analyzes were initially done with the inclusion of non-bacterial data but was also conducted with the mitochondria and chloroplast removed for comparison.

2.4.3. Bioinformatic Analysis

Bioinformatic analysis was conducted at Novogene Co. (Hong Kong, China). Sequence analysis was performed using Uparse software (Upvase V.7.0.1090) using all effective tags. Sequences with ≥97% similarity were assigned to the same OTU. Species annotation at each taxonomic rank (threshold 0.1~1) was performed in QIIME against the SSU rRNA database of SILVA138 database. Further phylogenetic relationships of OTUs were obtained using MUSCLE (Version 3.8.31). Relative abundances were normalized using a standard of sequence number corresponding to the sample with the least sequences.

2.5. Growth Assay of Isolates Obtained from Biltong Process

Select bacterial isolates collected from various meat suppliers including *C. divergens* GO R1B, *C. gallinarum* NB R1C, *C. gallinarum* NB R2A, and *L. sakei* GO R2D were tested in comparison to mesophilic bacteria including *Enterococcus faecium 201224-016* and *E. coli* ATCC BAA-1427 for growth at 5 °C and 30 °C. All strains were transferred twice from frozen stock and finally 50 uL was inoculated into into 5 mL TSB (*Carnobacterium*, *E. faecium*, *E. coli*) or MRS broth (*L. sakei* GO R2D) in spectrophotometer test tubes. The separate sets of inoculated pre-chilled or pre-warmed media tubes were incubated at both 5 °C (for 7 days) and 30°C (for 24 hours). Un-inoculated media tubes for use as 'blanks' were incubated along with the inoculated tubes. Absorbance (590 nm) of each tube was obtained using a Spectronic-20D+ spectrophotometer (model 333183, Thermo Fisher Scientific, Waltham, MA, USA). To maintain the incubation temperature for the short duration outside of the incubator during readings, each test tube rack was kept in a metal bin filled with an ice slurry to submerge the tubes to the level of the broth. Absorbance readings were obtained every hour for the samples incubated at 30 °C over the course of 10 hours. Samples incubated at 5 °C were read 10 hours after inoculation and then every 24 hours thereafter for 7 days.

3. Results and Discussion

3.1. Culture Dependent Microbiome Analysis

3.1.1. Bacteria Identified via Culture-Dependent Methodology

A total of 30 isolates were collected from the raw beef (10 from each processor). An additional 31 isolates were collected from the marinaded, dried beef (11 isolates were collected from Greater Omaha; 10 isolates each from Nebraska Beef and High River- JBS). The identification of the bacteria isolated during biltong processing via culture dependent methodology are compiled in Tables 1-3. It is generally accepted by taxonomists that % identity scores of ≥97% and ≥99% for 16S rRNA gene sequences are sufficient to identify organisms down to genus and species level, respectively (Janda & Abbott, 2007; Petti, 2007; Rossi-Tamisier, Benamar, Raoult, & Fournier, 2015).

Table 1. Bacterial isolates identified from Greater Omaha Packing Co. Inc. (GO) based on 16S rRNA gene sequencing analysis of isolates obtained from raw beef (Raw) and marinaded beef after 8 days of drying (D8) for trial 1 (R1) and trial 2 (R2).

Sample	Genus (species)	Sequence Length (bp)	Query Coverage (%)	Percent ID
GO Raw R1A[1,2]	*Serratia* sp.	1,477	99	97.6
GO Raw R1B[1,2]	*Carnobacterium* sp.	1,411	100	98.1
GO Raw R1C[1,2]	*Lactococcus piscium*	1,449	98	99.7
GO Raw R1D[1,2]	*Carnobacterium divergens*	1,421	100	99.8
GO Raw R1E[1,2]	*Lactococcus piscium*	1,449	98	99.7
GO Raw R2A[1,2]	*Carnobacterium divergens*	1,407	100	99.9

GO Raw R2B[1,2]	*Carnobacterium divergens*	1,467	98	99.7
GO Raw R2C[1,2]	*Carnobacterium* sp.	1,420	99	98.1
GO Raw R2D[1,2]	*Lactobacillus sakei*	1,382	100	99.9
GO Raw R2E[1,2]	*Lactobacillus sakei*	1,408	100	100.0
GO D8Mar R1A[1,3]	*Lactobacillus sakei*	1,489	99	99.7
GO D8Mar R1B[1,3]	*Carnobacterium divergens*	1,469	98	99.7
GO D8Mar R1C[1,3]	*Lactobacillus sakei*	1,494	99	99.7
GO D8Mar R1D[1,3]	*Carnobacterium divergens*	1,468	98	99.8
GO D8Mar R1E[1,3]	*Lactobacillus sakei*	1,489	99	99.7
GO D8Mar R2A[1,3]	*Lactobacillus sakei*	1,490	98	99.9
GO D8Mar R2B[1,3]	*Lactobacillus sakei*	1,493	98	99.9
GO D8Mar R2C[1,3]	*Lactobacillus sakei*	1,489	98	99.8
GO D8Mar R2D[1,3]	*Lactobacillus sakei*	1,475	98	99.8
GO D8Mar R2E-A[1,3]	*Lactobacillus sakei*	1,489	98	99.7
GO D8Mar R2E-B[1,3]	*Carnobacterium divergens*	1,467	98	99.6

[1] GO (Greater Omaha Packing Co. Inc.

[2] Raw (Raw Beef)

[3] D8Mar (Marinaded beef, dried eight days)

Table 2. Bacterial isolates identified from Nebraska Beef (NB2) based on 16S rRNA gene sequencing analysis of isolates obtained from raw beef (Raw) and marinaded beef after 8 days of drying (D8) for trial 1 (R1) and trial 2 (R2).

Sample	Genus (species)	Sequence Length (bp)	Query Coverage (%)	Percent ID
NB2 Raw R1A[1,2]	*Latilactobacillus* sp. (*sakei, graminis,* or *curvartus*)	1,501	100	99.7
NB2 Raw R1B[1,2]	*Latilactobacillus* sp. (*graminis,* or *curvartus*)	1,461	100	99.8
NB2 Raw R1C[1,2]	*Hafnia paralvei*	1,453	99	99.7
NB2 Raw R1D[1,2]	*Carnobacterium gallinarum*	1,436	99	98.5
NB2 Raw R1E[1,2]	*Hafnia paralvei*	1,472	99	99.6
NB2 Raw R2A[1,2]	*Latilactobacillus curvartus*	1,467	100	99.5
NB2 Raw R2B[1,2]	*Latilactobacillus curvartus*	1,467	100	99.7
NB2 Raw R2C[1,2]	*Carnobacterium gallinarum*	1,461	99	98.5
NB2 Raw R2D[1,2]	*Enterobacter mori*	1,472	100	99.3
NB2 Raw R2E[1,2]	*Hafnia paralvei*	1,473	99	99.6
NB2 D8Mar R1A[1,3]	*Latilactobacillus* sp. (*sakei* or *curvatus*)	1,473	100	99.6
NB2 D8Mar R1B[1,3]	*Latilactobacillus* sp. (*sakei* or *curvatus*)	1,503	100	99.7
NB2 D8Mar R1C[1,3]	*Carnobacterium gallinarum*	1,500	99	98.2
NB2 D8Mar R1D[1,3]	*Carnobacterium gallinarum*	1,472	100	98.2
NB2 D8Mar R1E[1,3]	*Latilactobacillus* sp. (*sakei* or *curvatus*)	1,498	100	99.7
NB2 D8Mar R2A[1,3]	*Leuconostoc mesenteroides*	1,441	100	99.4
NB2 D8Mar R2B[1,3]	*Leuconostoc mesenteroides*	1,445	98	99.2
NB2 D8Mar R2C[1,3]	*Latilactobacillus* sp. (*sakei* or *curvatus*)	1,524	100	99.7
NB2 D8Mar R2D[1,3]	*Latilactobacillus* sp. (*sakei* or *curvatus*)	1,500	100	99.6
NB2 D8Mar R2E[1,3]	*Latilactobacillus* sp. (*sakei* or *curvatus*)	1,467	100	99.7

[1] NB2 (Nebraska Beef)

[2] Raw (Raw Beef)

[3] D8Mar (Marinaded beef, dried eight days)

Table 3. Bacterial isolates identified from High River-JBS (HR) based on 16S rRNA gene sequencing analysis of isolates obtained from raw beef (Raw) and marinaded beef after 8 days of drying (D8) for trial 1 (R1) and trial 2 (R2).

Sample	Genus (species)	Sequence Length (bp)	Query Coverage (%)	Percent ID
HR Raw R1A[1,2]	*Carnobacterium divergens*	1,472	98	99.5
HR Raw R1B[1,2]	*Carnobacterium divergens*	1,519	96	98.6

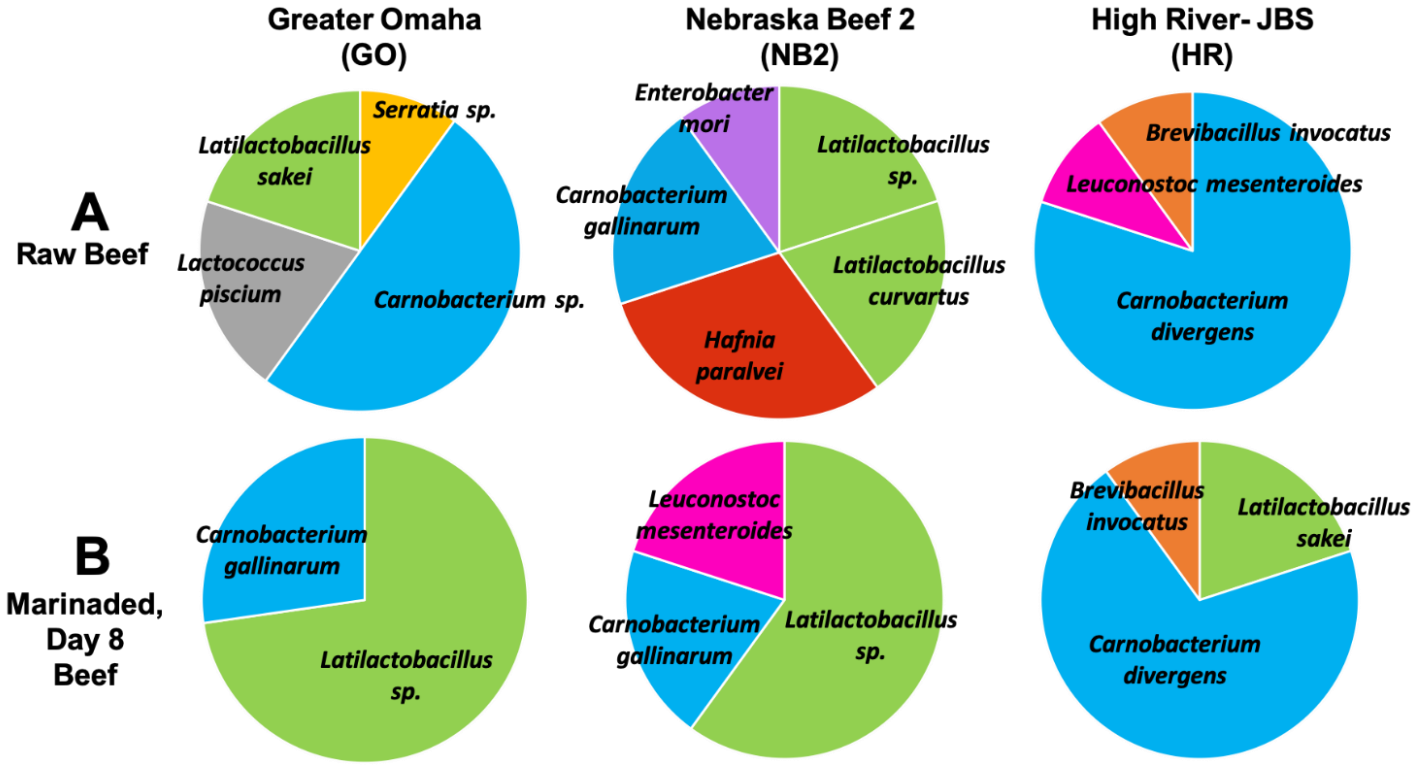
HR Raw R1C[1,2]	*Carnobacterium divergens*	1,440	96	100.0
HR Raw R1D[1,2]	*Carnobacterium divergens*	1,481	97	99.2
HR Raw R1E[1,2]	*Leuconostoc mesenteroides*	1,477	99	99.7
HR Raw R2A[1,2]	*Carnobacterium divergens*	1,504	96	99.3
HR Raw R2B[1,2]	*Brevibacillus invocatus*	1,467	99	98.8
HR Raw R2C[1,2]	*Carnobacterium divergens*	1,477	96	99.6
HR Raw R2D[1,2]	*Carnobacterium divergens*	1,510	96	98.2
HR Raw R2E[1,2]	*Carnobacterium divergens*	1,506	96	98.4
HR D8Mar R1A[1,3]	*Lactilactobacillus sakei*	1,494	100	99.5
HR D8Mar R1B[1,3]	*Carnobacterium divergens*	1,485	98	98.5
HR D8Mar R1C[1,3]	*Carnobacterium divergens*	1,478	98	98.8
HR D8Mar R1D[1,3]	*Carnobacterium divergens*	1,448	98	99.2
HR D8Mar R1E[1,3]	*Carnobacterium divergens*	1,441	99	99.3
HR D8Mar R2A[1,3]	*Latilactobacillus sakei*	1,512	100	99.3
HR D8Mar R2B[1,3]	*Carnobacterium divergens*	1,476	98	99.3
HR D8Mar R2C[1,3]	*Carnobacterium divergens*	1,546	96	98.9
HR D8Mar R2D[1,3]	*Brevibacillus invocatus*	1,471	99	98.9
HR D8Mar R2E[1,3]	*Carnobacterium divergens*	1,502	96	99.4

[1] HR (High River Angus-JBS)

[2] Raw (Raw Beef)

[3] D8Mar (Marinated beef, dried eight days)

A general trend among the identified isolates from all three processors is a greater variation in the bacterial species found on the raw beef and then decreases in variety after the beef had been marinated and dried for eight days (Figure 1). The remaining bacteria on the beef after the biltong process were predominantly members of the *Carnobacterium* or *Latilactobacillus* species.



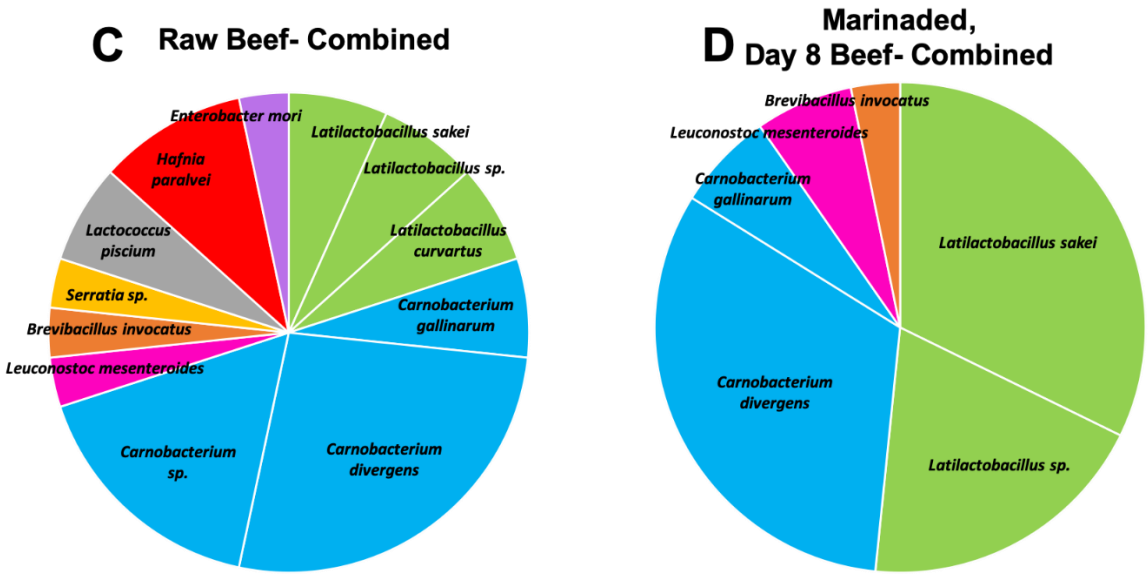


Figure 1. Culture dependent microbiome analysis. Bacteria isolated and identified using 16S rRNA PCR and sequencing from duplicate biltong trials with beef from each of the 3 processors: (**A**) raw beef and (**B**) biltong that had been dried for eight days. Pie chart analysis of all isolates from the three processors combined from (**C**) raw beef and (**D**) Day 8 dried beef.

Of the isolates recovered from raw beef obtained from Greater Omaha Inc., 50% of the isolates were identified as *Carnobacterium gallinarum*, 20% as *Lactococcus piscium*, 20% as *Latilactobacillus* sp. and 10% as *Serratia* sp. (Figure 1A). After processing, the only two bacteria identified from the selected isolates were *L. sakei* (72.7%) and *Carnobacterium gallinarum* (27.3%) (Figure 1B). The raw beef from Nebraska Beef also contained bacteria identified as *Latilactobacillus* sp. (20%) and more specifically *Latilactobacillus curvartus* (20%) as well as *C. gallinarum* (20%) (Figure 1A). Additionally, *Enterobacter mori* was identified as 10% of the isolates from the raw beef. After marination and drying, three different bacterial populations were recovered from the beef. They include *Latilactobacillus* sp. (60%), *C. gallinarum* (20%) and *Leuconostoc mesenteroides* (20%) (Figure 1B). Bacterial isolates from beef obtained from High River-JBS were primarily *Carnobacterium divergens* (80%), along with *L. mesenteroides* (10%) and *Brevibacillus inovatus* (10%) (Figure 1A). Similar domination of *Latilactobacillus* sp. and *Carnobacterium* sp. is observed in the marinated and dried beef (Figure 1B). Of the isolates recovered from the processed beef, 70% was identified as *C. divergens*, 20% as *L. sakei* and the remaining 10% as *B. invocatus*. These results are consistent with other studies in which spoilage-related bacteria found on raw, chilled beef stored in vacuum-packaged products included *Pseudomonas, Carnobacterium, Rahnella, Serratia, Hafnia,* and *Enterobacter* (Gram et al., 2002; Pennacchia, Ercolini, & Villani, 2011). In a similar culture-dependent microbiome analysis of dried biltong in Botswana, investigators found that *Staphylococcus* and *Bacillus* dominated the microflora but also identified *Leuconostoc, Enterobacter* and *Brevibacterium* as also present which is consistent with the findings in the marinated, dried biltong in this study (Matsheka et al., 2014).

3.1.2. Impact of Processing on Culture-Dependent Microbiome

There is a clear dominance of *Carnobacterium* sp. and *Latilactobacillus* sp. on the raw beef from all three processors. This dominance within the bacterial community on the raw beef can be attributed to the use of 'wet-aged' beef (also known as 'vacuum aging') during processing (Minks & Stringer, 1972; Terjung, Witte, & Heinz, 2021). Both *Carnobacterium* sp. and *Latilactobacillus* sp. are known psychrotrophic bacteria that can grow under refrigerated conditions during wet-aging of vacuum-packaged beef that is generated by the producer, stored,

sold to brokers, and then to end users who may hold the product in refrigerated storage until use in biltong or other types of beef processing (Chen et al., 2021; Laursen et al., 2005; Leisner, Laursen, Prévost, Drider, & Dalgaard, 2007). We compared the growth of 3 *Carnobacterium* strains (*C. divergens* GO R1B, *C. gallinarum* NB R1C, NB R2A) and 1 *L. sakei* GO R2D strain isolated during our biltong process along with typical contaminants (*E. faecium*, *E. coli*) that might be found on raw beef to demonstrate how these organisms can out compete mesophiles during refrigerated storage. A spectrophotometric growth assay was conducted to demonstrate the ability of the isolated lactic acid bacteria to selectively grow during colder temperatures while the mesophilic organisms are restricted from growing to higher levels (Figure 2A). At moderate temperatures (30 ◦C), the mesophiles grow faster than the psychrotrophs (Figure 2B). The data shows growth in separate nutrient environments (test tubes) whereas when mixed on the same meat surface, the psychrotrophs could grow fast, using up the available nutrient supply and the mesophiles would be even more hard pressed to increase in numbers. This experiment demonstrates the ability of the psychotropic bacteria like *Carnobacterium* sp. and *Latilactobacillus* sp. to grow better in the colder conditions such as the temperatures the beef is exposure to during wet-aging and storage prior to processing. The ability of *Carnobacterium* sp. and *Latilactobacillus* sp. to grow under refrigerated conditions underscores their ability to out compete mesophilic bacteria and demonstrates how they can dominate the microbial community on the raw beef leading into biltong processing. It would not be unusual for vacuum-packaged beef manufactured by a beef producer and obtained through a beef broker to be stored for several weeks at refrigeration temperature from fabrication to end use, thereby establishing a healthy psychrotrophic microbiota as evidenced by the data shown in Figure 2A.

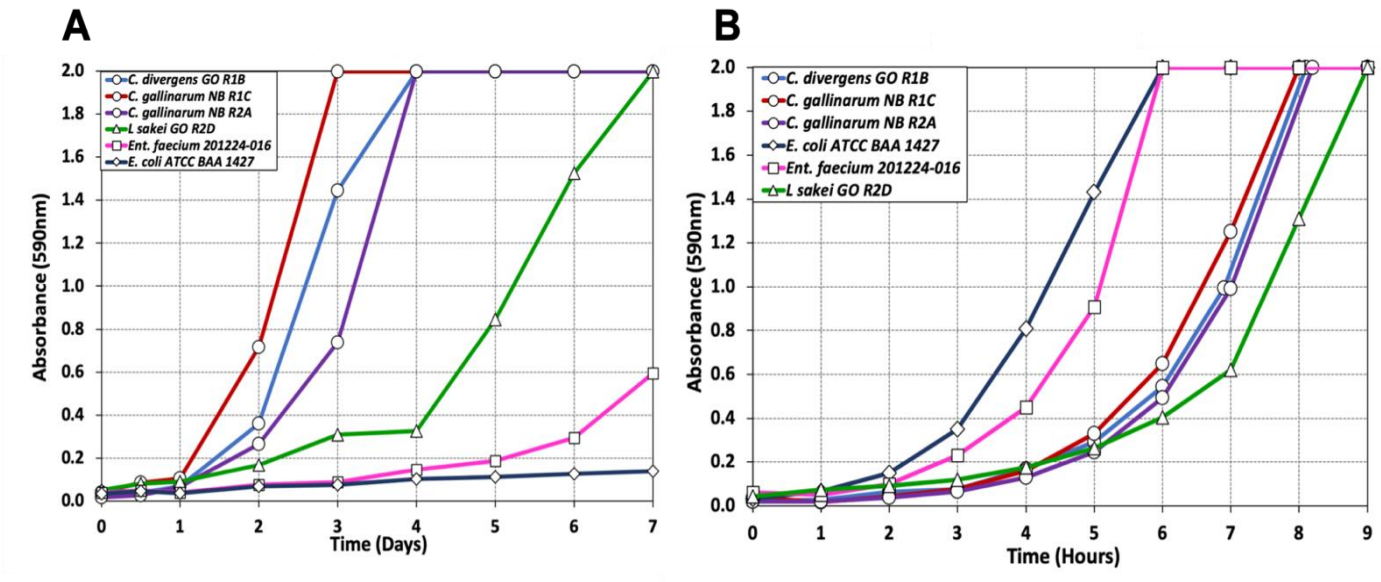


Figure 2. Spectrophotometric growth assay of bacteria isolated from marinated and dried biltong beef (*C. divergens* GO R1B; *C. gallinarum* NB R1C; *C. gallinarum* NB R2A; *L. sakei* GO R2D) compared to known mesophilic bacteria (*E. faecium* 201224-016; *E. coli* BAA ATCC 1427) at incubation temperatures (**A**) 5 °C and (**B**) 30 °C. Absorbance (at 590 nm) of each culture was measured every hour at 30 °C and every 24 hours at 5 °C.

In prior studies with pathogen-inoculated beef, we followed the decline of a pathogenic inoculum throughout the biltong process (Gavai, Karolenko, & Muriana, 2022; C. Karolenko & Muriana, 2020; C. E. Karolenko, Bhusal, Nelson, et al., 2020). In the current study, we enumerated APCs of the indigenous microbiota of the meat during the biltong process. Bacteria were enumerated from duplicate trials performed on beef from each of 3 processors at each step of the biltong process including the raw beef prior to processing, after vacuum-tumbling marination in salt, spice, and vinegar, and again after four and eight days of drying (Figure 3).

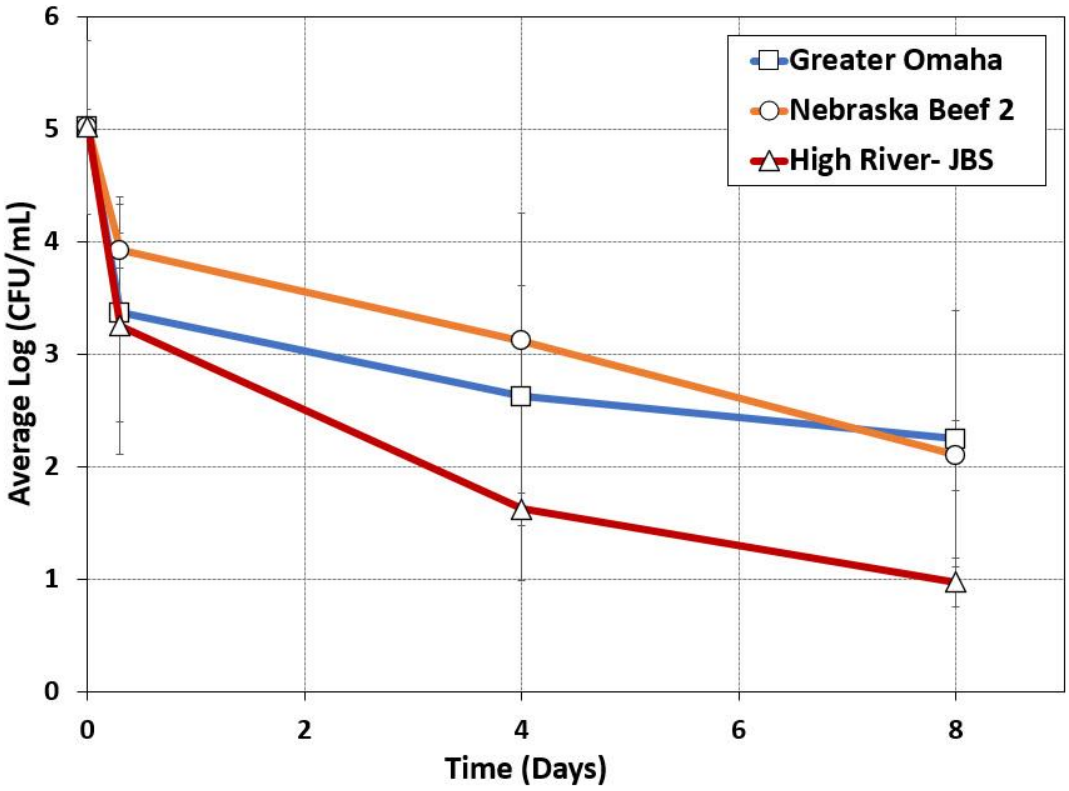


Figure 3. Aerobic plate count enumeration of bacteria recovered from biltong during duplicate processing trials of beef obtained from each of three beef producers (Greater Omaha Beef Co., Nebraska Beef, and High River/JBS). Surviving bacteria were enumerated at four different timepoints during biltong processing including: raw beef, beef after marination, and marinaded beef dried for 4 and 8 days at 24.9 °C (75 °F) and 55% RH. Samples were surface plated on TSA and incubated for 48 hrs at 30 °C prior to enumeration. The graph curves are averaged from duplicate trials sampled in triplicate at each timepoint (n = 6).

The initial reduction after the marinade step is due to exposure to low pH and high salt conditions from the vinegar and salt in the marinade killing sensitive bacteria. USDA-FSIS prefers validation studies to be performed with 'acid-adapted' cultures to ensure inoculum bacteria are not overly sensitive to acid treatments during processing (C. E. Karolenko, Bhusal, Gautam, & Muriana, 2020). The remaining bacteria were then further reduced during desiccation whereby up to 60% moisture loss is incured and the initial 2.2% salt concentration may reach upwards of 4% salt; the salt along with low humidity drying results in A_w levels below 0.85 A_w by the end of the biltong process. Similar reductions have been observed in previous biltong validation studies (C. Karolenko & Muriana, 2020). The meat from Nebraska Beef and Greater Omaha had the least overall reduction (APC counts), just shy of an overall process reduction of 2.8-2.9-log. This correlates to the culture dependent data that shows the dominate bacteria in the Day 8 marinaded beef was *L. sakei*. Alternatively, the beef from High River-JBS had a total reduction of just over 4-log while the dominate bacteria at the end of processing being *C. divergens*. This correlates to data obtained using *L. sakei* and *Carnobacterium sp.* as surrogate bacteria in biltong validation studies, whereby *Carnobacterium sp.* had an over 5-log process reduction while *L. sakei* only achieved a 1.8-2.0-log reduction by day 8 of drying (unpublished data).

3.1.3. Phylogenetic Relationship Between Isolates Obtained from Biltong Processing

Further analysis was done with the isolates to determine their relationship in respect to the origin of the beef (Figure 4). Sequence alignment and hierarchical cluster analysis is a useful tool for phylogenetic analysis (Woese, 1987). As expected, hierarchical clustering shows isolates originating from the same processor are more closely related to each other rather than to isolates from other

locations. This correlation is more defined in the Day 8 marinated samples where each bacterial species is more similarly related to other members of the same species from the same processor origins (Figure 4B). The prevalence of *Carnobacterium* sp. pre-process is likely a reflection of the fast growth rate during refrigerated incubation (wet aging, vacuum aging) whereas the prevalence of *L. sakei/Latilactobacillus* sp. post-process reflects the ability of this organism to survive the biltong process (i.e., ~2 log reduction) better than *Carnobacterium* sp. (i.e., ~4-5 log reduction).

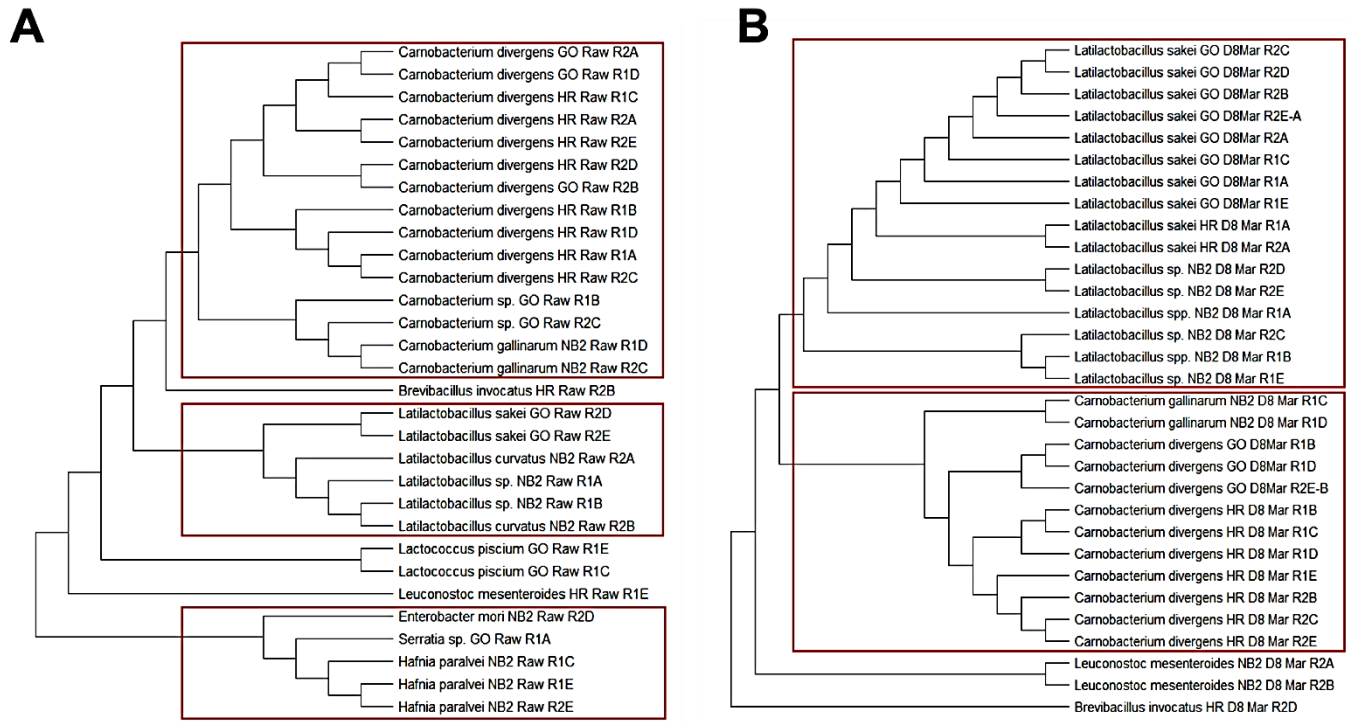


Figure 4. Dendrograms of all isolates obtained and identified from the (**A**) raw beef and (**B**) marinaded, day 8 beef from all three tested beef processors: Greater Omaha (GO), Nebraska Beef (NB2), and High River- JBS (HR). The phylogenic trees were constructed using the Maximum Likelihood method with pairwise distances estimated using the Maximum Composite Likelihood approach. Evolutionary analyses were conducted in MEGA X software. The red square highlight clades of importance related to the identified isolates and their processor of origin.

3.2. Culture Independent Microbiome Analysis

3.2.1. Bacterial Richness and Diversity in Biltong Processing

A total of 8,285,608 raw tags (3,047,152 tags from Greater Omaha samples; 3,085,216 from Nebraska Beef samples; 2,153,240 tags from High River-JBS) from 87 samples were sequenced using the Illumina platform. Following quality filtering and removal of non-bacterial sequences, a total of 7,952,944 clean tags remained. Additional chimera filtration steps resulted in a remaining 6,993,542 sequences used for further analysis. The determined number of OTUs and alpha diversity for each sample is described in Appendix I (Tables S1-S3). The environmental samples from the High River-JBS sample set did not have enough yields of nucleic acid extracted and therefore were not included further in library preparation and sequencing.

Alpha diversity analysis including observed species and Chao1 was conducted to determine differences in diversity between each timepoint of the biltong process from each processor separately. The alpha diversities are represented in the form of boxplots (Figure 5).

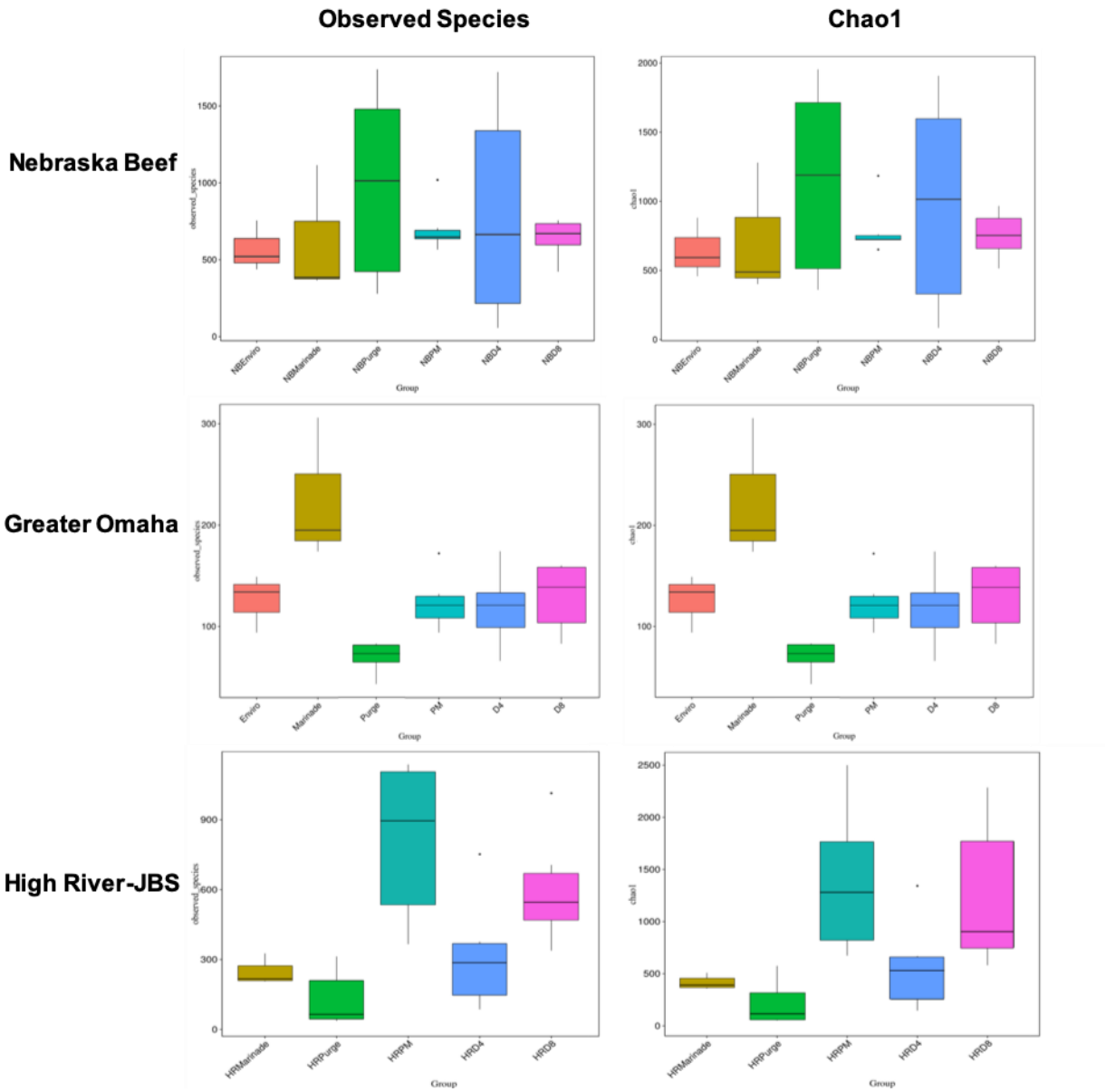


Figure 5: Box plot of differences between groups of observed species and Chao1 from different beef processors. Wilcox rank sum test and Tukey test were used to analyze the differences and significance ($p < 0.05$) in species diversity between groups.

Based on observed species data, the raw beef/purge samples from Greater Omaha and High River-JBS had the lowest diversity in all the steps of the biltong processing that were sampled (Figure 5). Following the marinade step, the marinated beef was significantly higher ($p < 0.05$) compared to the raw meat/purge samples in the Greater Omaha and High-River-JBS samples. After marination, there was no significant difference in diversity between the post-marinated (PM), Day 4 (D4) and Day 8 (D8) samples from Greater Omaha. The D4 bacterial community was significantly different ($p < 0.05$) from PM and D8 on the meat from High River-JBS. There was no significant difference in any of the indices with the samples from Nebraska Beef. The decrease in diversity at Day 4 with the meat from High River was unexpected. Both chemical (enzyme mediated) and physical (beads) lysing techniques were included in the DNA extraction process to achieve maximum yield and more accurate bacterial community structure (i.e. not favor Gram-negative bacteria since they are easier to lyse) (Li et al., 2020). However, the amount of DNA extracted from the Day 4 samples from High River-JBS was lower (data not shown) compared to the post-marinated and Day

8 samples from the same processor which could have contributed to a decrease in microbial diversity (Teng et al., 2018).

3.2.2. Changes in the Microbial Community During Processing

The bacterial diversity at the genus levels from all three processors is shown in Figure 5. Each group is an average of six samples (three samples taken from each of two separate trials) of each processor meat. The top ten most abundant genera identified were used in the relative abundance analysis. *Latilactobacillus* sp. was the dominate genus in samples taken from Nebraska Beef and High River-JBS (Figure 6A, 6E) representing 94.5% and 60.6% of the OTUs identified from each batch of samples respectively. The highest levels of *Latilactobacillus* sp. were observed in the meat-based samples (raw meat/purge, post-marinated beef, PM; beef after four days of drying, D4; beef after eight days of drying, D8; Figure 6B, 6D) in which the abundance increased during processing and reached a maximum level in the Day 4 samples. *Lactococcus* sp. (40.7%) and *Latilactobacillus* sp. (30.2%) were the most abundant in the samples from Greater Omaha (Figure 6C). *Lactococcus* sp. levels are initially higher compared to the *Latilactobacillus* sp. in the environmental samples and the initial raw meat/purge samples. As the meat is processed (marinated and dried), the levels of *Lactococcus* sp. decreases and levels of *Latilactobacillus* sp. increase (Figure 6D). *Lactococcus* sp. was identified in both High River-JBS and Nebraska Beef samples as well but at less than 20% abundance in all samples. *Latilactobacillus* sp. and *Lactococcus* sp. are both lactic acid bacteria that are commonly associated with spoilage and aged beef and were expected to be in high abundance due to the use of cold-aged meat and an extended drying process (Hilgarth, Behr, & Vogel, 2018). Similar trends were observed using culture-dependent methodology to identify bacteria during processing.

Genera that could contain pathogenic bacteria such as *Escherichia* sp. in the Day 4 meat samples and *Pseudomonas* sp. in the environmental samples from Greater Omaha were detected in low proportions (<1%). Additionally, low levels (<0.5%) of *Escherichia* sp. was detected in Day 4 and Day 8 meat samples from High River-JBS. The detection of these genera does not directly indicate the presence of a pathogenic organism in the food product. The short reads used in Illumina platform based 16S rRNA sequencing cannot be identified beyond the genus level (Claesson et al., 2010). Therefore, it is unknown if the sequence identified is pathogenic in nature like *E. coli* 0157:H7 or a non-pathogenic member of the same genus (Braz, Melchior, & Moreira, 2020). Furthermore, even if it was pathogenic, the biltong process has been shown to give ≥5-log reduction not only Salmonella [1], but also *E. coli* O157:H7, *L. monocytogenes*, and *S. aureus* (Gavai et al., 2022), and is considered sufficiently safe that USDA-FSIS does not require ingredient or end product pathogen testing if using a '5-log process' (C. E. Karolenko, Bhusal, Nelson, et al., 2020; Nickelson, Luchansky, Kaspar, & Johnson, 1996).

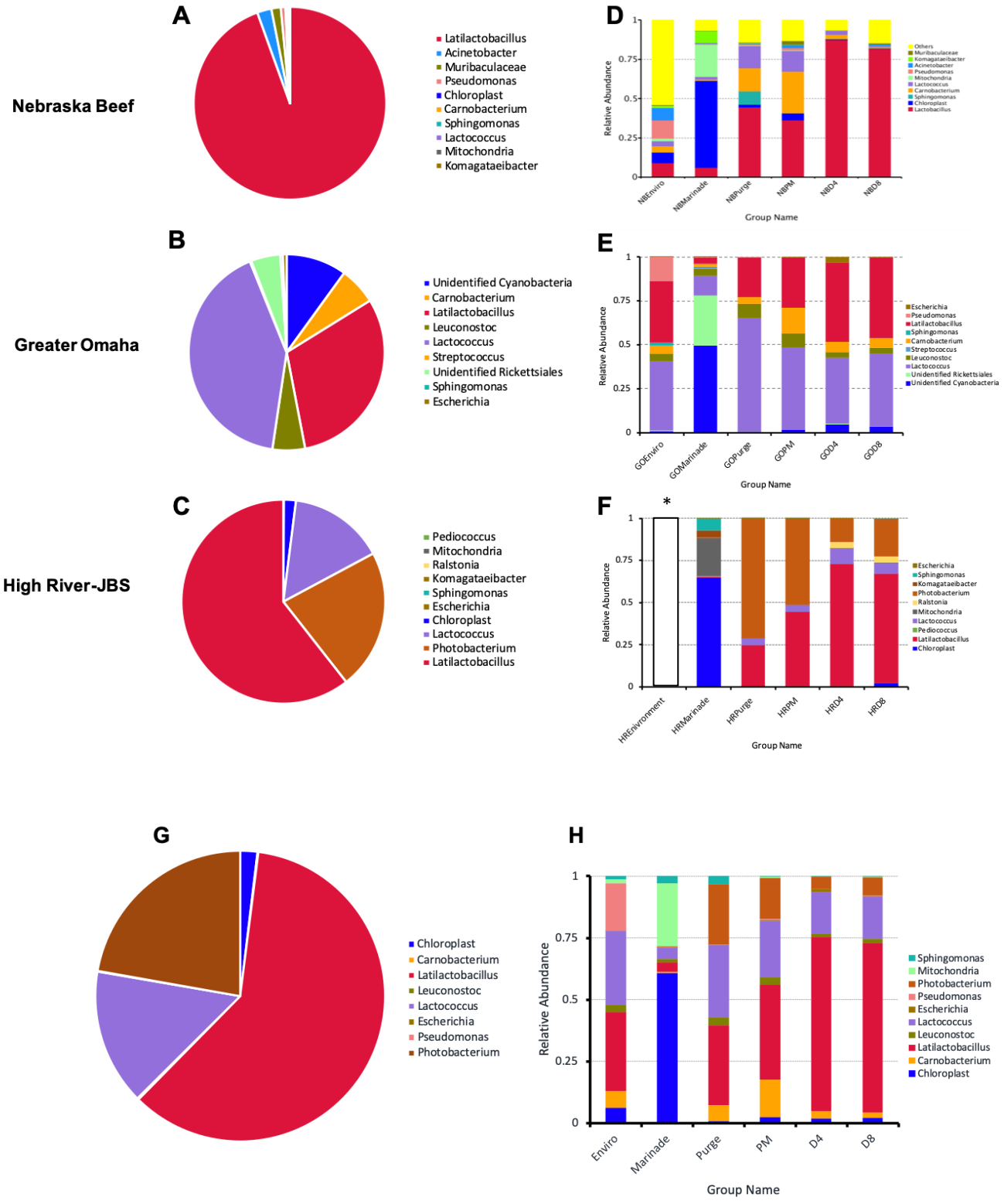


Figure 6. Relative abundance of total identified DNA from **(A)** Nebraska Beef, **(B)** Greater Omaha and **(C)** High River-JBS across all time points. OTUs were further related to specific sampling sites **(D, E, F)** during biltong processing based on abundance including non-bacterial data (mitochondria and chloroplast). Relative abundance of organisms among combined beef producers **(G)** and among combined common sampling sites **(H).** Groups are coded as: NB, Nebraska Beef; GO, Greater Omaha; HR, High River-JBS for meat processors and as Enviro, environmental samples; PM, post-marinade beef; D4, beef dried four days; D8, beef dried eight days for the process sampling points. *Insufficient DNA was recovered for subsequent sequencing

The findings of mainly lactic acid bacteria on the raw beef samples was to be expected as they are common spoilage organisms of aged vacuum-packaged beef as observed by Doulgeraki et al. (Doulgeraki, Ercolini, Villani, & Nychas, 2012). *Photobacterium* sp. was also identified in high proportions in the raw meat/purge samples (average of 70.8%) from High River- JBS. While commonly associated with cold marine environments, *Photobacterium* sp. have been identified in high numbers on packaged fresh beef and appear to play a role in the spoilage of meat (Fuertes-Perez, Hauschild, Hilgarth, & Vogel, 2019; Pennacchia et al., 2011). As is the case with all three processors, the initial diverse communities on the raw beef gives way to a few species that become more dominant by the end of the drying process as a result of processing conditions. This same trend was observed in the culture-dependent data as well.

The addition of the marinade during processing could have also played a role in the bacteria diversity. In marinated chicken breast (marinade was pH 3.7-4.2), the predominate lactic acid bacteria found were *Latilactobacillus plantarum, L. paracasei* subsp. *paracasei,* and *L. parabuchneri, and L. brevis* which are not the typical meat spoilage organisms commonly found such as *L. sakei, L. curvatus* and *Carnobacterium* sp. (Lundstrom & Bjorkroth, 2007). This suggest that the marinade may contribute is own source of lactic acid bacteria different from those lactic acid bacteria that are found on the beef from environmental contamination. It may also help select for bacteria from the meat itself to survive the marination, allowing the dominance of *Latilactobacillus* sp. on the meat during biltong processing. Additionally, the salt in the marinade may also contribute to the prevalence of the lactic acid bacteria that are halotolerant during processing. The marinade used during biltong processing for this study is 2.2% NaCl (w/w). In other reduced-sodium studies, sausage products with similar salt levels (2.0% w/w) that were vacuum packaged had a core community consisting of *Latilactobacillus sakei, Lactococcus piscium, C. divergens, C. maltaromaticum, Serratia proteamaculans, and Brochothrix thermosphacta* (Fougy et al., 2016). The high abundance of *Latilactobacillus* sp., particularly *L. sakei* as specifically identified in the culture-dependent data, on the salted marinated meat samples is not surprising given that it is a halotolerant bacterium found in many dried meat products (Chaillou, Chaulot-Talmon, Caekebeke, Cardinal, Christieans, Denis, Hélène Desmonts, et al., 2015; Champomier-Vergès, Chaillou, Cornet, & Zagorec, 2001).

Distinct clustering between meat samples across all processors was observed in UniFrac analysis (Figure 8A). This is particularly true for the beef that was marinated and dried for 4 and 8 days (Figure 8B, 8C, 8D). This correlated with the lack of significant differences between observed species in the individual processor data at the same drying timepoint (Figure 5). The marinade samples are clustered separately from the rest of the samples. Based on the relative abundance from each processor (Figure 6), the marinade samples had a drastically different microbial community composition compared to the other meat samples which could account for the separate clustering. The marinade was made up primarily of *Cyanobacterium,* chloroplast, and *Rickettsiales*, likely due to the marinade being made of primarily plant material (i.e., spices such as coriander and pepper) and was present in higher proportion within the marinade samples compared to the others collected. Given that the marinade is an acidic, vinegar-based marinade, it was expected that the bacterial load in the marinade samples would be low (microbial counts drop after marination, Figure 3) and that the plant material in the marinade would yield higher levels of chloroplasts. Although the chloroplast data was not initially removed from the bioinformatic analysis, it was a small proportion of the subsequent beef samples which is the mainstay of this study (Figure 6D, 6E, 6F, 6H). Plant chloroplast 16S rRNA and bacterial 16S rRNA genes share high sequence similarity as they are evolutionary descendants from bacteria (Hanshew, Mason, Raffa, & Currie, 2013). The universal primers targeting the 16S rRNA gene can influence non-specific binding and given the likely low population of bacterial material available in the marinade, the primers could then bind to the chloroplast rRNA instead given the similar homology (Rastogi, Tech, Coaker, & Leveau, 2010). Confirmation of the minimal impact of the marinade on the meat samples was done by subsequently removing chloroplast and mitochondrial data from the analysis (Figure 7).

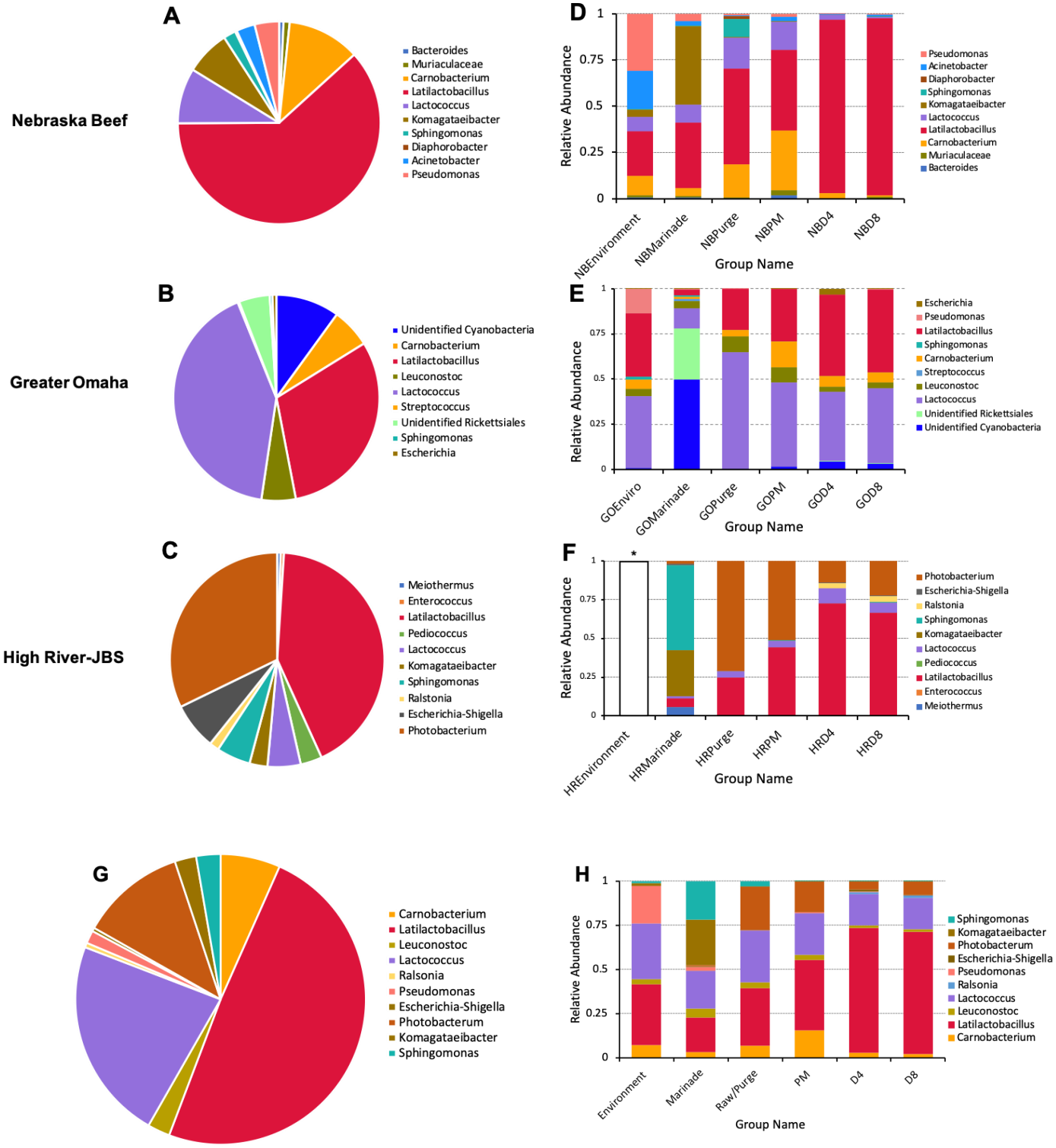
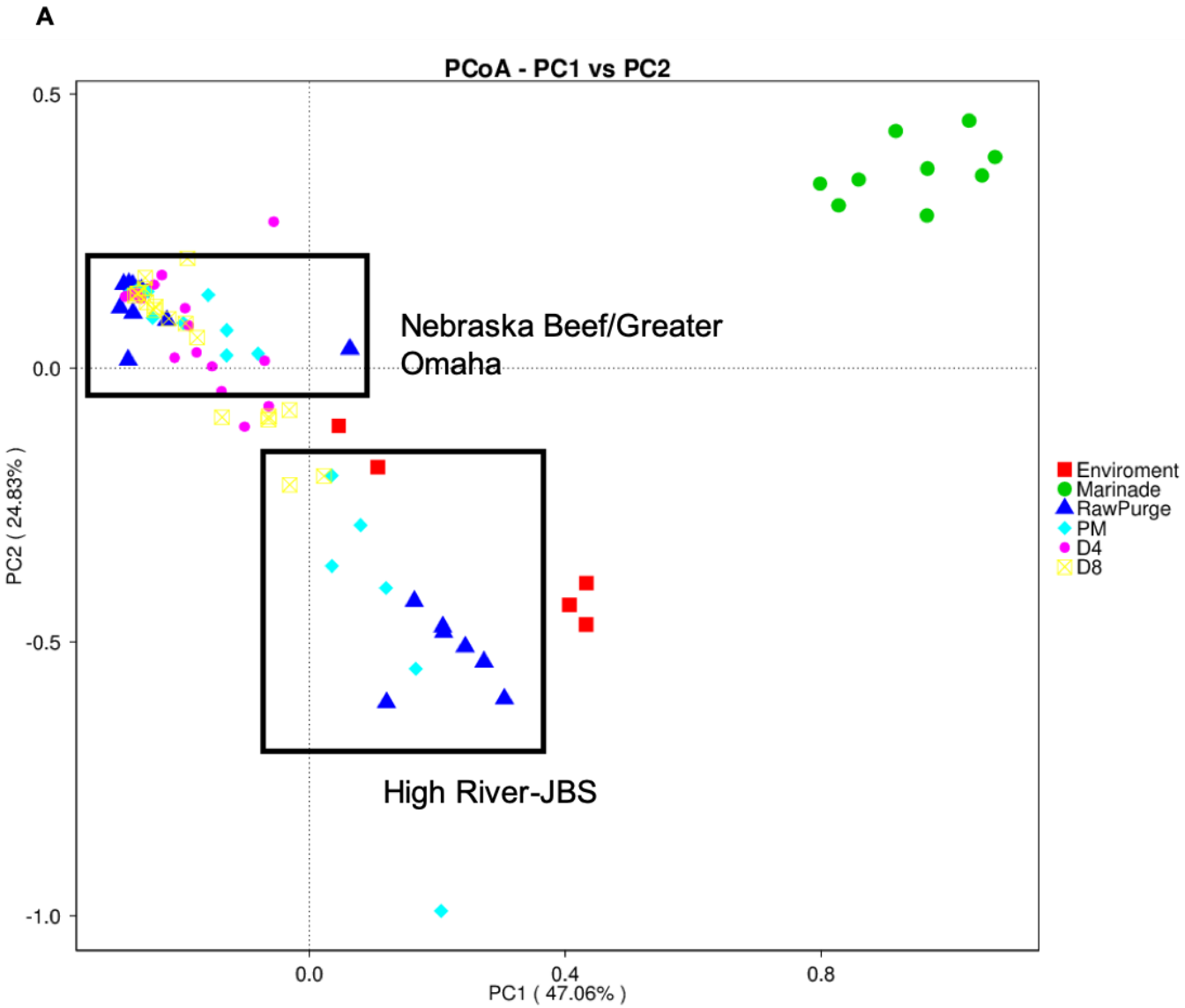


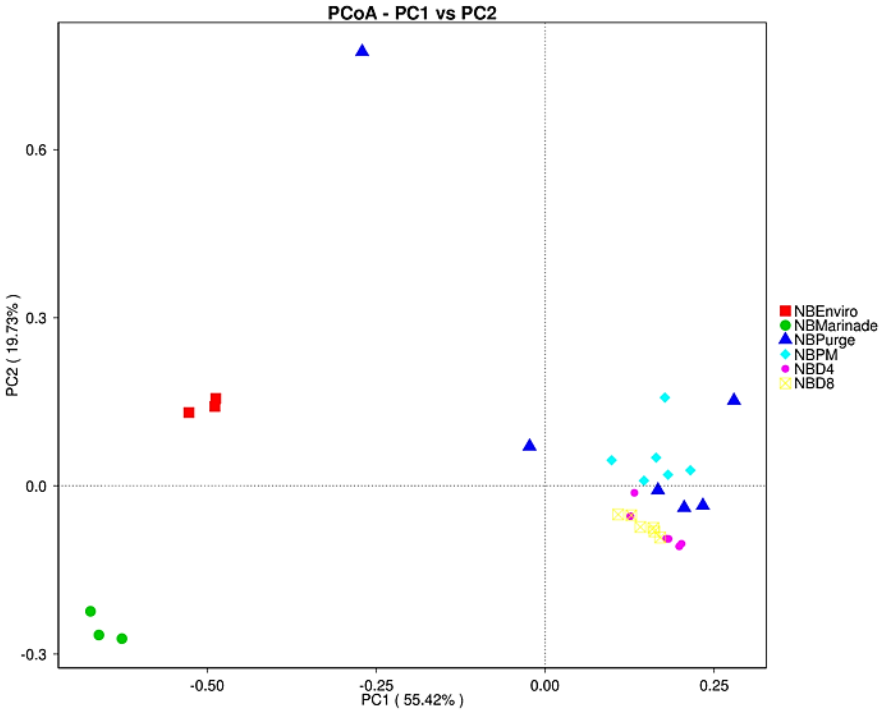
Figure 7. Relative abundance of total identified DNA from **(A)** Nebraska Beef, **(B)** Greater Omaha and **(C)** High River-JBS across all time points. OTUs were further related to specific sampling sites **(D, E, F)** during biltong processing based on abundance with non-bacterial (mitochondrial and chloroplast) data removed. Relative abundance of organisms among combined beef producers **(G)** and among combined common sampling sites **(H).** Groups are coded as: NB, Nebraska Beef; GO, Greater Omaha; HR, High River-JBS for meat processors and as Enviro, environmental samples; PM, post-marinade beef; D4, beef dried four days; D8, beef dried eight days for the process sampling points. *Insufficient DNA was recovered for subsequent sequencing

Upon removal of the non-bacterial data, a change in the total microbial profile can be seen for the samples from each of the individual processors (Figure 7A, 7B, 7C). However, the same dominate genera from the initial analysis remain including *Latilactobacillus* sp., *Lactococcus* sp., and *Photobacterium* sp. Further investigation of the microbial profile of the samples from each step in the biltong process (Figure 7D, 7E, 7F), reveals the marinade now has a different profile but still with minimal overlapping genera on the subsequent meat samples as previously seen with the analysis including the non-bacterial data. The microbial profile of the meat samples (purge, PM, D4, D8) are comprised of different bacteria then those observed in the marinade samples with an increase in the abundance of *Latilactobacillus* sp. by the end of biltong processing. The removal of the non-bacterial data still supports the overall outcome of the of the microbial profile on the final biltong product.



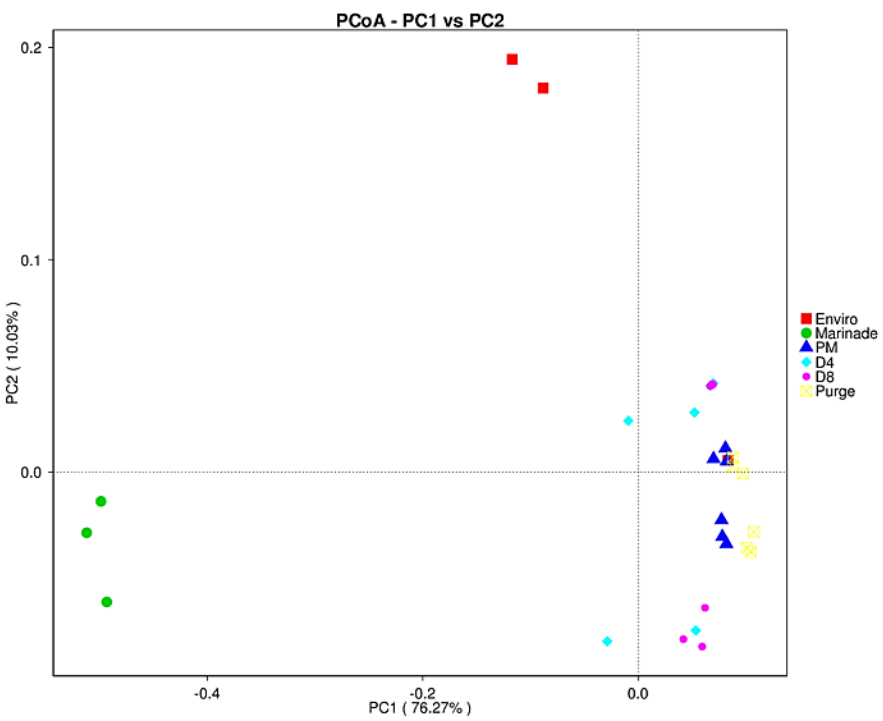
B

Nebraska Beef



C

Greater Omaha



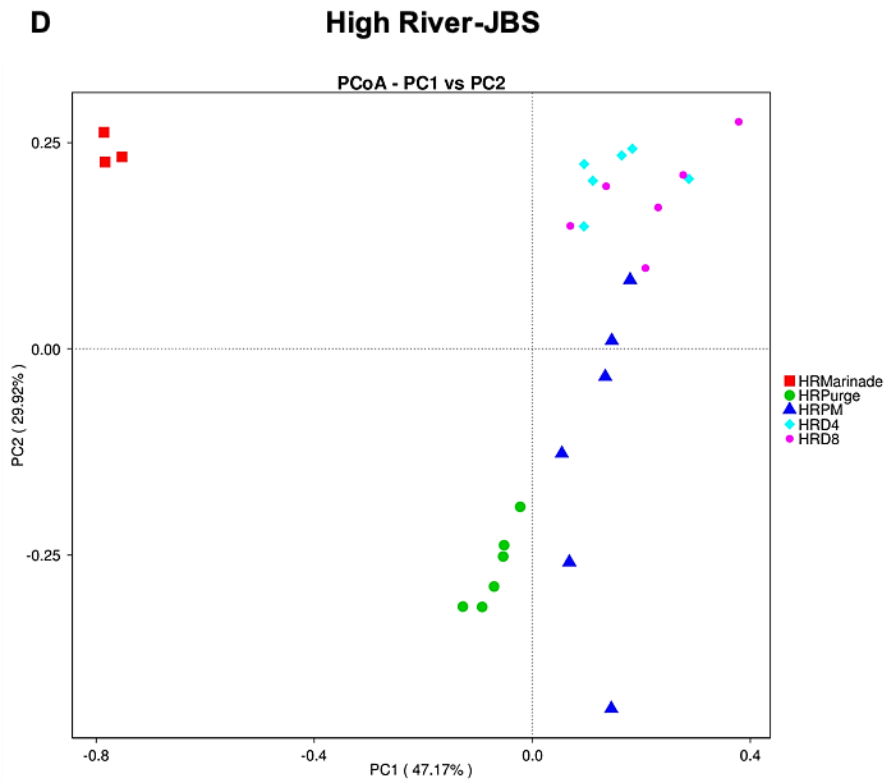


Figure 8: UniFrac analysis of the microbiome of **A)** all three processors combined, **B)** Nebraska Beef (NB), **C)** Greater Omaha (GO), and **D)** High River-JBS (HR). Legend: the lab environment (Enviro), biltong marinade (Marinade), raw meat/purge after fabrication (Purge), beef post-marination (PM), marinated beef dried four days (D4) and marinated beef dried eight days (D8).

5. Conclusions

Regardless of what bacteria are present on the raw beef as different meat processors had diverse initial microbial compositions on the raw beef, the biltong process results in domination by *Latilactobacillus* sp. and *Lactococcus* sp. based on culture-independent based analysis of the bacterial community. The culture-dependent analyses showed similar results with a wider diversity initially recovered from the raw beef and then dominated by *Latilactobacillus sakei* and *Carnobacterium* sp. after processing. The presence of these psychrotrophic meat spoilage bacteria are likely due to the initial vacuum-packaged refrigerated conditions ('wet aging', 'vacuum-aging') that the beef is stored at for an extended period of time prior to use in the biltong process. This study highlights how storage conditions of beef influences microbial populations prior to use in food processing and how manufacturing conditions can further cause a shift in the abundance of bacteria present on the final product.

Supplementary Materials: The following supporting information can be found in Appendix I.

Author Contributions: Conceptualization, P.M.; methodology, P.M., C.K.; software, P.M.; validation, C.K. and P.M.; formal analysis, C.K.; investigation, C.K.; resources, P.M.; data curation, P.M.; writing—original draft preparation, C.K.; writing—review and editing, P.M., C.K.; visualization, P.M., C.K.; supervision, P.M.; project administration, P.M.; funding acquisition, P.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The work performed in this study was performed under Protocol #17-8 approved by the OSU Institutional Biosafety Committee within the Office of University Research Compliance.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not Applicable

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Conflicts of Interest: The authors declare no conflict of interest.

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Appendix I

Supplemental Material for Chapter VII: Microbial Profiling of Biltong Processing Using Culture-Dependent and Culture-Independent Microbiome Analysis

Table S1: Sequencing data and alpha diversity estimation of the 16S rRNA gene libraries on Illumina HiSeq platform in biltong processing and environmental samples from Greater Omaha.

Sample Name	Effective Tags	Average Length	OTUs	Shannon	Simpson	Chao1	ACE	Goods Coverage	PD Whole tree
GOEnv1	91,818	429	84	1.642	0.575	142.583	132.81	0.998	25.481
GOEnv2	59,854	427	139	3.338	0.847	156.4	160.737	0.999	33.807
GOEnv3	78,166	428	130	2.945	0.79	156.464	172.803	0.998	21.896
GOMar1	104,652	411	153	2.897	0.776	200.045	200.136	0.998	23.107
GOMar2	93,652	411	178	3.09	0.806	218.182	229.474	0.998	25.378
GOMar3	96,764	412	279	3.857	0.849	327.75	333.666	0.997	33.022
GOPurR1A	84,369	429	52	1.581	0.54	83.625	85.164	0.999	17.78
GOPurR1B	85,112	429	55	1.475	0.476	65.909	68.906	0.999	18.637
GOPurR1C	82,586	429	64	1.473	0.464	95.5	105.092	0.999	27.354
GOPurR2A	82,948	429	70	1.431	0.527	155	143.539	0.998	17.293
GOPurR2B	84,153	429	56	1.392	0.537	87.625	86.339	0.999	14.251
GOPurR2C	82,644	429	36	1.209	0.486	43.333	51.885	0.999	5.527
GOPMR1A	69,103	428	87	2.184	0.707	116.176	121.208	0.999	56.691
GOPMR1B	81,909	428	111	2.168	0.642	144.056	145.286	0.998	63.073
GOPMR1C	83,134	428	149	2.325	0.667	200.037	211.091	0.998	64.816
GOPMR2A	75,327	427	102	1.906	0.618	125.8	139.452	0.998	59.139
GOPMR2B	90,241	428	95	1.75	0.596	118.625	119.305	0.999	77.585
GOPMR2C	93,682	428	81	1.633	0.577	106.833	122.603	0.999	67.775
GOD4R1A	87,879	428	147	2.297	0.664	262.556	233.373	0.997	69.662
GOD4R1B	89,593	426	128	2.08	0.524	158	164.199	0.998	76.726
GOD4R1C	70,843	425	115	2.251	0.62	154.545	141.153	0.999	74.42
GOD4R2A	81,210	428	50	1.478	0.44	78.5	86.511	0.999	20.048
GOD4R2B	87,423	428	71	1.184	0.364	89.071	96.456	0.999	18.961
GOD4R2C	88,240	429	89	1.261	0.382	118.75	130.605	0.998	25.676
GOD8R1A	79,962	426	148	2.198	0.566	221.5	195.342	0.998	64.518
GOD8R1B	86,548	426	147	2.177	0.539	171.231	177.51	0.998	98.184
GOD8R1C	87,785	426	150	2.119	0.522	191.25	199.7	0.998	111.954
GOD8R2A	91,106	429	94	1.107	0.334	129	137.343	0.998	35.344
GOD8R2B	89,287	429	87	1.1	0.34	99	111.894	0.999	34.749
GOD8R2C	78,457	429	76	0.96	0.298	119.154	125.118	0.998	42.281

Table S2: Sequencing data and alpha diversity estimation of the 16S rRNA gene libraries on Illumina HiSeq platform in biltong processing and environmental samples from Nebraska Beef.

Sample Name	Effective Tags	Average Length	OTUs	Shannon	Simpson	Chao1	ACE	Goods Coverage	PD Whole tree
NBEnv1	83,005	426	521	2.968	0.676	888.604	964.54	0.987	89.094
NBEnv2	76,176	426	357	3.033	0.655	484.53	556.053	0.993	76.555
NBEnv3	63,694	426	338	3.874	0.87	556.226	628.667	0.992	84.794
NBMar1	103,518	409	279	2.994	0.762	420.942	445.82	0.994	44.833
NBMar2	96,134	409	274	3.013	0.776	387.056	423.166	0.995	35.275
NBMar3	92,151	410	729	3.733	0.814	1080.088	1217.547	0.984	84.975
NBPurR1A	80,512	413	536	3.119	0.722	995.579	1087.423	0.986	214.884
NBPurR1B	87,620	424	1216	4.198	0.81	2076.235	2204.448	0.971	285.131
NBPurR1C	79,819	425	1117	3.845	0.742	1640.555	1776.356	0.977	563.588
NBPurR2A	90,372	426	856	3.328	0.685	1206.625	1348.719	0.983	255.023
NBPurR2B	99,167	428	199	1.673	0.546	347.154	408.867	0.995	179.81
NBPurR2C	84,758	428	178	1.544	0.538	290.579	351.385	0.996	184.038
NBPMR1A	75,083	426	727	3.229	0.736	1027.471	1109.214	0.985	309.371
NBPMR1B	70,493	424	478	4.282	0.847	718.081	744.854	0.991	244.9
NBPMR1C	84,535	425	426	3.475	0.779	633.68	662.388	0.992	300.53
NBPMR2A	76,337	425	475	3.465	0.77	666.032	722.07	0.991	232.163
NBPMR2B	77,549	427	535	3.092	0.738	758.009	808.604	0.99	237.87
NBPMR2C	69,815	427	477	3.094	0.749	618.127	687.624	0.992	296.521
NBD4R1A	93,907	426	1126	2.52	0.398	1700.459	1903.353	0.975	163.328
NBD4R1B	99,898	428	1180	3.071	0.56	1817.162	1942.428	0.974	194.308
NBD4R1C	97,787	428	409	0.867	0.141	657.351	805.226	0.99	89.152
NBD4R2A	97,338	429	350	0.799	0.133	703.5	757.837	0.991	78.284
NBD4R2B	105,226	429	48	0.214	0.047	106	125.764	0.999	68.475
NBD4R2C	90,231	429	37	0.23	0.053	67.6	60.542	0.999	25.11
NBD8R1A	90,231	429	263	0.693	0.129	457.381	542.034	0.993	77.8
NBD8R1B	89,056	427	542	2.653	0.467	753.579	764.158	0.991	126.401
NBD8R1C	99,167	427	568	2.346	0.397	763.709	801.779	0.99	96.642
NBD8R2A	99,753	427	457	1.887	0.32	638.176	673.062	0.992	104.757
NBD8R2B	93,351	426	573	2.594	0.453	723.634	774.239	0.991	107.003
NBD8R2C	89,968	427	459	2.164	0.39	630.067	665.613	0.992	102.893

Table S3: Sequencing data and alpha diversity estimation of the 16S rRNA gene libraries on Illumina HiSeq platform in biltong processing and environmental samples from High River-JBS.

Sample Name	Effective Tags	Average Length	OTUs	Shannon	Simpson	Chao1	ACE	Goods Coverage	PD Whole tree
HRMar1	57,283	404	366	3.017	0.761	554.138	652.778	0.992	92.456
HRMar2	86,490	406	226	2.72	0.75	439.529	469.479	0.994	39.977
HRMar3	88,958	406	235	2.665	0.738	356.528	431.708	0.995	42.177
HRPurR1A	73,603	426	293	2.596	0.706	447.967	487.081	0.994	111.502
HRPurR1B	76,761	428	82	2.175	0.677	121.375	143.339	0.998	43.374
HRPurR1C	81,288	429	55	2.194	0.698	92.5	105.022	0.999	24.232
HRPurR2A	87,842	429	39	2.023	0.691	74	61.397	0.999	14.414
HRPurR2B	96,957	429	46	2.012	0.683	59.6	70.762	0.999	13.398
HRPurR2C	79,862	428	391	2.408	0.708	772.857	918.396	0.989	71.056
HRPMR1A	43,146	353	1471	4.188	0.798	1694.27	1650.357	0.982	481.227
HRPMR1B	79,585	426	381	2.801	0.754	711.304	773.623	0.99	228.113
HRPMR1C	52,996	425	512	2.865	0.759	771.375	847.887	0.989	207.788
HRPMR2A	38,306	422	482	2.574	0.628	736.857	787.363	0.99	218.424
HRPMR2B	39,136	420	608	2.961	0.693	946.754	1009.195	0.987	289.185
HRPMR2C	52,083	411	799	2.718	0.675	1877.045	2231.841	0.975	347.118
HRD4R1A	57,446	418	502	2.558	0.651	769.541	889.725	0.988	260.732
HRD4R1B	86,259	428	125	1.857	0.523	204.565	245.028	0.997	39.242
HRD4R1C	86,393	428	97	1.956	0.589	144.045	180.657	0.998	30.861
HRD4R2A	50,605	425	226	2.097	0.573	358.222	443.841	0.994	177.634
HRD4R2B	69,674	427	160	1.432	0.351	321.889	366.382	0.996	117.647
HRD4R2C	74,953	428	432	1.656	0.36	729.359	911.928	0.989	80.026
HRD8R1A	41,540	423	307	2.238	0.559	533.246	640.998	0.992	282.163
HRD8R1B	73,703	425	475	2.834	0.678	758.454	808.271	0.989	170.687
HRD8R1C	62,547	424	570	2.946	0.71	880.508	962.891	0.987	201.356
HRD8R2A	42,099	407	950	3.515	0.698	1577.029	1826.372	0.977	376.526
HRD8R2B	53,930	423	541	2.603	0.555	790.31	849.398	0.989	207.038
HRD8R2C	83,344	428	349	2.015	0.517	594.959	700.505	0.991	58.966

Appendix II

Predictive Modeling of Biltong Processing

Methods

Microbial reduction data was compiled from previous biltong validation studies that evaluated the ability of the process to reduce *Salmonella* to sufficient levels during processing. The key treatments of interest were various drying temperatures (73°F, 75°F, 77°F), salt concentration in the marinade formulation (1.7%, 2.2%, 2.7%; w/w), salt used in the marinade (sodium chloride, potassium chloride, calcium chloride), and vinegar concentration in the marinade (2%, 3%, 4%). Thermal kinetic parameters and D-values for each treatment was calculated using the United States Department of Agriculture Global Fit Integrated Predictive Modeling Program (USDA IPMP) Global Fit software fitted to the Weibull model according to procedures described in Huang (2014) and Huang (2017). The D-values of each treatment were analyzed with STAT software here using analysis of variance supplemented with Tukey's test at a significance level $p<0.05$ to determine significant differences between different paraments within each treatment.

Results and Discussion

The Weibull-Mafart model (Equation 1) was selected as the best fit for the biltong data(Mafart, Couvert, Gaillard, & Leguerinel, 2002).

$$\text{Equation 1: } \log(N) - \log(N_0) = y_0 - (\frac{t}{D})^K$$

This model is a nonlinear model to analyze thermal inactivation data that is traditionally used to determine different D-values (D) for each temperature condition tested factoring in the dependent variable, the log reduction ($\log(N) - \log(N_0)$ (Figure 1).

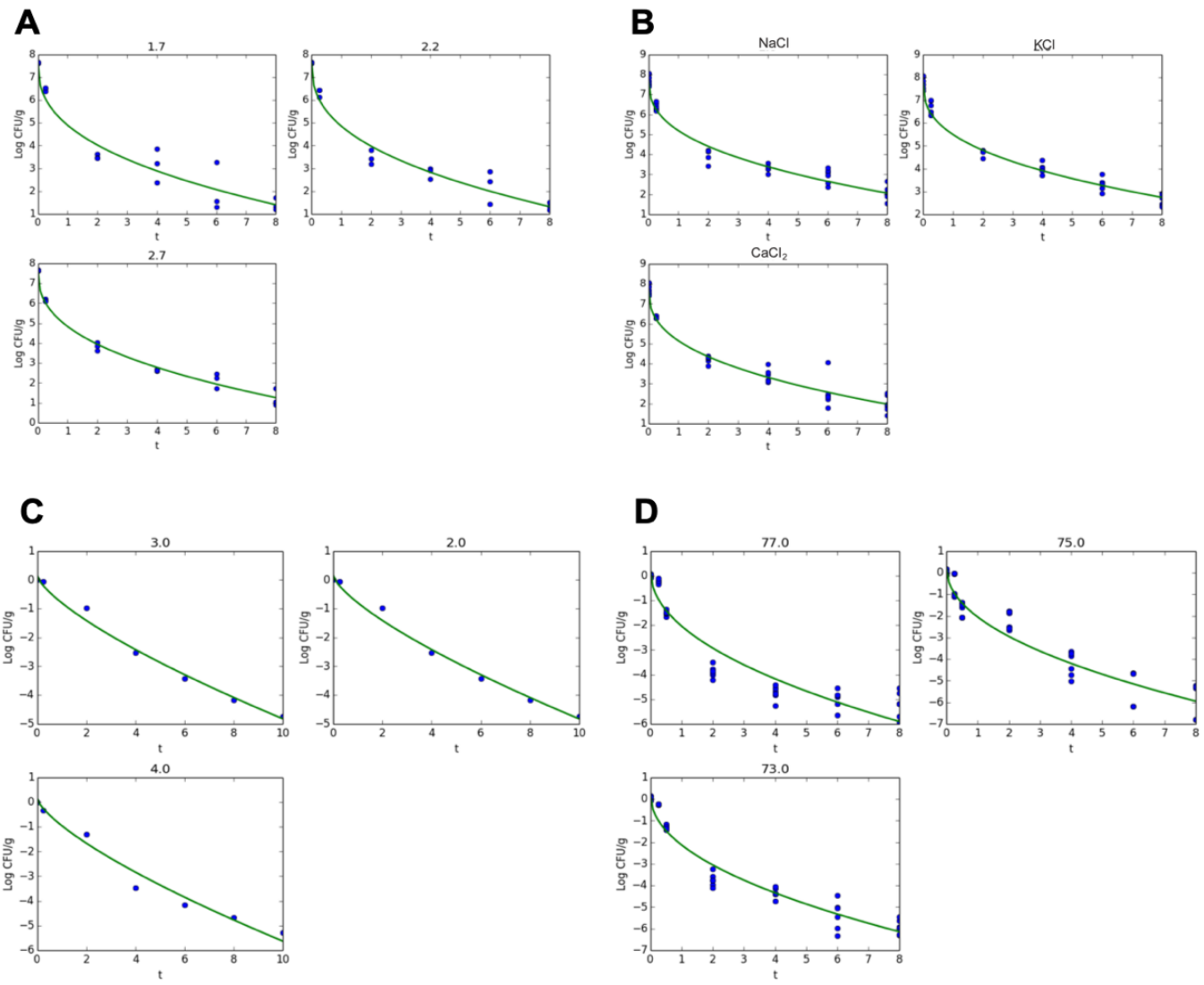

Figure 1. Plots of best fit generated by USDA-IPMP Global fit software using the Weibull model for A) salt concentration, B) salt type used in marinade C) vinegar concentration in marinade and D) drying temperature.

The model in this case was used to determine D-values at different processing conditions (parameters) during biltong processing including varying salt concentration in the marinade (Figure 1A), alternative salts used in the marinade (Figure 1B), varying vinegar concentrations used in the marinade formulation (Figure 1C) and varying drying temperatures (Figure 1D).

Table 1. Weibull model calculated D-values of *Salmonella* in biltong with different salt concentrations and type in the marinade, varying vinegar concentrations in the marinade and different drying temperature during processing. Calculations were done using USDA-IPMP Global Fit software.

	Parameters	Value	Std-Error	t-value	p-value	L95CI	U95CI
Salt Percentage in Marinade	D, T1.7	0.07	0.05	1.27	0.21	-0.04	0.18
	D, T2.2	0.07	0.05	1.27	0.21	-0.04	0.17
	D, T2.7	0.06	0.05	1.26	0.21	-0.04	0.17
	K	0.39	0.06	6.48	0.00	0.27	0.51
	y0	0.08	0.33	0.24	0.82	-0.58	0.73
Salt Used in Marinade	D, NaCl	0.08	0.05	1.62	0.11	-0.02	0.18
	D, KCl	0.11	0.07	1.69	0.09	-0.02	0.24
	D, CaCl2	0.08	0.05	1.62	0.11	-0.02	0.17
	K	0.38	0.05	7.98	0.00	0.29	0.47
	y0	0.04	0.23	0.18	0.86	-0.42	0.50
Vinegar Concentration Used in Marinade	D, T3.0	1.08	0.79	1.37	0.19	-0.60	2.77
	D, T2.0	1.08	0.79	1.37	0.19	-0.60	2.77
	D, T4.0	0.88	0.68	1.30	0.21	-0.55	2.31
	K	0.72	0.22	3.29	0.00	0.26	1.19
	y0	0.15	0.49	0.30	0.77	-0.90	1.19
Drying Temperature	D, T77.0	0.19	0.08	2.33	0.02	0.03	0.34
	D, T75.0	0.18	0.08	2.33	0.02	0.03	0.34
	D, T73.0	0.17	0.07	2.31	0.02	0.02	0.32
	K	0.48	0.05	9.67	0.00	0.38	0.58
	y0	0.22	0.23	0.96	0.34	-0.23	0.66

The D-values for each parameter is reported in Table 1. The is no difference in D-values for any of the parameters evaluated during biltong processing. This indicates that the drying temperature, rather than the evaluated parameter, is the main effect on the microbial reductions observed. The lack of differentiation between D-values could be the result of parameters that too similar to each other. A wider variation in drying temperature or concentration of salt used in the marinade for example might show more of an effect by the parameters on the D-values.

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Appendix III

Additional Published Work

microorganisms

MDPI

Article

Effect of Biltong Dried Beef Processing on the Reduction of *Listeria monocytogenes*, *E. coli* O157:H7, and *Staphylococcus aureus*, and the Contribution of the Major Marinade Components

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Abstract: Biltong is a dry beef product that is manufactured without a heat lethality step, raising concerns of whether effective microbial pathogen reduction can occur during biltong processing. Raw beef inoculated with 4-strain cocktails of either *E. coli* O157:H7, *Listeria monocytogenes*, or *Staphylococcus aureus*, and processed with a standard biltong process, were shown to incur a >5-log reduction in 6–8 days after marination by vacuum-tumbling for 30 min in vinegar, salt, spices (coriander, pepper) when dried at 23.9 °C (75 °F) at 55% relative humidity (RH). Pathogenic challenge strains were acid-adapted in media containing 1% glucose to ensure that the process was sufficiently robust to inhibit acid tolerant strains. Internal water activity (A_w) reached < 0.85 at 5-log reduction levels, ensuring that conditions were lower than that which would support bacterial growth, or toxin production by *S. aureus* should it be internalized during vacuum tumbling. This was further confirmed by ELISA testing for staphylococcal enterotoxins A and B (SEA, SEB) after marination and again after 10 days of drying whereby levels were lower than initial post-marination levels. Comparison of log reduction curves obtained for *E. coli* O157:H7, *L. monocytogenes*, *S. aureus*, and *Salmonella* (prior study) showed that microbial reduction was not significantly different ($p < 0.05$) demonstrating that even without a heat lethality step, the biltong process we examined produces a safe beef product according to USDA-FSIS guidelines.

Keywords: *Listeria monocytogenes*; *E. coli* O157:H7; *Staphylococcus aureus*; biltong; log reduction; acid adaptation; water activity

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1. Introduction

Dried beef products are found worldwide, and although they may have originated as a way to preserve perishable meat products [1], they have now become accepted as nutritious high protein 'meat snacks' for sportsmen, campers, and hikers [2]. They are also included in specialized 'paleolithic diets' to simulate foods our hunter-gatherer ancestors consumed, serving as a modern means of reducing weight [3]. Popular dried beef products such as beef jerky, kippered beef, and biltong can be found in nearly every supermarket and convenience store. A recent newcomer to the manufacturing of biltong, Stryve Foods (Plano, TX, USA), recently announced record annual sales of $30 million USD (2021), representing an increase of 77% over 2020 and projected sales of $43–48 million USD for 2022. It is just one of many biltong beef processors experiencing increased sales of this new product line in US markets that have been dominated by traditional American style beef jerky.

Although biltong is noted to have originated in South Africa as the result of early Dutch settlers attempting to preserve meat, there are no South African regulatory guidelines for its manufacture. As biltong has grown in popularity in the UK and US, efforts to export the product directly from South Africa have been thwarted. In prior years, it was difficult to export biltong out of South Africa into developed countries where food safety is

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