PACKAGING AND STORAGE EFFECTS ON *LISTERIA MONOCYTOGENES* REDUCTION AND ATTACHMENT ON READY-TO-EAT MEAT SNACKS

by

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B.S., University of the Philippines, Los Baños, Laguna, 2005

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A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

A total of three studies were conducted to evaluate the effects of different packaging systems and storage times on reduction of *Listeria monocytogenes* on ready-to-eat meat snacks. Study 1 was conducted to determine the effects of four packaging systems [heat sealed (HS), heat sealed with oxygen scavenger (HSOS), nitrogen flushed with oxygen scavenger (NFOS), and vacuum (VAC)] and storage times (24, 48, and 72 h, and 14 and 30 d) on reduction of *L. monocytogenes* in turkey jerky in the presence or absence of sodium nitrite. Inclusion of sodium nitrite in turkey jerky did not affect (P>0.05) *L. monocytogenes* log reductions regardless of packaging type or storage time. After 14 d of storage in HSOS, NFOS, or VAC, and 48 or 72 h in HS, a reduction of >1.0 log CFU/cm^2 of *L. monocytogenes* was achieved. Processors could use HS in conjunction with 48 h of ambient storage and be in compliance with the United States Department of Agriculture Food Safety and Inspection Service *Listeria* Rule of post-lethality treatment in achieving at least 1 log reduction of *L. monocytogenes*. Study 2 was conducted to investigate attachment of *L. monocytogenes* to uncured and cured turkey jerky packaged in HS, HSOS, NFOS, or VAC using scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS). The SEM examination showed that *L. monocytogenes* is capable of adhering to uncured or cured turkey jerky surfaces. Elemental maps from EDS analysis revealed that no element was unique or elevated at sites of *L. monocytogenes* attachment. Elemental composition showed the presence of elemental sulfur and could be an indication of the presence of sulfur-containing amino acids in turkey jerky. Finally, Study 3 evaluated the affects of two packaging types (HSOS and NFOS) and four ambient storage times (24, 48, and 72 h, and 14 d) on reduction of *L. monocytogenes* on five commercial RTE meats and poultry snacks (beef tenders, beef jerky, beef sausage sticks, pork jerky, and turkey sausage sticks). A mean reduction of >1.0 log CFU/cm^2 of *L. monocytogenes* was achieved on all products, regardless of packaging or storage time. Correlation analysis provided some indication that reduction of *L. monocytogenes* increased with fat content. However, the strength of linear correlation was not sufficient to account for the differences in log reduction in *L. monocytogenes*. In study 1, a holding time of 24, 48, or 72 h for HSOS or NFOS packaging of was not effective for reducing *L. monocytogenes* by at least 1 log on turkey jerky. In contrast, packaging beef tenders, beef jerky, beef sausage sticks, pork jerky, and turkey sausage sticks in HSOS or NFOS for at least 24

h ambient storage was sufficient to achieve at least 1 log reduction in *L. monocytogenes* population. Specific components such as sulfur-containing amino acids in turkey jerky might be contributing to <1 log reduction of *L. monocytogenes* population on turkey jerky after 24, 48, or 72 h of ambient storage. Overall, nitrite was not an effective ingredient to control *L. monocytogenes* in turkey jerky. However, packaging such as HS, HSOS, NFOS or VAC and at least 24 h holding time were effective hurdles for controlling *L. monocytogenes* at post-lethality.

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Chapter 1 - Introduction

Listeria monocytogenes is a gram positive bacterium that occurs ubiquitously in nature. It is a multi-systemic and invasive intracellular pathogen responsible for listeriosis (Kuhn and others 2008; McLaughlin and others 2011). It was recognized as a foodborne pathogen in humans more than 30 years ago. It has been noted that the majority (99%) of human listeriosis cases in the U.S. are foodborne (Swaminathan and others 2007; Scallan and others 2011b). *Listeria monocytogenes* is an opportunistic pathogen that often affects those individuals with impaired immune systems, pregnant women, unborn or newly delivered infants, and the elderly. Unlike other foodborne illnesses such as those caused by *Campylobacter jejuni* or *Salmonella* spp., listeriosis has a low incidence rate of 0.25%; however, the hospitalization and case-fatality rates are high among immune-compromised individuals (Scallan and others 2011b; CDC 2013b). In 2000-2008, listeriosis caused an estimated 1,662 illnesses annually in the U.S., with 94% and 16% hospitalization and case-fatality rates, respectively (Scallan and others 2011b; CDC 2013a). Scallan and others (2011b) reported that among the 31 foodborne pathogens, *L. monocytogenes* is the third leading cause of death from food poisoning in the U.S. in 2000-2008. An estimated 1,651 invasive listeriosis cases with 292 deaths or fetal losses were reported in 2009-20011 by the active surveillance network of the CDC, FoodNet (CDC 2013b). In European countries, about 1,476 confirmed cases of human listeriosis and 134 deaths were reported in 2011 (EFSA and ECDC 2013). In 2012, *L. monocytogenes* exhibit an incidence rate of 0.25% per 100,000 people in the U.S., which is 0.05% more than the Healthy people 2020 objective which has a target of 0.20% per 100,000 people for *L. monocytogenes* (CDC 2013a).

The ubiquitous and pervasive nature of *L. monocytogenes* enables this organism to be introduced into food processing facilities. Post-processing contamination is a major food safety concern, especially for ready-to-eat (RTE) (Appendix C) meat and poultry products that are exposed to post-lethality environments (Appendix C) such as slicing and packaging. Previous reports showed that *L. monocytogenes* contamination from the post-processing environment was the principal source of *L. monocytogenes* contamination of RTE meat and poultry products (Tompkin 2002; Kornacki and Gurtler 2007). Ready-to-eat meat and poultry products have been previously linked to major listeriosis outbreaks in 1998, 2000, and 2002 (CDC 1998; CDC 1999;

CDC 2000; CDC 2002). As a result, federal regulators issued stringent rules, called the *Listeria* Rule, designed to prevent *L. monocytogenes* contamination of RTE meat and poultry products (USDA FSIS 1999; USDA FSIS 2003; USDA FSIS 2012).

Under the *Listeria* Rule, the U.S. Department of Agriculture Food Safety and Inspection Service (USDA FSIS) has a "zero tolerance" policy for *L. monocytogenes* in post-lethality exposed RTE meat and poultry products (USDA FSIS 1999; USDA FSIS 2003; USDA FSIS 2012). The "zero tolerance" policy has been in existence since 1987, after *L. monocytogenes* emerged as a foodborne human pathogen in the 1980's (Schlech and others 1983; Fleming and others 1985; Linnan and others 1988). This policy means that detection of *L. monocytogenes* in an RTE meat and poultry product or on a food-contact surface renders the product adulterated under the Federal Meat Inspection Act or the Poultry Products Inspection Act [21 USC 453 (g) or 601 (m)]. This policy suggests that no detectable level of *L. monocytogenes* is permitted on any RTE meat and poultry product.

In addition, the *Listeria* Rule states that *L. monocytogenes* is a hazard in post-lethality exposed products and must be controlled by establishments. In 2003, the *Listeria* Rule established three alternatives that establishments can use to control *L. monocytogenes* contamination of post-lethality exposed RTE products (USDA FSIS 2003; USDA FSIS 2012). An establishment under alternative 1 must use a post-lethality treatment (PLT) to reduce or eliminate *L. monocytogenes* and an antimicrobial agent or process (AMA or AMP) to suppress or limit the growth of the pathogen. Under alternative 2, an establishment must use either a PLT or AMA or AMP. Establishments using alternative 1 and 2 are required to validate the effectiveness of the PLT and AMA or AMP for eliminating or reducing and suppressing *L. monocytogenes*, respectively. This validation should show that the PLT and AMA or AMP demonstrate at least a 1-log decrease in *L. monocytogenes* (before product distribution) and that no more than 2-logs of *L. monocytogenes* growth occurs throughout product shelf life, respectively.

To date, RTE meat and poultry snacks such as jerky and sausage sticks have not been linked to foodborne human listeriosis outbreaks; however, these products were subjects for recalls due to *L. monocytogenes* detected through routine sampling by processors or regulators (USDA FSIS 1998; USDA FSIS 1999; USDA FSIS 2000; SPCNETWORK 2000; Just-food.com 2002; USDA FSIS 2004; USDA FSIS 2005b; Parker Waichman LLP 2008). Controlling *L. monocytogenes* in these types of products is significant because of the medical and production

losses associated with *L. monocytogenes*. Ivanek and others (2004) had estimated a total of $2.3 billion cost of medical treatment and production losses associated with *L. monocytogenes*. Scharff (2012) and Hoffmann and others (2012) had estimated about $2-2.5 million cost of listeriosis in 2009 and 2010.

Since *L. monocytogenes* emerged as an important foodborne human pathogen in the 1980's (Schlech and others 1983; Fleming and others 1985; Linnan and others 1988), several studies were conducted in order to explore strategies for controlling *L. monocytogenes* on RTE meat and poultry. Previous studies have investigated the antilisterial activity of antimicrobials used as formulation ingredients (Schlyter and others 1993a; 1993b; Wederquist and others 1994; Blom and others 1997; Bedie and others 2001; Islam and others 2002; Mbandi and Shelef 2001; Samelis and others 2002; Porto and others 2002; Stekelenburg 2003; Choi and Chin 2003; Barmpalia and others 2004; 2005; Glass and others 2007) or as spraying or dipping solutions (Schlyter and others 1993a; 1993b; Palumbo and Williams 1994; Ariyapitipun and others 2000; Samelis and others 2001a; Glass and others 2002; Barmpalia and others 2004; Uhart and others 2004; Geornaras and others 2005; Lu and others 2005; Luchansky and others 2006). Other practical alternatives that rely on water activity (a_w), packaging, and ambient storage times in controlling *L. monocytogenes* were found to inhibit and reduce *L. monocytogenes* populations on RTE meat and poultry products (Ingham and others 2004; Ingham and others 2006a; Ingham and others 2006b; Lobaton-Sulabo and others 2011; Uppal and others 2012). Although results of these studies may be valuable to RTE meat processors in identifying antilisterial interventions for their products, more research is needed to evaluate other ingredients or product chemical properties that could enhance the efficacy of antilisterial interventions. This would give processors more alternatives to improve the margin of safety for *L. monocytogenes* control. For instance, addition of at least 30 ppm nitrite in a meat formulation could enhance the antilisterial activity of lactate-diacetate in restructured sliced turkey product (Glass and others 2008). Models developed to predict *L. monocytogenes* growth have shown improved effectiveness of antimicrobials like sorbic acid, sodium lactate and sodium diacetate in the presence of nitrite (Sofos and others 1979b; Schlyter and others 1993b; Duffy and others 1994; Buchanan and others 1997; McClure and others 1997; Gill and Holley 2003; Legan and others 2004). In addition, there was conflicting evidence that product characteristics such as fat content decreased

or enhanced resistance of *L. monocytogenes* in RTE meat model systems (Hu and Shelef 1996; Barmpalia-Davis and others 2009; Hack-Youn 2012; Perumalla and others 2013).

The capacity of *L. monocytogenes* to attach and colonize on biotic and abiotic surfaces (Mafu and others 1990; Hood and Zottola 1997; Smoot 1998; Chae and others 2001; Kalmokoff and others 2001; Silva and others 2008; Caly and others 2009) could explain its persistence in diverse and hostile environments. It was confirmed by several studies that association of *L. monocytogenes* to these surfaces was governed by complex processes that involved both physiochemical properties of bacterial cell and solid surfaces, substrate composition (Chavant and others 2002; Cunliffe and others 1999; Dickson Koomarie and others 1989; Mafu and others 1991; Smoot 1998), and chemotaxis (Galsworthy and others 1990; Katsikogianni and Missirlis 2004). Results from these studies were of great value especially in understanding that in the absence of antimicrobial intervention, *L. monocytogenes* attachment on RTE meat and poultry products could pose a food safety risk. This is due to the fact that once *L. monocytogenes* is immobilized on the surface, interactions with its environment, as affected by pH, a_w, temperature, salt content, concentration of salts of organic acids, package atmospheres, and storage times could influence its survival and growth (Glass and others 2002; Ingham and others 2004; Membré and others 2004; Ingham and others 2006b; Glass and others 2007; Zuliani and others 2007; Lobaton-Sulabo and others 2011; Uppal and others 2012).

Studying substrate or substratum composition in attachment studies may be valuable in studying survival of *L. monocytogenes* since certain elements such as Na, Cl, Fe, Mg, and Ca present in the attachment substrates can influence bacterial growth and stress response, as well as surface chemistry and reactivity (Barnes and others 1999; Jensen and others 2007; Andrews and others 2003; Kerchove and Elimelech 2008; McLaughlin and others 2011). For instance, Fe is known to be a cofactor in numerous cellular enzymes and its uptake by *L. monocytogenes* supports growth and survival in many diverse environments (McLaughlin and others 2011).

Thus, this research aimed the following: (1) determine the effect of different packaging treatments and storage times in the presence or absence of sodium nitrite in a turkey jerky formulation on *L. monocytogenes* reduction; (2) characterize *L. monocytogenes* attachment onto turkey jerky surfaces under different packaging treatments and times of storage using SEM and EDS analysis; and lastly, (3) determine the effect of packaging systems and storage times on *L. monocytogenes* reduction on five commercial ready-to-eat meat and poultry snacks.

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Chapter 2 - Literature Review

History and Biological Characteristics of *Listeria monocytogenes*

History

Listeria monocytogenes has been recognized as an animal pathogen for 90 years. It was first observed and described (named *Bacterium monocytogenes*) in 1926 in Cambridge, United Kingdom, as a cause of monocytosis in laboratory rodents (Murray and others 1926). The same bacterium was observed by Pirie (1927) to infect wild gerbils in South Africa, and he proposed the name *Listerella* for the bacterium in honor of the surgeon Lord Lister. The name *Listerella monocytogenes* was coined by Murray and Pirie after realizing that they were dealing with the same bacterium, which later changed to *Listeria monocytogenes* due to taxonomic reasons (Pirie 1940; Bell and Kyriakides 2005).

The discovery, isolation and description of *L. monocytogenes*, as well as its recognition as a human pathogen have been documented since the mid 1920's (Murray and others 1926; Pirie 1927; Nyfeldt 1929; Pirie 1940; Seeliger 1961; Murray 1963; Gray and Killinger 1966). It was not until the last two to four decades that it was considered a significant foodborne pathogen and received much attention from the food industry. During these periods, concerns have been focused on the role of food in the transmission of human listeriosis and on *L. monocytogenes* as a cause of febrile gastroenteritis (Bell and Kyriakides 2005).

Morphology and nutrition

Listeria monocytogenes is a short (0.4-0.5 x 0.5-2.0 μm), gram-positive, non-spore-forming rod that can grow in the presence of oxygen, but is capable of switching to fermentation in the absence of oxygen (Rocourt and Cossart 1997; Farber and Peterkin 2000; Bell and Kyriakides 2005). Wagner and McLauchlin (2008) described that *L. monocytogenes* usually occurs as a single cell or in short chains with parallel sides and blunt ends. In addition, virulent filaments of 6 μm in length may be observed in older or rough cultures (Wagner and McLauchlin 2008). Glucose and glutamine are required as primary sources of carbon and nitrogen, respectively, in a chemically defined media (Premaratne and others 1991). Thus, *L. monocytogenes* favors food as a vehicle of transmission. It reaches stationary phase after 12 h in

brain heart infusion (BHI) at 30°C and motility can be observed for cultures maintained at 20 and 25°C, but is non-motile at 37°C (Wagner and McLauchlin 2008).

In five separate experiments, none of the minimal media tested was able to support growth all *L. monocytogenes* strains such as, 10403, EGD-e, LO28, NCTC 7973, NCTC 5214-m; 4155, LM; C-286, C-294, Scott A, V7, CA, OH, ATCC 19115, and 28 strains isolated from dairy plants (Friedman and Roessler 1961; Welshimer 1963; Premaratne and others 1991; Phan-Thanh and Gormon 1997; Tsai and Hodgson 2003). Lungu and others (2009) suggested that differences in the nutritional requirements for *L. monocytogenes* growth reported from these experiments could be attributed to genetic and ecological diversity, as well as the initial physiological state of the bacterium. Premaratne and others (1991) reported that *L. monocytogenes* Scott A required glucose, glutamine, leucine, isoleucine, arginine, methionine, valine, cysteine, riboflavin, biotin, thiamine, and thioctic acid for growth; while fructose, mannose, glucosamine, *N*-acetylglucosamine and *N*-acetylmuramic acid supported growth in the absence of glucose. Tsai and Hodgson (2003) reported that only cysteine and methionine were essential amino acids for *L. monocytogenes* 10403. They found that *L. monocytogenes* 10403, in the absence of glutamine, could use inorganic nitrogen sources, such as ammonium salts, in contrast to the strains used by Premaratne and others (1991). The requirements for cysteine, valine, leucine, and isoleucine were also observed by Siddiqi and Khan (1989). They also found that phenylalanine was stimulatory for all six *L. monocytogenes* strains evaluated (NCTC 7973, NCTC 5214-m, 4155, LM, C-286, and C-294), while tryptophan was only required by NCTC 5214-m, 4155, and C-294. In addition, none of the strains required asparagine, glutamine, proline, histidine, and tyrosine as essential growth factors. In an earlier study, Friedman and Roessler (1961) reported that valine, leucine, isoleucine, and cysteine were absolute requirements for growth of *L. monocytogenes* A4413. Based on four experiments (Friedman and Roessler 1961; Siddiqi and Khan 1989; Paramatne and others 1991; Tsai and Hodgson 2003), it was evident that cysteine was an absolute requirement for *L. monocytogenes* growth. This supported the conclusion of Slaghuis and others (2007) who stated that extracellular replicating *L. monocytogenes* will depend on cysteine or methionine (in the absence of cysteine) supply from the host due to its inability to use oxidized sulfur and nitrogen sources.

Harper and others (2011) reported that addition of NaCl, KCl, $CaCl_2$, and $MgCl_2$, or replacement salt at a 2% level in ground turkey formulation did not decrease ($P>0.05$) growth of

L. monocytogenes during 5 d storage at 4°C. In contrast, a 0.41 log decrease (*P*<0.05) in *L. monocytogenes* populations was observed in ground beef formulated with the same salt types used at a 2% level with ground turkey. Difference in growth behavior of *L. monocytogenes* in beef could be due to its sulfur-containing amino acid components. Brandsch and others (2006) reported that pork and turkey meat fillets contained higher levels of the amino acid cysteine with 1.7 g/kg (dry matter), while beef fillet contained 1.5 g/kg cysteine. In addition, turkey fillet exhibit higher methionine content of 5.1 g/kg than pork and beef fillets, with 4.7 and 4.8 g/kg, respectively.

Attachment and biofilm formation

Generally, bacterial cells attach to solid surfaces, colonize, and form protective substances, such as extra-polymeric substances (EPS) in order to survive in hostile environments. This protected mode of growth characterizes biofilm formation, which Marshall and others (1971) first described more than 40 years ago. Benefits of biofilms include nutrient entrapment (Scott and Zottola, 1997) and protection for bacterial cells from ultraviolet exposure (Espeland and others 2001), metal toxicity (Teitzel and Parsek 2003), acid exposure (McNeill and Hamilton 2003), dehydration and salinity (Le Magrex-Debar 2000), phagocytosis (Leid and others 2002), and several antibiotics and antimicrobial agents (Mah and O'tootle, 2001; Stewart and Costerton, 2001; Gilbert and others 2002). Marshall and others (1971) described essential steps of biofilm formation that involves reversible attachment of bacterial cells on the solid surface held by weak electrostatic forces as the first step. Lastly, cells attach on the surface irreversibly through time as the bacterial cells produce EPS as observed by Zottola (1991). Zottola (1994) and Cunliffe and others (1999) described biofilm formation in detail and divided it into five stages: nutrient transport, reversible adsorption step, primary adhesion or attachment of bacterial cells to surface, bacterial metabolism and colonization, and detachment.

Attachment is an essential step in bacterial colonization in food since following this step is subsequent growth and biofilm formation. Duffy and Sheridan (1997) reported that the optimum media conditions for *L. monocytogenes* attachment were growth at 30°C and pH 4.76. After attachment, *L. monocytogenes* has the ability to proliferate in cold wet environments, ideal for biofilm formation (Chmielewski and Frank 2003). It can form biofilms in pure culture and can survive and grow in the presence of multispecies (Charlton and others 1990; Mafu and others

1990; Blackman and Frank 1996; Chae and Schraff 2001). It forms biofilms on stainless steel, plastic, and polycarbonate surfaces, in addition to many other food-contact surfaces (Frank and Koffi 1990; Mafu and others 1990; Nelson 1990; Helke and others 1993; Dhir and Dodd 1995; Jeong and Frank 1994). Generally, bacteria in biofilms are more resistant to cleaning and disinfection than bacteria in plaktonic state, and biofilms may act as a reservoir for *L. monocytogenes* contamination in food processing facilities.

Previous studies showed that initial attachment of *L. monocytogenes* to solid and meat surfaces was influenced by several factors such as hydrophobicity, hydrophilicity, strain, flagella, initial cell numbers, adhesion medium, and surface types (Chung and others 1989; Briandet and others 1999; Cunliffe and others 1999; Norwood and Gilmour 1999; Vatanyoopaisarn and others 2000; Kalmokoff and others 2001; Jensen and others 2007). Earlier studies showed that attachment of *L. monocytogenes* to stainless steel was influenced by strain differences that showed serotype 1/2c exhibited the highest attachment followed by serotype 4b, then 1/2a (Chung and others 1989). On the contrary, in an experiment by Foong and Dickson (2004), attachment of *L. monocytogenes* on the basis of S_R values—which is defined as the ratio of strongly attached sessile *L. monocytogenes* to the total *L. monocytogenes* cells (sessile and planktonic cells)— was not affected ($P>0.05$) by strain differences and RTE meat types. The RTE meat types included frankfurters, sliced bologna, sliced chopped ham, and sliced deli-style roast beef. A contact time of 20 min was required for *L. monocytogenes* to attach to test surfaces at 20°C, while at least 1 h contact time was required for attachment to test surfaces at 4°C (Mafu and others 1990). Foong and Dickson (2004) found that *L. monocytogenes* had an even shorter (5 min) contact time at room temperature for attachment to RTE meat products. This suggests that as ambient temperature increases, attachment time for *L. monocytogenes* decreases.

The capacity of *L. monocytogenes* attachment to stainless steel at different temperatures could be attributed to flagella expression of the organism at specific temperatures. Vatanyoopaisarn and others (2000) found that wild-type strains of *L. monocytogenes* attached 10-fold higher than flagellin-mutant *L. monocytogenes* strains at 22°C, while both strains showed similar attachment capacity at 37°C, where flagella in both strains were not present. Results of this study suggest that flagella act as an adhesive structure during early stages of attachment under static conditions (Vatanyoopaisarn and others 2000). Based on the studies reviewed, attachment of *L. monocytogenes* to solid and meat surfaces is governed by a complex interaction

of several factors, including temperature, flagella, initial bacterial cell numbers, strain, surface type of substrates, adhesion medium, hydrophobicity, and hydrophilicity.

Factors affecting growth

Temperature

   Listeria monocytogenes is a versatile organism that has common characteristics of both psychrotrophic and mesophilic bacteria. In general, temperatures below 0°C are bacteriostatic or moderately prevent *L. monocytogenes* growth (Ryser and Marth 1999). However, given a suitable medium*, L. monocytogenes* can grow in a wide range of temperatures (-0.15-45°C) (Liu and others, 2005), with optimum growth at 38°C (Wilkins and others 1972) and slower growth occurring at lower temperatures. Rosenow and Marth (1987) reported a generation time ranging from 1-2 d for *L. monocytogenes* in dairy products stored at 4°C. This range of generation time for *L. monocytogenes* agreed with the values reported by Hudson and Mott (1993) who evaluated growth of *L. monocytogenes* in vacuum packaged beef stored at 4°C. When stored at -1.5°C, Hudson and Mott (1993) found the generation time of *L. monocytogenes* in vacuum packaged beef to be 100 h. Barbosa and others (1994) reported that the average generation time for 39 *L. monocytogenes* strains in tryptic soy broth with 0.6% yeast extract was 43, 6.6, and 1.1 h at 4, 10, and 37°C, respectively.

   Thermal inactivation of *L. monocytogenes* was affected by several factors. First, strain variation under similar experimental conditions demonstrated variable thermal resistance. Golden and others (1988) reported that D values in tryptose phosphate broth at 56°C of *L. monocytogenes* strains Brie-1 (from Brie cheese), LCDC 81-861 (from cabbage implicated in a Canadian outbreak), and DA-3 (human isolated from a 1983 milk outbreak), and DA-3 (human isolate) were, 16.0, 10.4, 7.4, and 5.7 min, respectively. In addition, cells at stationary phase seemed to be more heat resistant to thermal stress compared to cells in log phase. At 60°C, the D-value for *L. monocytogenes* strain 13-249 in stationary phase was four times higher than in log phase (2.22 min compared to 0.6 min) (Jørgensen and others 1999). At 56°C, the D-value for strain Sott A in stationary phase was eight times higher than log phase cells (8.6 min compared to 1.0 min) (Lou and Yousef 1996). Presence of food or media component can protect thermal damage by stabilizing membranes or by stimulating stress response. Presence of 1.5 M salt in the

suspending medium caused elongation in *L. monocytogenes* cell structure and had increased its thermal resistance compared to cells suspended in 0.09 M. Growth in tryptic phosphate broth containing 0.09, 0.5, 1.0, or 1.5 M NaCl followed by testing in media with the same salt concentration resulted in D-values of 1.6, 2.5, 7.4, and 38.1 min, respectively, at 60°C (Jørgensen and others 1995). *Listeria monocytogenes* in food is more heat resistant compared to when it is suspended to laboratory media (Boyle and others 1990; Casadei and others 1998; Jørgensen and others 1999). For example, at 60 and 65°C, D-values of *L. monocytogenes* in 20% ground beef slurry was 3-4 times higher compared to *L. monocytogenes* in phosphate buffer (Boyle and others 1990). In addition, fat content increased thermal resistance of *L. monocytogenes*. For instance, Fain and others (1991) reported that at 57.2 and 62.8°C, D-values for *L. monocytogenes* in ground beef with 30.5% fat were two times higher (5.8 and 1.2 min, respectively) compared to D-values of *L. monocytogenes* in ground beef with 2% fat (2.6 and 0.6 min, respectively). Thus, thermal inactivation of *L. monocytogenes* is dependent on the strain, age of microorganism, and growth media.

pH

    Listeria monocytogenes is a neutrophile, capable of growing at a minimum, optimum, and maximum pH of 4.4, 7.0, and 9.4, respectively (Hill and others 1995; ICMSF 1996; Abee and Wouters 1999). According to the Bergey's Manual of Systematic Bacteriology (Seeliger and Jones 1986), *L. monocytogenes* can grow at pH 5.6-9.6 with optimal growth at neutral to slightly alkaline conditions in laboratory media. In a high acid environment, it was observed to grow at pH 4.4 in HCl acidified nutrient medium at 20 and 30°C (George and others 1988). Its growth in a high acid environment is attributed to the incubation temperature and the type of acid. Farber and others (1989) evaluated the effect of different acidulants on the survival of *L. monocytogenes* in brain heart infusion (BHI) broth. They observed that BHI broth acidified by acetic acid demonstrated an inhibitory effect on *L. monocytogenes* growth at pH<5.6 and pH<5.0, incubated at 4 and 30°C, respectively. These values fall within the pH range where a larger proportion of acetic acid would be in an undissociated form, which is the form where the acid exhibits its antimicrobial action (ICMSF 1980). On the other hand, BHI acidified with HCl demonstrated inhibitory activity at pH 3.0 and pH 5.0 at 30 and 5°C, respectively. Furthermore, Swaminathan and others (2007) noted that, experimentally, inhibition of *L. monocytogenes* growth in tryptose

broth increased as incubation temperature decreased in the presence of acetic, citric, and lactic acids up to a concentration level of 0.1%. At the same pH, the antimicrobial activity of these acids against *L. monocytogenes* was related to their degree of dissociation, with citric and lactic acid being less detrimental for the pathogen (Swaminathan and others 2007). In meat products, with pH near or above 6.0 the organism grows well, but at pH below 5.0, it grows at a slower rate or not at all (Glass and Doyle 1989).

Water activity

The water requirement for microbial growth can be expressed in terms of water activity (a_w). From a microbiology standpoint, it is defined as the amount of water available for microbial growth and survival (Jay and others 2005; Farkas 2007). It is expressed as the ratio of water vapor pressure of a food substrate to the vapor pressure of pure water at constant temperature. It characterizes the intensity to which the water is "bound" in food and affects its availability to act as a solvent, to participate in chemical/biochemical reactions and growth of microorganisms (Fontana 1998). It is considered as an important factor for the microbiological stability of food. The application of this concept to food preservation is a strategy used for producing microbiologically stable and safe food by controlling spoilage and pathogenic bacteria. Under normal conditions, if the a_w of food is less than the minimum a_w requirement of a specific microorganism, the growth of that microorganism is inhibited (Fontana 1998). Foods that have an $a_w \leq 0.85$ are considered to be shelf stable and do not require refrigeration. The a_w of a food product is lowered by drying or addition of solutes, such as NaCl and sucrose. Troller (1989) suggested that the antimicrobial effect of lowering a_w extends the lag phase or suppresses the logarithmic phase, consequently decreasing the total number of viable microorganisms at its growth stage. Ingham and others (2006b) reported that drying beef jerky to an a_w of ≤ 0.87 will ensure that bacterial pathogens, including *L. monocytogenes*, cannot grow in vacuum-packaged product stored at 21°C.

The optimum a_w requirement for *L. monocytogenes* growth is approximately >0.97 (Petran and Zottola 1989; ICMSF 1996; Bell and Kyriakides 2005), with the minimum a_w of 0.90 for some strains, and 0.93 for most strains (Swaminathan and others 2007). The latter reported minimum a_w value supports the findings of Vermeulen and others (2007) that most *L. monocytogenes* strains were not able to grow at $a_w < 0.93$. However, according to ICMSF (1996) and Fontana (2008), the minimum a_w of the bacterium is 0.92. This is the value that the USDA

FSIS compliance guidelines has been using as a growth limit for *L. monocytogenes* in post-lethality exposed RTE meats and poultry products (USDA FSIS 2012). This value is supported by earlier reports that *L. monocytogenes* growth was inhibited at a_w<0.92 in tryptic soy broth (TSB) with varying levels of carbohydrates (Petran and Zottola 1989). Growth of *L. monocytogenes* in solute-adjusted complex laboratory media, such as TSB and BHI, can reach up to $\geq$ 6.5 log CFU/mL at minimum a_w of 0.90, 0.92, 0.92, and 0.97 in the presence of glycerol (30% w/w), sucrose (39.4% w/w), NaCl (11.5% w/w), and propylene glycol (16.7% w/w), respectively (Miller 1992; Nolan and others 1992). It was also observed that lag phase and generation time increases as a_w of the medium decreases (Miller 1992; Nolan and others 1992).

A group of scientists (Shahamat and others, 1980) found that *L. monocytogenes* survived at an a_w of approximately 0.83 in 25.5% NaCl adjusted TSB for at least 132 days at 4°C. It also demonstrated a similar survival behavior in an RTE meat system (Johnson and others 1988; Lado and Yousef 2007). It survived in fermented dry salami with an a_w of 0.79-0.86 for at least 84 days at 4°C in the presence of 156 ppm $NaNO_2$, 5-7.8% NaCl, and pH of 4.3-4.5 (Johnson and others 1988). Growth of *L. monocytogenes* can be inhibited in reduced a_w conditions, but growth can rapidly resume when a_w increases (Lado and Yousef 2007).

Resistance of *L. monocytogenes* may increase in a reduced a_w environment. An inverse relationship exists between the thermal resistance of *L. monocytogenes* and the a_w of sucrose/phosphate buffer solution in which it was suspended (Sumner and others 1991). The bacterium demonstrated four times more heat resistance in buffer with 0.90 a_w than in buffer with 0.98 a_w (Sumner and others 1991).

Fat

Fat content of meat products may or may not influence lethality and survival of *L. monocytogenes*. One study showed that *L. monocytogenes* growth was not affected when the fat content of cheddar cheese was reduced from 48% to 36% (fat-in-dry matter) at 7°C (Mehta and Tatini 1994). Hu and Shelef (1996) investigated the influence of fat content (22%, 37%, 52%, and 67% by weight) and preservatives (sodium lactate, sorbic acid, potassium sorbate and sodium propionate) on the growth behavior of *L. monocytogenes* in pork liver sausage batter stored up to 50 and 14 d at 4°C and 10°C, respectively. Results showed that increasing fat content of liver sausage batter from 22 to 67% resulted in reductions of <1.5 log CFU/g in *L. monocytogenes* populations by the end of each storage period (7.1 to 6.5 CFU after 50 d at 4°C;

9.9 to 9.36 CFU/g after 14 d at 10°C). In addition, efficacy of preservatives increased with increasing fat content.

A group of scientists (Barmpalia-Davis and others 2009) evaluated the potential gastrointestinal survival of *L. monocytogenes* on beef frankfurters with 4.5 and 32.5% fat content. In this study, *L. monocytogenes* inoculated beef frankfurters were exposed to a simulated gastrointestinal system (gastric fluid and intestinal fluid) at 37°C and were stored at 7°C for up to 55 d (Barmpalia-Davis and others 2009). *Listeria monocytogenes* populations were determined after 1, 6, 20, 39, and 55 d of storage. Results showed that *L. monocytogenes* populations on 4.5% and 32.5% fat frankfurters exceeded 8.0 log CFU/g after 39 and 55 d of storage, respectively. This suggests that fat content delayed the growth of *L. monocytogenes* by 16 d.

A recent study showed that the level of fat in hotdogs had variable effects depending on meat type and whether a post-packaging heat treatment was applied, regardless of the type of antimicrobial agent (2% potassium lactate, 0.15% sodium diacetate, and 25% of partial replacement of chemical preservatives by green tea and grape seed extracts) present in the formulation (Perumalla and others 2013). Both non-heated and heated chicken hotdogs containing 20% fat had higher (*P*<0.05) growth than chicken hotdogs formulated with 5% fat. Perumalla and others (2013) noted that overall, fat provides thermo-insulation and a protective effect against heat treatment and salts of organic acids. Furthermore, they also noted that fat increases hydrophobicity in the meat system, limiting the accessibility of organic acid salts to provide antimicrobial effects. On the other hand, non-heated 5% fat turkey hotdogs had higher growth than 20% fat turkey hotdogs. Regardless of the fat content, no survivors were found on turkey hotdogs subjected to post-packaging heat treatment (Perumalla and others 2013).

Salt

Salt (NaCl) is an important food preservation ingredient due to its antimicrobial properties. At high concentrations, salt demonstrated antilisterial capacity due to its dehydrating effect lowering the a_w of food (Petran and Zottola 1989) and electrochemical potential across the cell membrane (Patchett and others 1994). The dehydrating effect of salt has been equated to its bacteriostatic properties. Conversely, other researchers believe that the antimicrobial effect of salt is not due to its dehydrating effect, but is due to its capacity to interfere with substrate

utilization to stop cellular functions (Woods 1982; Erecinska and Deutsch 1985; Smith and others 1987; Csonka 1989).

Listeria monocytogenes is halotolerant capable of surviving at high salt concentrations. Jay (2005) reported that growth of *L. monocytogenes* is inhibited at 10% NaCl. However, there are strains that have been observed to grow in 10% NaCl at 35°C and 12% NaCl at 10 and 25°C in tryptic soy broth (Sorrels and Enigl 1990). Lado and Yousef (2007) reported that growth of *Listeria* ceases in the presence of >12% NaCl. Hudson (1992) reported that *L. monocytogenes* growth was not affected in the presence of 16.5% NaCl in BHI broth for at least 33 days at $\leq$4°C. In the same study, presence of 26.5% NaCl decreased *Listeria monocytogenes* in the same medium by 2 and 4 logs at 0-4 and 10°C, respectively, for a similar storage period. Incubation in the presence of 14% NaCl for 36 days at 10 and 25°C decreased *L. monocytogenes* populations by approximately 2 and $\geq$6 logs, respectively (Sorrels and Enigl, 1990). Based on these findings, the presence of extremely high salt condition (>12%) is not a reliable method to eliminate *L. monocytogenes*. In addition, *L. monocytogenes* was observed to survive in a commercial cheese brine with 23.8% NaCl and pH 4.9 stored for 259 days at 4°C (Larson and others 1999). The ability of *L. monocytogenes* to tolerate and survive in an extremely high salt environment can be attributed to its ability to utilize compatible solutes and osmoregulation of stress proteins for osmoadaptation (Stack and others 2008).

Nitrite

Functions and chemistry

Nitrite (NO_2^-) is a multifunctional important curing ingredient used in cured meats. It is used to achieve a desirable bright reddish pink color in cured meats, while producing meaty cured flavor, controlling oxidation of lipids, and serving as an effective antimicrobial by itself or synergistically with other ingredients (Sebranek and Fox 1985; Townsend and Olson 1987; Pegg 2004; Sebranek 2009). Nitrite can be directly added to meat or derived from nitrate through bacterial enzymatic or chemical reduction.

Nitrite chemistry for cured color development in meat is a very complex process that involves several different reactions influenced by environmental factors such as pH and temperature. Reactions involving nitrite are accomplished via oxidation, reduction, and nitrosylation, resulting in conversion of nitrite to nitrous acid and nitric oxide (Sebranek and

Bacus 2007). The production of nitric oxide (NO) from nitrite is a significant step for meat curing because NO will subsequently react with myoglobin to produce the final red/pink color meat pigment (Sebranek 2009). The nitrite conversion to NO involves series of intermediate reactions resulting in production of highly reactive compounds, such as nitrous acid (HNO_2) and dinitrogen tri-oxide (N_2O_3). The HNO_2 is formed from nitrite under acidic conditions (Reaction 1) (Fox and Thomson, 1963; Pegg and Shahidi, 2000). Aberle and others (2001) noted that <1% of nitrite is present as HNO_2 in fresh meat with pH 5.5-5.6 while the rest is present as dinitrogen trioxide (N_2O_3). Scientists believe that N_2O_3 is formed from dissociation of nitrous acid (Reaction 2) and will subsequently be reduced to NO (Reaction 3) by endogenous or by other ingredients in a meat mixture (Fox and Thompson 1963; Aberle and others 2001; Honikel 2004).

Reaction 1: $NO_2^- + H^+ \rightarrow HNO_2$

Reaction 2: $2\ HNO_2 \leftrightarrow N_2O_3 + H_2O$

Reaction 3: $N_2O_3 \leftrightarrow NO + NO_2$

One of the likely ingredients used in cured meat products that will serve as a substrate for N_2O_3 is ascorbate (H-ASC) or erythorbate. This ingredient is a reductant and is often used as a cure accelerator. Reaction of H-ASC or erythorbate with N_2O_3 yields NO (Reaction 4) (Fox and Thomson, 1963; Møller and Skibsted, 2002), thus providing another source of nitric oxide in cured meat.

Reaction 4: $N_2O_3 + 2\ H\text{-ASC} \leftrightarrow 2\ dehydro\text{-ASC} + H_2O + 2\ NO$
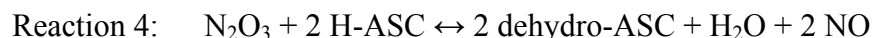
Then, the NO formed will react with the iron of both myoglobin ($MbFe^{+2}$) and metmyoglobin ($MMbFe^{+3}$) to produce cured meat pigments and cured color. There are several reaction pathways by which nitrite can be converted to NO. For example, when nitrite is added to comminuted meat, nitrite acts as a strong heme oxidant and reduces to NO (Reaction 5). As a result, meat turns brown (Sebranek and Bacus 2007).

Reaction 5: $MbFe^{+2}$ (red) $+ NO_2^- \rightarrow MMbFe^{+3}$ (brown) $+ NO + OH^-$
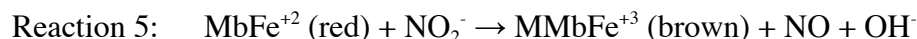
Then, NO reacts with metmyoglobin, and subsequent reduction reactions convert the oxidized heme to reduced nitric oxide myoglobin before processing and will be stabilized upon heating. Heat denatures and separates the protein portion of myoglobin (globin) from the nonprotein heme and contributes to the visual color change to the final pink cooked cured color of the nitrosylhemochrome pigment (Sebranek 2009). The resulting pigment, called the

nitrosylhemochromogen, is responsible for the bright pink color of cured meat (Aberle and others 2001; Sebranek and Bacus 2007).

Reaction 6: $MMbFe^{+3}$ (brown) $+ NO \rightarrow MMbFe^{+3}\text{-}NO \xrightarrow{\text{reductant}} MbFe^{+2}\text{-}NO$ (red) $\xrightarrow{\text{heat}}$

Nitrosylhemochromogen (Pink Fe^{+2})
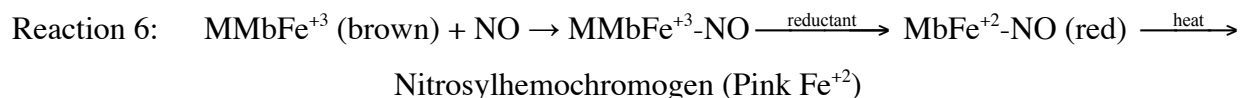
Antimicrobial properties

Sindelar and others (2011) noted that before 2000, it was widely recognized that the main antimicrobial impact of nitrite in cured meats was *Clostridium botulinum* control. Bacterial sporeformers are inhibited by nitrite by preventing outgrowth of germinating spores (Duncan and Foster 1968). There are two antimicrobial effects of nitrite on *C. botulinum* in thermally processed meat products. The first antibotulinal effect of nitrite is the inhibition of vegetative cells emerging from surviving spores (Sindelar and others 2011). The second is prevention of vegetative cell division emerging from surviving spores (Pierson and Smooth, 1982). Residual nitrite of 40-80 ppm in meat products is the minimum level of nitrite necessary to inhibit spores of *C. botulinum* (Aberle and others 2001). Steinke and Foster (1951) were the first scientists to observe the antibotulinal properties of nitrite in cured meats. In the past 60 years, antibotulinal effects of nitrite have been exhaustively studied (Cassens 1995; Cassens 1997; Davidson 1997). The nitrite antimicrobial effect on the outgrowth of spores is dependent on concentration. Inhibition of spore germination can only be inhibited effectively at high nitrite concentrations (Davidson and Taylor 2007). Sofos and others (1979a) noted that only about 25 ppm or less is needed for color development while the main portion of nitrite added to cured meat is used for *C. botulinum* control. However, nitrite level is directly proportional to the growth and toxin production control of *C. botulinum* (Sofos and others 1979a).

It has been suggested that inhibition of *Clostridial* ferrodoxin and/or pyruvate-ferredoxin oxidoreductase is the ultimate mechanism of growth inhibition for clostridia (Carpenter and others 1987; Tompkin 2005). These findings are supported by the fact that excess available iron in perishable canned cured meat reduces the inhibitory effect of nitrite against *C. botulinum* (Tompkin and others 1978).

The mechanism for inhibition for aerobic and non-sporeformer bacteria might be different. Yarbrough and others (1980) suggested that antibacterial effects of nitrite involve three mechanisms. First, nitrite inhibits active transport, oxygen uptake, and oxidative phosphorylation

in aerobic bacteria by oxidizing ferrous iron of an electron transport carrier (e.g., cytochrome oxidase) to ferric iron. These observations are similar with the findings of Rowe and others (1979). Second, nitrite acts as an uncoupler, causing a collapse of the proton gradient also reported by Meijer and others (1979). Thirdly, certain metabolic enzymes such as aldolase are inhibited which was previously observed by O'Leary and Solberg (1976). In addition, Tompkin and others (1978) suggested that nitrite can react with ferredoxin, an essential iron-containing enzyme necessary for energy production in some germinated botulinal cells and prevents outgrowth. Furthermore, Woods and others (1989) suggested that nitrite inhibition of aerobic bacteria is through the binding of nitrite to heme iron of cytochrome oxidase.

Some compounds derived from nitrite, such as HNO_2, can render a bacteriostatic effect. Tarr (1941) showed that the preservative action of nitrite in fish was greatly increased by acidification, suggesting that HNO_2 was the active form. In bacteriological media, the inhibitory action of nitrite on several species of bacteria was shown to increase with decreasing pH, particularly at pH 6.0 and below. This effect was confirmed in other bacteria, including vegetative cells of *C. sporogenes* (Perigo and others 1967) and spores of *C. botulinum* (Christiansen and others 1975).

Antilisterial properties

Overall, antilisterial effects of nitrite is influenced by concentration, pH, presence of NaCl and salts of organic acid, temperature, and a_w. Antilisterial effect increases when nitrite addition is combined with acidic pH, low oxygen level, high salt concentrations, and low temperature (Buchanan and others 1989).

At pH above 6.0, $NaNO_2$ had little effect in delaying visible growth except at higher concentrations and also at lower incubation temperatures (McClure and others 1991). These findings have been supported by several studies. For instance, listeriostatic activity of 30 ppm $NaNO_2$ was not increased in turkey slurries at pH 6.2 even in the presence of sodium diacetate (Schlyter and others 1993). At pH 6.3, 103 ppm $NaNO_2$ did not inhibit *L. monocytogenes* in sausage batter with 3.5% NaCl at 32°C (Glass and Doyle 1989). However, at 156 ppm $NaNO_2$ under similar conditions, growth of *L. monocytogenes* was controlled (Glass and Doyle 1989). Buchanan and others (1989) indicated that $NaNO_2$ was more effective against *L. monocytogenes* when the incubation temperature of the media was lowered to 5°C. In addition, NaCl

concentration also increases the inhibitory effect of $NaNO_2$. Raising the NaCl level from 0.5% to 4.5% also increased the inhibitory effect of $NaNO_2$ against *L. monocytogenes* (Buchanan and others, 1989). Shahamat and others (1980) suggested that at levels permitted in meat products, $NaNO_2$ would have significant inhibitory activity only in refrigerated products containing at least 3% NaCl and having a pH of 5.5 or less. These observations agree with the findings of Johnson and others (1988). They found that *L. monocytogenes* growth at 4°C was suppressed for $\geq$12 weeks in hard salami (pH 4.3-4.5) that contained 5.0-7.8% NaCl and had an ingoing $NaNO_2$ level of 156 ppm. But, the interactive effects of low a_w and low pH were more important than the addition of 156 ppm $NaNO_2$.

The mode of action elicited by nitrite to inhibit *L. monocytogenes* in processed foods is unclear and there are inconsistencies in the published research describing inhibition mechanisms. For instance, a lower concentration of sterilized nitrite (50 ppm) was more effective than 200 ppm nitrite in preventing *L. monocytogenes* growth at pH 5.0 within 48 h at $\leq$20°C (McClure and others 1991). Cammack and others (1999) suggested that the listeriostatic activity of nitrite maybe attributed to reactive species derived from nitrite rather than nitrite itself. Lado and Yousef (2007) suggested that inhibition of *L. monocytogenes* at low pH may be attributed to low production of HNO_2. They also suggested that under aerobic conditions, peroxynitrite is formed upon reaction of nitric oxide with a superoxide or nitrosyl ion with oxygen. Cui and others (1992) reported that the minimum inhibitory concentration of Roussin's black salt ($NH^{4+}[Fe_4S_3(NO_7]-$), an iron-sulfur-nitrosyl complex derived from nitrite, against *L. monocytogenes* was 3 μmol/L (1.66 ppm).

Overall, the ability of $NaNO_2$ to inhibit growth of *L. monocytogenes* in many meat and poultry RTE products depends on its interactions with pH, temperature, NaCl, and other factors. The interactive effects of nitrite with other factors can either enhance or reduce its capacity to control *L. monocytogenes* in RTE products.

Health concerns and regulations

The use of nitrite in food is strictly regulated because of its toxicity when used in excessive amounts. Nitrite poisoning can cause methemoglobinemia which can be fatal by causing abnormal buildups of hemoglobin in the blood (NIH 2011c). A single dose of nitrite in excess of 15 to 20 mg/kg of body weight may cause death (Aberle and others 2001) while earlier literature noted that the lethal dose of nitrite for humans was estimated to be about 1.0 g (Ellenhorn and Barceloux 1988).

The USDA recognized the toxicity of nitrite, thus, secure storage and written records of use are required of meat processors who utilize nitrite for meat curing (Sebranek 2009). In addition, nitrite level in processed meat is also regulated. Section 172.175 of Title 21 of the Code of Federal Register (CFR) states that sodium/potassium nitrite levels cannot exceed 200 ppm in whole muscle products, 156 ppm in comminuted meat products and bacon levels must be below 120 ppm based on the green weight of the meat block (CFR 2011b).

The toxicity of nitrite is only one of the reasons for concern in cured meats. The high reactivity of nitrite is a concern because of the potential formation of carcinogenic nitrosamines in cured meat or in the stomach after ingestion (Sebranek 2009). Nitrosamines can be formed in food products by reactions between NO and secondary or tertiary amines under high temperatures or low pH (Aberle and others 2001). Protein foods always contain secondary and tertiary amines as amino acid side chains of proline, hydroxyproline, histidine, arginine, and tryptophan, thus, formation of nitrosamine in cured meats is possible (Aberle and others 2001). However, clear evidence demonstrated that the use of nitrite for meat curing was not a safety issue (Sebranek 2009). Less than 5% of ingested nitrite is derived from cured meat and it does not pose a risk (Cassens 1997; Archer 2002; Sebranek 2009).

The use of salt/nitrite mixture, "curing blends" (usually 6.25% nitrite), rather than using pure nitrite is a common processing practice in meat industry (Sebranek 2009). In addition, a red or pink coloring agent is used to make the mixture distinct from the pure form of sodium nitrite. This strategy prevents mistaken overuse because of the high salt content (Sebranek 2009).

Listeria monocytogenes **Ecology**

Listeria monocytogenes is widely known to be a ubiquitous organism and capable of surviving over a long period of time in the environment. It can be found in many environments

such as water, soil, sewage, silage, and animal surfaces (Farber and Peterkin 2000; Jay 2000). Its natural habitat is mostly found in soil, water, and on plant material, particularly those that undergo decay (Rocourt and Seeliger 1985). Numerous cases of listeriosis in farm animals have been attributed to *L. monocytogenes* infection from decayed vegetation, such as aerobically spoiled silage (Fenlon 1999). This natural habitat was suspected to be the source of *L. monocytogenes* that enter the food chain. *L. monocytogenes* poses a particular threat to the health and food industry due to its resistance and persistence on food processing equipment. It can survive longer than many other non-spore forming bacteria under adverse environmental conditions (Jay 2000).

The persistence of *L. monocytogenes* in various natural and farm environments from different studies are summarized in Table 2-1. Base on these studies, *L. monocytogenes* can survive over long periods in different environments and conditions. These natural environments can act as reservoirs of *L. monocytogenes*, capable of spreading contamination to animal and plant food products (Rocourt and Seeliger 1985).

Table 2-1. Survival of *Listeria monocytogenes* in various environmental samples.

Sample	Temperature (°C)	Survival (days)
Soil		
Sterile soil (I)[a]	Outside—winter/spring	154
Clay soil (I)	24-26	225
sealed tubes	24-26	67
Fertile soil (I)		
sealed tubes	24-26	295
cotton-plugged tubes	24-26	267
Top soil (I)		
exposed to sunlight	NG[b]	12
not exposed to sunlight	NG	182
Moist soil	NG	~497
Dry soil	NG	>730
Soil	12-Apr	240-311
Soil	18-20	201-271
Fecal material		
Cattle feces (NC)[c]	5	182-2190
Moist horse/sheep feces (I)	Outside	347
Dry horse/sheep feces (I)	Outside	730
Sheep feces	Outside	242
Liquid manure	Summer	36
Liquid manure	Winter	106
Sewage		
Sewage sludge cake (NC)		
Surface	28-32	35
Interior	48-56	49
Sprayed on field	Outside	>56
Water		
Sterilized pond water (I)	Outside	7
Unsterilized pond water (I)	Outside	<7-63
Pond water	35-37	346
Pond water	15-20	299
Pond water/ice	8-Feb	790-928
Pond/river water	37	325
Pond/river water	5-Feb	750
Water	Outside	140-300
Distilled water	4	<9
Animal feed		
Silage (NC)	4	450

Table 2-1. Survival of *Listeria monocytogenes* in various… (continued)

Silage (NC)	5	180-2190
Mixed feed (I)	Outside	188-275
Oats (I)	Outside	150-300
Hay (I)	Outside	145-189
Straw (NC/I)	ca.22	365
Straw (I)	Outside	47-207
Straw	Outside-summer	23
Straw	Outside-summer	135

[a]Inoculated

[b]Not given

[c]Naturally contaminated

Source: Al-Ghazali and Al-Azawi 1988; Amtsberg 1979; Mitscherlich and Marth 1984.

Post-processing Contamination

Previous reports showed that contamination from the post-processing environment was the principal source of *L. monocytogenes* contamination to RTE meat and poultry products (Tompkin 2002; Kornacki and Gurtler 2007). Two separate studies demonstrated that food processing plant environments have shown to be a greater source of *L. monocytogenes* contamination for finished products (Fenlon and others 1996; Lin and others 2006). Fenlon and others (1996) found processing (e.g., cutting, mincing, and grinding) to be a predominant influence on the extent of *Listeria* contamination. They found that 21 out of 23 minced beef samples from a variety of retail outlets were positive for *L. monocytogenes*. Their results suggested that processing significantly increased the level of contamination compared to whole carcasses (Fenlon and others 1996).

There was a correlation observed between contamination of deli meat during slicing with initial load of *L. monocytogenes* on a slicer blade and the type of meat being sliced (Lin and others 2006). The greatest number of *L. monocytogenes*-positive samples was observed in sliced oven-roasted turkey (Lin and others 2006). Although there has been considerable focus in preventing *L. monocytogenes* contamination, it is nearly impossible to completely eliminate the organism from the food processing environment (Tompkin and others 1999; Tompkin 2002). *Listeria monocytogenes* can be found on and in various equipment and sites in food processing environments such as gaskets, conveyor belts, slicing, dicing and packaging machines,

containers, knives, tables, drains, floors, and walls (Nelson 1990; Wenger and others 1990; Klausner and Donnelly 1994; Salvat and others 1995; Destro and others 1996; Holah and Gibson 2000). Therefore, the risk of *L. monocytogenes* contamination in RTE meat and poultry products can come from contamination after heat treatment, owing it to the organism's pervasiveness and persistence in the environment.

The United States Department of Agriculture Food Inspection and Service began testing for *L. monocytogenes* in hot dogs and luncheon meats in 1987 as part of their monitoring program (USDA FSIS 2000). There were eight product categories covered in the monitoring program which included beef jerky, roasted beef/cooked beef/corned beef, sliced ham and luncheon meat, small-and large-diameter sausage, cooked/uncured poultry products, salads and spreads, and dry/semi-dry sausages. Of the 2,547 samples tested for *Listeria* spp. in 1998, about 90 (2.5%) were positive for *L. monocytogenes* (USDA FSIS 2000). Data from the USDA FSIS monitoring program showed that overall prevalence of *L. monocytogenes* in the eight RTE meat and poultry product categories decreased over a 10-year period from 1990-1999 (Levine and others 2001). Overall prevalence in jerky and fermented sausages was 0.52 and 3.25%, respectively, which are relatively lower compared to overall prevalence of *L. monocytogenes* in sliced ham and luncheon meats (5.16%). In 2008, results of the *L. monocytogenes* risk-based sampling study by the USDA FSIS showed that 0.5% (5/959 samples) of the food products, 0.6% (19/3,322 samples) of the contact surface samples, and 2% (35/1,725) of the environmental samples contained *L. monocytogenes* (Mamber 2010). This involved five *L. monocytogenes* positive products from four separate establishments. The five positive products included three chicken products, one beef, and one pork product.

Public Health Impact

Listeriosis

Listeria monocytogenes causes listeriosis in humans. Unlike other foodborne illnesses caused by *Salmonella* spp., *Escherichia coli* O157:H7, and *Campylobacter jejuni*, reported foodborne listeriosis is rare. Only about 3 per 100,000 people each year (0.25% of all foodborne illnesses each year) becomes ill because of listeriosis (CDC 2013b). However, the severe form of the infection in high risk individuals has a high mortality rate. Scallan and others (2011) reported

a case-fatality rate of 15.9% for listeriosis, which made *L. monocytogenes* as the 3rd foodborne pathogen, behind *Vibrio vulnificus* (34.8%) and *Clostridium botulinum* (17.3%), to cause death.

The infective dose of *L. monocytogenes* is not clearly established. It is believed to vary with the strain and host susceptibility (FDA 2012). Jay and others (2005) noted that 100 bacterial cells can cause foodborne listeriosis (Jay and others 2005). On the other hand, the Food and Drug Administration (FDA 2012) indicated that fewer than 1,000 cells may cause disease in susceptible individuals. Swaminathan and others (2001) noted that the infective dose of *L. monocytogenes* in contaminated food was usually more than 100 CFU/g; however, Mead (1999) reported that <0.3 CFU/g of *L. monocytogenes* was present in a food vehicle associated with a listeriosis outbreak in the U.S. in 1998. One of the challenges with epidemiological studies of *L. monocytogenes* outbreaks is that the bacterium has an incubation period of 3-90 days (CDC 2011a; FDA 2012). This means that it could take up to 90 days after ingestion of the bacterium before the patient shows clinical signs of listeriosis, especially the invasive type (NHS 2011). This also suggests that tracing the product linked to the infection is difficult.

Foodborne listeriosis causes two forms of disease in humans, namely, non-invasive and invasive listeriosis (FDA, 2012). The non-invasive type of listeriosis is characterized by gastrointestinal or flu-like symptoms and is considered as the mild type (FDA 2012; NHS 2011). Symptoms of non-invasive listeriosis are loss of appetite (especially in babies), muscle pain, fever, seizures, vomiting, diarrhea, and lethargy (FDA 2012; NIH 2009; NHS 2011). On the other hand, the invasive type is characterized by potentially fatal complications such as bloodstream infection, central nervous system infection, materno-fetal infection, or focal infection (Teodor and others 2012). Severe complications of invasive listeriosis can cause stillbirths or miscarriages in pregnant women, endocarditis, pneumonia, septicemia, meningitis or meningoencephalitis, encephalitis, local purulent lesions, and keratoconjunctivitis (Painter and Gitter 1985; Slutsker 2007; NIH 2009).

The risk of listeriosis is greatest among certain well-defined groups, including pregnant women, neonates, elderly patients, and immunocompromised adults, but may occasionally occur in persons who have no predisposing underlying condition (Painter and Slutsker 2007). Examples of immunocompromised adults are those with Acquired Immunodeficiency Syndrome (AIDS) or diabetes, or those who are under immunesuppressive medications for treatment of malignancy and management of organ transplantation (Jay and others 2005; Painter and Slutsker

2007). People who suffer from alcoholism, cardiovascular disease, or renal issues also belong to the immunocompromised group (Jay and others 2005).

Outbreaks

The first reported foodborne outbreak of listeriosis occurred in Canada between 1981-1982 and was linked to consumption of *L. monocytogenes* (serotype 4b) contaminated coleslaw (Schlech and others 1983). Between 1980 and 2012, there were 20 foodborne outbreaks of listeriosis published or reported to the Centers for Disease Control and Prevention (CDC) (Table 2-2). From these reported outbreaks, one of the biggest occurred in 1998 and involved 22 states where *L. monocytogenes* was linked to consumption of processed meats. This caused 101 cases of listeriosis, 15 deaths, and 6 miscarriages due to serotype 4b strain of *L. monocytogenes*. The CDC and state and local health departments identified the vehicle for transmission as hot dogs and deli meats produced under many brand names by one manufacturer, Bil Mar Foods (CDC 1999). Brand names that were affected included Ball Park, Bil Mar, Bryan Bunsize, Bryan 3-lb. Club Pack, Grillmaster, Hygrade, Mr. Turkey, Sara Lee Deli Meat, and Sara Lee Home Roast (CDC 1999).

In the latest estimates of foodborne illnesses acquired in the U.S., Scallan and others (2011a) reported that 31 major pathogens cause an estimated 9.4 million illnesses annually in the U.S. *Listeria monocytogenes* is an important foodborne pathogen, estimated to cause approximately 1,662 illnesses, 1,520 hospitalizations, and 266 deaths in the U.S. each year (Scallan and others 2011b). *Listeria monocytogenes* exhibit 0.25% incidence rate in 100,000 people, which is 0.05% more from the Healthy People 2020 objective that target 0.20% incidence rate for *L. monocytogenes* (CDC 2013b).

Table 2-2. Listeriosis outbreaks from known food vehicles, 1983-2012.

Year of outbreak	State(s)	No. of cases	No. of deaths	Miscarriages	Vehicle	Reference(s)/source
1983	Massachusetts and Connecticut	62	14	9	Pasteurized milk	Fleming and others 1985;
1985	California	86	8	21	Unknown	CDC 1985
1986	Pennsylvania	36	14	2	Mexican-style cheese	Schwartz and others 1989
1989	Connecticut	10			Shrimp	Reido and others 1994
1994	Illinois, Michigan, and Wisconsin	69	0	0	Pasteurized chocolate milk	Dalton and others 1997
1998	22 states	101	15	6	Processed meats	CDC 1998; CDC 1999
1998	Colorado	4	-	-	Hot dogs	CDC 2003
1998	Ohio	3	1	0	Unknown	CDC 1998
1999	Florida	2	-	-	Delicatessen turkey, ham, and roast beef	CDC 2003
1999	Connecticut, Maryland, and New York	11	-	-	Paté	CDC 2003
1999	Minnesota	5	-	-	Delicatessen meats	CDC 2003
1999	New York	4	-	-	Hot dogs	CDC 2003
1999	New York	6	-	-	Unknown	CDC 2003
2000	11 states	30	4	3	Delicatessen turkey	CDC 2000
2000	North Carolina	12	0	5	Homemade Mexican-style cheese	CDC 2001
2002	8 states	46	7	3	Turkey deli meat	CDC 2002
2007	Massachusetts	5	3	1	Pasteurized milk	CDC 2008
2010	Louisiana	14	2	0	Hog head cheese	CDC 2011a
2011	19 states	147	33	1	Cantaloupes	CDC 2011b
2012[a]	15 states	22	4	1	Ricotta Cheese	CDC 2012

[a]Updated as of November 21, 2012, Centers for Disease Control and Prevention.

USDA FSIS Regulations and Guidelines for *Listeria monocytogenes* Control in RTE Meat and Poultry

Control efforts in reducing *L. monocytogenes* in RTE foods has decreased the annual number of hospitalizations and associated deaths in the past decade (Scallan and others 2011b; USDA FSIS 2011). The USDA FSIS (2012b) noted that though the levels of *L. monocytogenes* in RTE meat and poultry products have decreased over the years as shown by FSIS testing, the pathogen continues to contaminate RTE products at low levels. The USDA FSIS has maintained a "zero tolerance" policy for *L. monocytogenes* in post-lethality exposed RTE products under the *Listeria* Rule (USDA FSIS 1999; USDA FSIS 2003; USDA FSIS 2012b). This policy means that detection of *L. monocytogenes* in RTE meat and/or poultry product or on a food-contact surface makes the product adulterated. This policy states that no detectable level of *L. monocytogenes* is permitted in any RTE meat and poultry product. The *Listeria* Rule also stated that *L. monocytogenes* is a hazard in post-lethality exposed products and must be controlled by establishments. *Listeria monocytogenes* can be controlled through their Hazard Analysis and Critical Control Points (HACCP) plans or the organism can be controlled in post-lethality environments through Sanitation Standard Operating Procedures (SSOP) or other prerequisite programs (USDA FSIS 2012b).

The *Listeria* Rule only applies to products that are RTE and exposed to post-lethality environments after cooking, heating, drying, or fermentation (USDA FSIS 2012b). A RTE product is defined as "a meat or poultry product that is in the form that is edible without additional preparation to achieve food safety and may receive additional preparation for palatability or aesthetic, epicurean, gastronomic, or culinary purposes" by the USDA FSIS (2003). In addition, USDA FSIS (2003) defined post-lethality exposed product as a "RTE product that comes into direct contact with a food contact surface after lethality treatment in post-lethality environment."

The *Listeria* Rule established three alternatives that establishments can use to control *L. monocytogenes* contamination on post-lethality exposed RTE products (USDA FSIS 2003; USDA FSIS 2012b). An establishment under alternative 1 must use a post-lethality treatment (PLT) to reduce or eliminate *L. monocytogenes,* and an antimicrobial agent or process (AMA or AMP) to suppress or limit the growth of the pathogen. Under alternative 2 (2a and 2b), an

establishment must use either PLT (2a) or AMA/AMP (2b). In addition, establishments using alternative 1 and 2 must validate the effectiveness of the PLT and AMA/AMP for eliminating or reducing and suppressing *L. monocytogenes*, respectively. This validation should show that PLT and AMA/AMP demonstrate at least a 1-log decrease in *L. monocytogenes* (before product distribution) and no more than 2-logs of *L. monocytogenes* growth throughout the product shelf life, respectively. Furthermore, establishments must document the use and effectiveness of PLT and/or AMA/AMP in the establishment's HACCP plan, SSOP, or other pre-requisite programs. Alternative 2b requires testing of food-contact surfaces (FCS) in the post-lethality processing area for *L. monocytogenes* or indicator organisms such as *Listeria* spp. or *Listeria*-like organisms. This is to ensure that surfaces in direct contact with a RTE product are sanitary and free from *L. monocytogenes*. Under alternative 3, an establishment must use sanitation measures to control *L. monocytogenes*. The establishment may incorporate the use of sanitation measures in its HACCP plan, SSOPs, or other prerequisite programs. In addition, just like Alternative 2b, the establishment must test FCS in the post-lethality processing area to ensure that surfaces in direct contact with RTE products are sanitary and free from *L. monocytogenes*.

Stringency of *L. monocytogenes* control decreases from Alternative 1 to Alternative 3. Thus, establishments operating under Alternative 3 are subject to the highest frequency of testing by FSIS (USDA/FSIS 2012b).

RTE Meat Categories

Jerky

Jerky is a dried meat snack usually produced as a whole muscle type or restructured (formed) type. The USDA FSIS (2012a) defines meat or poultry jerky as a dried product that is considered shelf stable and has been processed through slicing (whole muscle) or restructuring the meat or poultry, marinating, cooking, and then drying. In addition to a lethality step such as cooking, antimicrobial interventions before and after marinating the raw meat product can be added to ensure the reduction of pathogens in the product (USDA FSIS 2012a). The finished product must also meet the moisture-to-protein ratio (MPR) product standard of 0.75:1 (USDA FSIS, 2003) to label the product "jerky."

The compliance guideline for small and very small establishments producing meat and poultry jerky (USDA FSIS, 2012a) describes cooking and drying as two essential steps in jerky processing that will ensure product safety and shelf life stability. The cooking step serves as a lethality treatment intended to kill pathogenic microorganisms that are reasonably likely to occur in meat or poultry jerky, such as, *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* (USDA FSIS 2012b). *Salmonella* spp. population in meat jerky must be reduced by 5.0 log through lethality treatments. At least a 7.0 log reduction of *Salmonella* spp. must be achieved by lethality treatment of poultry jerky as required in 9 CFR 381.150. Sufficient reductions in other bacterial pathogens must also be achieved through lethality treatment of meat jerky. USDA FSIS (2012a; 2012c) recommends at least a 5.0 log reduction for *E. coli* O157:H7 in meat jerky, especially beef jerky, and a 3 log reduction or greater for *L. monocytogenes*. A 5.0 log reduction or greater is recommended by USDA FSIS for providing an even greater safety margin for ensuring that *L. monocytogenes* does not grow during cold storage to detectable levels (USDA FSIS 2012a). Regulations do not require establishments to validate that their process achieves reduction in *L. monocytogenes* if it achieves sufficient reductions in *Salmonella* because *Salmonella* is considered an indicator of lethality (USDA FSIS, 2012a). This is provided that supporting documents are available and show that sufficient *Salmonella* spp. reduction was achieved (USDA FSIS 2012c).

In addition, important critical operational parameters for jerky processing must be achieved in an establishment's actual process and procedure, regardless of the scientific support documentation used (USDA FSIS 2012a). It is critical that jerky product is cooked at an adequate temperature and time combination shown to be effective in Appendix A to ensure that

adequate lethality is achieved. In addition, the relative humidity in the smokehouse or oven is also critical for achieving adequate lethality. High relative humidity in the early stages of jerky cooking reduces evaporative cooling that will result in higher product surface temperature, thus, greater reduction in microorganisms (USDA FSIS 2012a). Furthermore, humidity adds moisture to the product that prevents concentration of solutes such as salt and sugar. Research has demonstrated that a decrease in moisture and increase in solute concentration (especially sugar) could increase the heat resistance of bacteria (USDA FSIS 2012a). Published literature has shown that at least 27-32% relative humidity should be present and a wet bulb temperature of at least 125-130°F for 1 h or more must be achieved during cooking to attain adequate lethality (Buege 2006; Harper 2009). However, it is critical that processors control *Salmonella* and *E. coli* O157:H7 during jerky production by using validated research documenting humidity parameters during cooking.

Drying in jerky processing is intended to remove water from the product to achieve an a_w level for food safety purposes. This process makes the product shelf stable and controls the growth of microorganisms. For shelf stable products such as jerky that will be stored under aerobic or an oxygen containing environment, a critical limit of ≤ 0.85 a_w should be achieved to control all bacterial pathogens, including *Staphylococcus aureus*. On the other hand, for shelf stable products that will be anaerobically vacuum packaged, the critical limit for a_w could be ≤ 0.91. USDA FSIS (2012b) recommends these limits based on the growth and toxin production limits for *S. aureus* under optimal and anaerobic conditions (ICMSF 1996).

Shelf stable sausage

Non-refrigerated semidry shelf stable sausage must have an MPR of <3.1 and a pH value of <5.0; or alternately, must have a pH of <4.5 (or 4.6 with an a_w <0.91), an internal brine concentration of <5%, and must be intact (or vacuum packaged if sliced), cured, and smoked (USDA FSIS 2005a).

Packaging

Kropf (2004) described three major functions of packaging. First, packaging protects the product from environmental variables such as temperature, moisture or humidity, oxygen, airborne particles, and light. Second, it protects the product against biological contamination such as microorganisms, rodents, and insects. Lastly, it serves as a marketing tool since it must provide space for a label that contains the information such as product identification, ingredients, nutritional information, net weight, verification of inspection, cooking instructions, promotional material, company name and location, and instructions on how to contact the processors. These three functions work to achieve the primary purpose to maintain the product quality and to assure the safety of the product to the consumer at the point of ultimate consumption.

Effects of modified atmosphere packaging and storage times on *Listeria monocytogenes*

Modified atmosphere packaging definition

There are two benefits of modifying atmosphere through oxygen reduction surrounding a product including an increase in product shelf life due to a reduction in the rate of chemical oxidation and reduction in the growth of spoilage microorganisms (Parry 1993; Phillips 1996). Modifying packaging atmosphere through oxygen reduction is specifically beneficial for shelf stable RTE products with low a_w because reduced oxygen concentration can inhibit mold growth (Jay and others 2005). Modified atmosphere packaging (MAP) is the technique used to reduce or exclude oxygen. This can be done by altering food packaging, flushing different gas mixtures of CO_2, N_2, and/or O_2 into a package, or using oxygen scavengers or other techniques (Phillips 1996; Jay and others 2005). Modified atmosphere packaging includes vacuum packaging, controlled atmosphere packaging (CAP), and MAP systems or 'dynamic MAP' (Kropf 2004; Phillips 1996).

Antilisterial effects of MAP and storage times

Generally, foods that are subjected to MAP should possess one or more of the following hurdles: $a_w < 0.93$, $pH \leq 4.6$, cured with $NaNO_2$, contain high levels of nonpathogenic competing organisms, maintained frozen, maintained at 4.0°C or below, and have a definitive shelf life (Jay 2005). The food industry employs the use of MAP to inhibit spoilage and pathogenic organisms (Lungu and others 2009).

The presence of increased CO_2 in packaging systems have been shown to inhibit *L. monocytogenes*. Daniels and others (1984) noted that the effectiveness of CO_2 in inhibiting microorganisms generally increased as the concentration of the gas increased. A CO_2 concentration of 5-10% (with 5% O_2/95% N_2; 10% O_2/90% N_2) had no inhibitory effect compared to atmospheric air on growth and survival of pure *L. monocytogenes* cultures (Francis and O'Bierne 2002). Farber and Daley (1994) reported that MAP containing at least 70% CO_2 could protect turkey roll slices against potential growth of *L. monocytogenes* throughout 30 d shelf life at 4°C. A 100% CO_2 atmosphere for chicken breast meat stored at 6°C and 15°C inhibited the growth of *L. monocytogenes* through 15 d and 4 d storage period (Hart and others 1991). This experiment showed that 100% CO_2 was more effective than an atmosphere containing 30% CO_2 plus air, or a 30% CO_2 and 70% N_2 environment. There was no difference between 30% CO_2/70% air and the 30% CO_2/70% N_2 atmosphere.

Previous studies have shown that *L. monocytogenes* is capable of surviving under anaerobic and reduced oxygen conditions. Gounadaki and others (2007) investigated post-process contamination of sliced salami with *L. monocytogenes* stored under air and vacuum packaging at 5, 15, and 25°C. Results showed that longer storage days were needed for 2-4 log reduction of *L. monocytogenes* on sliced fermented sausage packaged under vacuum compared to air packaging. In addition, days required for *L. monocytogenes* reduction increased as the temperature decreased for both packaging systems. Bunic and others (1995) reported that *L. monocytogenes* was able to grow in vacuum packaged frankfurters at 4°C. An atmosphere of 100% N_2 allowed survival of pure *L. monocytogenes* cultures, but populations did not change during storage, whereas a 3% O_2/95% N_2 atmosphere allowed significant growth of *L. monocytogenes*. In a different experiment demonstrated by Buchanan and Klawitter (1990) using tryptose phosphate broth, it was observed that O_2 and temperature interacted to influence survival of *L. monocytogenes* in low pH environments. They found that at pH 4.5, *L. monocytogenes* under aerobic conditions survived for extended periods at 5 and 10°C, while *L. monocytogenes* growth was observed at 19 and 28°C. At a higher temperature (37°C), *L. monocytogenes* was inactivated (Buchanan and Klawitter 1990). However, under anaerobic conditions, *L. monocytogenes* recovered and survived for extended periods at 37°C, while restriction of oxygen enhanced *L. monocytogenes* growth at 19°C (Buchanan and Klawitter 1990).

Data from a survey of Midwestern meat processors showed that respondents used a variety of packaging methods such as vacuum (78%), no vacuum (32%), and gas flush (14%) packaging for jerky products (Lonnecker and others 2010). Most products were stored at room temperature (38%) or refrigerated (32%). *Listeria monocytogenes* is of great concern in RTE products packaged using these packaging systems due to its facultative nature that enables it to survive in anaerobic and reduced oxygen conditions. Thus, application of multiple hurdles will increase the margin of safety for *L. monocytogenes* control in RTE meat and poultry products.

Previous studies have shown that packaging in combination with at least 24 h ambient storage was effective as a post-lethality treatment and antimicrobial process for RTE meat and poultry products with $\leq$0.87 a_w (Ingham and others 2004; Ingham and others 2006b; Lobaton-Sulabo and others 2011; Uppal and others 2012). Lotaton-Sulabo and others (2011) studied the effects of packaging (vacuum, heat seal without oxygen scavenger, heat seal with oxygen scavenger, and nitrogen flushed) on *L. monocytogenes* populations in a reduced a_w (<0.79) product, beef jerky, over a 30 day storage period at 25.5°C. Results showed that all packaging types achieved a 1 log CFU/cm^2 reduction of *L. monocytogenes* after 48 h of storage and a 3.5 log CFU/cm^2 reduction after 30 days of storage. Uppal and others (2012) performed a similar study on kippered beef steak and turkey tenders. Samples were stored for 72 h at 25.5°C. Kippered beef steak had an a_w of 0.81 while turkey tenders had an a_w of 0.77. For kippered beef steak, *L. monocytogenes* was reduced by >1 log CFU/cm^2 after 24 h of storage and 2.1 log CFU/cm^2 after 72 h of storage for all packaging treatments. *L. monocytogenes* populations on turkey tenders were reduced by >1 log CFU/cm^2 after 24 h of storage for all packaging treatments except vacuum packaged (0.9 log CFU/cm^2). After 72 h of storage, all packaging treatments caused a reduction of >1 log CFU/cm^2 for turkey tenders.

Earlier research has shown that counts of *L. monocytogenes* on vacuum-packaged beef jerky (a_w=0.75) decreased by 2.4 log CFU/sample in the first week of 21°C storage, and no surviving cells were detected after 4 weeks (Ingham and others 2006b). Another study evaluated survival of *L. monocytogenes* on 15 beef jerky and related products (Ingham and others 2004). Results showed that vacuum packaged beef jerky and related products with $a_w \leq 0.87$ and stored for 1 week at 21°C were sufficient to control *L. monocytogenes*. The population of *L. monocytogenes* on these products decreased by 0.60 to 4.70 log CFU/sample after 1 week, and decreased by 2.30 to 5.60 log CFU/sample after 5 weeks. Results suggest that a_w was not the

main compositional factor affecting the survival of *L. monocytogenes* on vacuum packaged beef jerky (Ingham and others 2004).

Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy (EDS)

Scanning electron microscopy

The scanning electron microscope (SEM) utilizes a focused beam of high-energy electrons or primary electrons (usually operating at <1-30 kV) with significant amounts of kinetic energy to interact with a solid sample (Hafner 2007; Swapp 2013). This electron-sample interaction generates multiple signals such as photons, secondary electrons (SEs), backscattered electrons (BSEs), and characteristic x-rays (used in EDS), and Auger electrons (used in EDS) (Egerton 2005). These signals are collected by detectors and processed to generate an image (Hafner 2007). The primary signals that are responsible for SEM image generation are SEs and BSEs. The SEs are atomic electrons ejected from the specimen as a result of interaction between the fast electron and atomic electrons (inelastic scattering) (Egerton 2005) and the signal generated displays surface structure (topography) of a specimen (Egerton 2005; Swapp 2013).

On the other hand, BSEs are incident electrons elastically scattered at a deflection angle of more than 90 degrees (Egerton 2005). Elastic scattering results from interaction between incoming fast electrons and the atomic nucleus (Egerton 2005), and the signal generated displays an image that illustrates contrasts in a specimen with variations in chemical composition (Egerton, 2005; Swapp 2013). Image formation is achieved by a scanning principle by focusing primary electrons into a small-diameter electron probe that is scanned across the specimen in two perpendicular directions (right angles to the incident beam), covering a square or rectangular area of specimen (a raster) (Egerton 2005). Signals such as SEs or BSEs from each point on the specimen are collected for image generation.

Scanning electron microscopes are equipped with at least one detector (usually a secondary electron detector), and most have additional detectors for BSEs and X-rays. Specific capabilities of a particular SEM are dependent on which detectors it contains. High vacuum imaging detectors such as ETD (Everhart-Thornley detector) and TLD (through-lens SE and BSE detector) are examples of SEs detectors. The vCD (low voltage high contrast detector) is an example of a detector that operates at high vacuum and low voltage mode that detects BSEs to

obtain images of the composition and topography of the surface (Swapp 2013; Anonymous 2013).

Scanning electron microscopy has been used in studying morphology (Ritz and others 2001; Bereksi and others 2002; Zaika and Faneli 2003), attachment and adhesion of bacterial cells to biotic and abiotic surfaces (Wu-Yuan and others 1995; Hsu and others 2013). It has been used in examining *L. monocytogenes* attachment and biofilm formation on RTE meat, food-contact surfaces, and on materials used for food processing equipment (Mafu and others 1990; Arnold and Silvers 2001; Kalmokoff and others 2001; Bremer and others 2002; Chavant and others 2002; Marsh and others 2003; Foong and Dickson 2004; Silva and others 2008).

Energy dispersive X-ray spectroscopy

Energy dispersive X-ray spectroscopy (EDS) is a technique that utilizes the physical principle of characteristic X-ray generation. It is used in microanalysis for qualitative and quantitative analyses of elements present in a sample. Qualitative analysis involves the identification of the lines in the X-ray spectrum while quantitative analysis involves measuring of these line intensities for each element in the sample and for the same element in calibration standards of known composition (Anonymous 2013).

Characteristic X-rays are produced by inelastic collisions of energetic electrons from an incident beam with electrons in discrete orbitals (e.g., K, L, M, and N shells) of atoms in a sample (Goldstein and others 2003; Swapp 2013). The inelastic collisions initially result in ejection of electrons from inner atomic shell of an atom, generating an ion in excited state. Then, this is followed by a de-excitation process that allows the excited ion to give up energy to return to a normal ground state or low energy state (Vaughan 1999). This process yields electromagnetic radiation X-rays that are of a fixed wavelength that is related to the difference in energy levels of electrons in different shells for a specific element (Swapp 2013). The energy of the radiation emitted during de-excitation is determined by the electronic structure of the atom, which is unique to the element where it came (Vaughan 1999), thus called characteristic X-rays in the form of photons. However, de-excitation of an excited atom undergoes an alternative process, called the Auger process, which generates Auger electrons. The Auger process involves emission of a characteristic X-ray, re-absorption of the characteristic X-ray within the same atom, and ejection of a low energy electron, or Auger electron (Vaughan 1999; Goldstein and

others 2003). Elements with low atomic numbers favor Auger emission, while higher atomic numbers favor characteristic X-ray emission (Vaughan 1999).

Energy levels or shells in Bohr model of an atom

The Bohr model suggests that the electrons are arranged in particular energy levels or shells (also known as 'lines' in X-ray microanalysis) around an atom that have specific energies. In X-ray microanalysis, the shell or level closest to the nucleus is the K shell and requires the most energy to remove an electron from this shell (Goldstein 2003). The next closest level is the L shell and then the M shell, where each of these shells is divided into subshells. The energy of a shell and a subshell is exactly and uniquely defined, where the minimum ionization energy necessary to remove an electron from a shell is distinct to that specific shell or subshell (Goldstein 2003).

Excitation occurs when beam electrons of sufficient energy (excitation energy, E_c) interacts with the tightly bound inner shell electrons of a specimen atom, ejecting an electron from a shell (Goldstein 2003). The accelerating voltage applied to the electron gun causes electrons to accelerate down the electron optic column (Oxford Instruments 2006). The amount of accelerating voltage applied influences the spatial resolution of the X-ray signal, the efficiency with which the X-ray lines (shells) are excited from the samples, and the relative intensities of these X-ray lines (Vaughan 1999).

Principle of X-rays detection and analysis

An EDS system consists of seven components: electron column, detector, preamplifier, pulse processor, energy-to-digital converter, multi-channel analyzer, and mini-computer. An EDS system utilizes pulse height analysis where a detector giving an output pulse with a height proportional to the energy of the X-ray (Vaughan 1999). The detector commonly used in an EDS system is manufactured from a single crystal of silicon, such as lithium-compensated silicon [Si(Li)] or silicon drift detector (SDD) (Newbury and Ritchie 2013). X-ray photons produced from the bombardment of focused beam electrons with the sample cause ionization in the detector, producing an electrical charge, which is amplified by a preamplifier located close to the detector. The pulse processor then converts the signal to a well-shaped voltage pulse with a height proportional to the energy of the X-ray (Vaughan 1999). The height of the voltage pulse is measured and assigned a channel number in the energy-to-digital converter. The signals from the

energy-to-digital converter will be accumulated and assembled into a spectrum in the multi-channel analyzer (Vaughan 1999).

Qualitative analysis in EDS involves identification of X-ray lines in the X-ray spectrum using tables of energies or wavelengths (CFAMM 2013). Modern analyzers have an automated process in which software routines detect the location of spectral peaks and compare these peaks with tabulated energy values. Then, a full report can be generated that includes X-ray spectral components of the sample with a full list of elements present.

X-ray intensities are measured by counting pulses generated in the detector by X-ray photons, which are emitted randomly from the sample (CFAMM 2013). To obtain the adequate intensity, accelerating voltage (kV) must not be less than twice the E_c (KeV) of any element present in the sample. Concentration of each element in a sample is usually reported in weight % (Wt. %) or Atomic %. Oxford Instruments (2006) has described the definition of Wt. and Atomic % in their INCA software operator's manual as:

Wt. % is calculated using the formula:

$$Wt.\,\% = \frac{Apparent\,concentration}{Intensity\,correction}$$

The intensity correction for each element is the ratio of the combined correction for the sample to the combined correction for the standard used for that element.

Atomic % is calculated from measured Wt. %:

$$Atomic\,\% = \frac{Wt.\,\%}{Atomic\,wt.}$$

Apparent concentration of an element A was the first estimate of the concentration before any matrix corrections were calculated and applied is defined by the formula:

$$Apparent\,concentration = \frac{Intensity\,of\,A_{sample}}{Intensity\,of\,element\,A_{standard}} \times Wt.\,\%\,element\,A_{standard}$$

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Chapter 3 - Effects of Packaging and Storage Times on *Listeria monocytogenes* Survival on Uncured and Cured Turkey Jerky

ABSTRACT

To validate how packaging, storage time, and sodium nitrite reduce *Listeria monocytogenes* on whole muscle turkey jerky, four packaging systems [heat sealed (HS), heat sealed with oxygen scavenger (HSOS), nitrogen flushed with oxygen scavenger (NFOS), and vacuum (VAC)], five storage times (24, 48, and 72 h, and 14 and 30 d), and two sodium nitrite treatments (uncured and cured) were evaluated. Cured jerky contained 147.7 ppm ingoing nitrite. Laboratory produced ready-to-eat turkey jerky was aseptically cut into 4 x 4 cm pieces, dipped into a five-strain *L. monocytogenes* cocktail, and then air dried for 1-2 h at 21.5°C until an approximate ≤ 0.80 water activity was achieved. *Listeria monocytogenes* population was enumerated at day 0 for initial count. Jerky was then packaged and stored at 21.5°C until enumerated for *L. monocytogenes* at 24, 48, and 72 h, and 14 and 30 d after packaging. The initial *L. monocytogenes* populations on uncured and cured turkey jerky were 5.93 and 5.94 log CFU/cm^2, respectively. Inclusion of sodium nitrite in turkey jerky did not affect ($P > 0.05$) *L. monocytogenes* log reductions regardless of packaging system or storage time. A two-way interaction ($P < 0.05$) was found for packaging system by storage time for *L. monocytogenes* log reductions. After 14 d of storage in HSOS, NFOS, or VAC, and in HS after 48 or 72 h of storage, a mean of >1.0 log CFU/cm^2 reduction of *L. monocytogenes* was achieved. The greatest ($P < 0.05$) log reductions of *L. monocytogenes* were found by packaging turkey jerky in HS for 14 and 30 d (2.23 and 2.51 log CFU/cm^2) and in VAC for 30 d of storage (2.19 log CFU/cm^2) at 21.5°C. Processors could use a 48 h holding time for HS packaging and be in compliance with the current *Listeria* Rule of post-lethality treatment in achieving at least 1 log reduction of *L. monocytogenes*. Addition of sodium nitrite in jerky products did not affect reduction of *L. monocytogenes* in turkey jerky.

INTRODUCTION

Listeria monocytogenes remains one of the leading causes of death in foodborne infections, estimated to cause 1,662 illnesses and 1,520 hospitalizations, and 16% case-fatality rate annually (Scallan and others 2011). Contamination of ready-to-eat (RTE) turkey products with *L. monocytogenes* is a food safety concern, especially since these products were implicated in listeriosis outbreaks in 2000 and 2002 (CDC 2000; CDC 2002). The 2002 outbreak resulted in one of the largest recalls in the U.S. history, involving more than 13.5 M kg of deli turkey meat products and resulted in 46 cases and 7 deaths (CDC 2002; Gottlieb and others 2006). Following this outbreak, federal regulators issued stringent rules designed to prevent *L. monocytogenes* contamination of RTE meat and poultry products (USDA FSIS 1999; USDA FSIS 2002; USDA FSIS 2012). To date, turkey jerky products have not been associated with listeriosis outbreaks; however, jerky products have been subject to recalls due to *L. monocytogenes* contamination detected through routine sampling conducted by processors or regulators (USDA FSIS 1996; SPCNETWORK 2000; USDA FSIS 2000; USDA FSIS 2004; Parker Waichman LLP 2008;).

Listeria monocytogenes is ubiquitous in nature, so it is considered to be an environmental contaminant. It is a tenacious organism capable of growing in cool, damp, or dry environments where other bacteria might not be able to survive and inactivation is difficult (Gravani 1999). Since *L. monocytogenes* is an environmental contaminant, it can contaminate products in post-lethality environments where further processing or packaging occurs. It is a significant concern for meat processors producing RTE meat and poultry because these products are not heated or cooked prior to consumption. The U.S. Department of Agriculture Food Safety and Inspection Service (USDA FSIS) has maintained a "zero tolerance" policy for this pathogen under the *Listeria* Rule (USDA FSIS 1999; USDA FSIS 2003). Under the *Listeria* Rule, the agency mandates establishments producing post-lethality exposed RTE meat and poultry to employ one of the three alternatives to control *L. monocytogenes* (USDA FSIS 1999; USDA FSIS 2003). The agency continues to strengthen *L. monocytogenes* intervention programs, policies, and recommendations to reduce or eliminate *L. monocytogenes* in RTE products (Gottlieb and others 2006; USDA FSIS 2012).

To increase the level of safety of RTE meat and poultry products for *L. monocytogenes* control, post-lethality antilisterial interventions must be able to inhibit growth or inactivate the

organism in order to increase the level of safety of the product throughout its shelf life. The USDA FSIS is expecting that post-lethality treatments and antimicrobial agents or processes achieve at least 1-log lethality and no more than 2-logs of *L. monocytogenes* growth throughout shelf life, respectively (USDA FSIS 2012). Common antilisterial interventions that have been evaluated and used in RTE meat and poultry products include incorporation of organic acids, nitrite, salt, sulfites, sorbates, lactates, and diacetates in the formulation, as well as drying, fermentation, freezing, and packaging (Williams and Golden 2001; Seman and others 2002; Mbandi and Shelef 2002; Gill and Holley 2003; Zhu and others 2005; Uppal and others 2012; Lobaton-Sulabo 2011)

Previous studies showed that packaging and storage time can be used as post-lethality treatment or antimicrobial process in RTE meat and poultry products (Ingham and others 2004; Ingham and others 2006; Lobaton-Sulabo and others 2011; Uppal and others 2012). It was reported that vacuum packaging of cured beef jerky for 24 h or 1 week at ambient storage can generate a 1 log *L. monocytogenes* reduction and the organism did not survive throughout 4 weeks of ambient temperature shelf life (Ingham and others 2006; Lobaton-Sulabo and others 2011). Lobaton-Sulabo and others (2011) reported that beef jerky packaged in heat-sealed low-linear-density polyethylene (LLPDE) with an oxygen scavenger (OS) or vacuum LLPDE and stored at 25.5°C for 24 h could reduce >1 log CFU/cm^2 of *L. monocytogenes* populations. Similar findings reported by Uppal and others (2012) showed that a combination of a packaging system (i.e., vacuum, nitrogen flushed with OS, and heat sealed with OS) and a holding time of 24 h generated ≥1 log reduction of *L. monocytogenes* on kippered beef and turkey tenders.

Nitrite added to cured meats contributes to pink color development (Killday and others 1988), flavor (Pierson 1982), antioxidant activity against rancidity (Pierson 1982), and inhibition of *Clostridium botulinum* growth and toxin production (Sofos and others 1979; Pierson 1982; Roberts and others 1991). Studies have shown that sodium nitrite demonstrated an antilisterial effect when added in brain heart infusion broth, tryptone soya broth, and sliced deli turkey (McClure and Kelly 1991; Buchanan and others 1994; Roenbaugh 2011). Glass and others (2008) concluded that a minimum of 30 ppm nitrite could enhance the antilisterial activity of lactate-diacetate in sliced turkey product (restructured). Models developed to predict *L. monocytogenes* growth have shown improved effectiveness of antimicrobials like sodium lactate and sodium diacetate in the presence of nitrite (Sofos and others 1979; Schlyter and others 1993;

Duffy and others 1994; Buchanan and others 1997; McClure and others 1997; Gill and Holley 2003; Legan and others 2004). These findings could help determine whether addition of sodium nitrite in turkey jerky formulations could enhance antilisterial efficacy of turkey jerky stored using different packaging systems and holding times. The objective of this study was to evaluate the effect of sodium nitrite, packaging systems, and holding times in reducing *L. monocytogenes* in whole muscle turkey jerky.

MATERIALS AND METHODS

Treatments, experimental design, and statistical analysis

Four packaging treatments were used including heat seal (HS), heat seal with oxygen scavenger (HSOS), nitrogen flush with oxygen scavenger (NFOS), and vacuum (VAC). All treatments were subjected to five different storage times that included 24, 48, and 72 h, 14 and 30 d. Turkey jerky was formulated with (cured) and without (uncured) sodium nitrite. Turkey jerky that was not inoculated with *L. monocytogenes* was used as a negative control. The experimental design was a completely randomized design (CRD) using a 4 x 5 x 2 factorial treatment structure with turkey jerky sample as the experimental unit. Analysis of variance for the mean log reduction of *L. monocytogenes* population was performed using the PROC MIXED procedure of SAS version 9.2 (SAS Inst. Inc., Cary, NC). The fixed effects for the statistical analysis were packaging treatment, storage time, formulation type, packaging type x storage time, packaging type x formulation type, and packaging type x storage time x formulation type. Random effect was the replication. Least square means were calculated for each independent variable. Statistical significance and tendencies were set at *P*<0.05 level of significance. The experiment was done in triplicate and each replication consisted of duplicate samples in each treatment (total of n=40). Two samples were enumerated for initial *L. monocytogenes* population at day 0, and two samples as negative control.

To determine the effect of storage time (0, 24, 48, and 72 h, 14 and 30 d) and formulation type on water activity (a_w), pH, and moisture protein ratio (MPR) of non-inoculated turkey jerky pieces packaged in HSOS, a CRD with 6 x 2 factorial treatment was used. Two samples per treatment were used for a_w and pH determination, while, four composite samples per treatment were used for MPR. Analysis of variance was performed using the PROC MIXED procedure of SAS

version 9.2 (SAS Inst. Inc., Cary, NC). Least square means were calculated for each independent variable. Statistical significance and tendencies were set at *P*<0.05 level of significance.

Sample preparation

Three batches (18 kg each) of frozen raw sliced turkey breast for each uncured and cured formulation were obtained from a meat processing facility (Kent, WA). The first batch was processed separately, while the second and the third batch were processed on the same day. Each batch of turkey breast received at the Kansas State University meat laboratory was immediately thawed upon receipt in a cooler at 4-7°C for at least 8 h prior to processing. Each batch of raw turkey breast slices were manually separated and weighed into two equal portions of 9.09 kg for each uncured and cured formulation. A list of ingredients present in the marinade for the uncured and cured formulations is shown in Table 3-1. The percent of each ingredient was proprietary and all ingredients were supplied by a commercial company (Kent, WA). The cured jerky formulation was formulated with 0.76% modern cure in the curing solution to achieve an ingoing concentration of 147.7 ppm sodium nitrite. A small slice of turkey breast was used to measure pH (see pH determination procedure described in proximate and chemical analyses section).

All turkey jerky processing and preparation was conducted in a 7.0°C USDA FSIS inspected meat processing laboratory at Kansas State University. Dry ingredients were received as pre-weighed premixes except for brown sugar. Dry and liquid ingredients for uncured or cured formulation were mixed together until dry ingredients were completely dissolved in liquid ingredients. For the cured formulation, modern cure was added last to the marinade before mixing in the turkey slices. Turkey slices were then added to the marinade (0.33:1 marinade to meat ratio) and were hand mixed until all turkey slices were coated with the marinade. The marinade-coated meat mixture was then vacuum tumbled (Model VTS-42, Biro Manufacturing, Marblehead, OH) under 508 mm Hg at 10 rpm for 6 min. After tumbling, the meat and marinade mixture was transferred to a plastic lug and a single piece of plastic wrap was placed over the marinade-coated turkey slices to serve as a temporary seal to prevent drying of those pieces on the top layer. Turkey slices were held in marinade for 48 h at 4.0-7.0°C in a cooler. After 48 h, turkey slices and marinade were hand mixed and tumbled again under 508 mm Hg at 10 rpm for 2 min. Marinated turkey slices were then weighed to determine percent pickup and resulting ingoing nitrite. Ingoing nitrite was calculated using the formula (USDA FSIS 1995) below:

$$\text{Ingoing nitrite (ppm)} = \frac{\text{weight of modern cure (lb) x 0.0625 x \% pickup x 1,000,000}}{\text{weight of pickle solution (lb)}}$$

Next, marinated turkey slices were laid by hand on metal screens placed on a smokehouse truck. Turkey slices were then thermally processed using a two stage cooking cycle in a smokehouse (Maurer; Northbrook, Ill., U.S.A.) cycle. In the first stage, jerky was cooked using 85°C dry bulb (DB) and 52.8°C wet bulb (WB) (20% relative humidity) for 45 min. For the second stage, jerky was cooked at 85°C DB and 48.3°C WB (15% relative humidity) for 140 min. Dampers were open/closed (auto) and the fan speed was 1200 rpm for all stages.

A total yield of approximately 10-12 packages of uncured and cured turkey with each package containing 454 g cooked turkey jerky products were obtained after cooking. Packaging was done by placing 454 g of cooked turkey jerky into an oxygen impermeable resealable, 5-mil-thick, low-linear-density polyethylene (LLDPE) clear pouch (oxygen transmission rate: 17 ml/100 in^2 [~645 cm^2] in 24 h, moisture vapor transmission rate of 0.084 g/100 in^2 [~645 cm^2] in 24 h) (19 cm x 36 cm; TPG Co., LTD, Gyeonggi-do, Korea), flushed with 100% food grade nitrogen for 10 s or until residual oxygen was at most 0.2% (measured using oxygen content procedure under chemical and proximate analyses), were sealed and stored at room temperature of 24.0°C for at least 2 weeks prior to inoculation and analyses.

Table 3-1. Uncured and cured turkey jerky marinade ingredients.

Dry ingredients
Brown sugar
Corn syrup
Granulated sugar
Salt
Garlic powder
Ginger powder
Black Pepper
Liquid ingredients
Liquid smoke
Soy sauce
Concentrated pineapple juice
Worcestershire sauce

Bacterial culture preparation

Bacterial culture preparation, media preparation, sample inoculation, packaging, and enumeration of *L. monocytogenes* were all conducted in a BL2 laboratory facility at Kansas State University, under IBC #291. Lyophilized reference stock cultures of five *L. monocytogenes* strains (Table 3-2) were obtained (Kwik-Stik, Microbiologics, Inc., Grenobel Cedex, France). A pure subculture of each strain was prepared by transferring a loopful of stock culture to 10 mL of pre-sterilized tryptic soy broth (TSB; Difco BD and Company, Sparks, MD) and incubated at $35 \pm 2°C$ for 24 h. One liter of the five-strain *L. monocytogenes* cocktail was prepared aseptically by first transferring 0.5 mL of each pure culture isolate to 200 mL pre-sterilized TSB, and then incubating at $35 \pm 2°C$ for 24 h. Next, each 200 mL inoculum was transferred into a sterile 1 L beaker to obtain a liter of the five-strain cocktail. A total of 2.0 L of *L. monocytogenes* cocktail per replicate was prepared. One liter was used for uncured turkey jerky and the other liter was used for cured turkey jerky inoculation. Each liter of the five-strain *L. monocytogenes* cocktail contained 8-9 log CFU/mL inoculum level. Inoculum level of the five-strain cocktail was

determined by spread-plate enumeration technique of *L. monocytogenes* populations on modified oxford media (MOX; see media preparation and enumeration of *Listeria monocytogenes* procedure).

For confirmation of *L. monocytogenes* cultures, a BD BBL Crystal ™ Gram-Positive Identification system (Difco BD and Company, Sparks, MD) was used and performed at least 1 week before inoculation. The kit contains 29 dried biochemical and enzymatic substrates that can be used to identify *L. monocytogenes*. The kit also contains a fluorescence negative control in addition to the 29 dehydrated substrates. Color changes or presence of fluorescence, as a result of *L. monocytogenes* metabolic activities, were examined using a BBL panel viewer. The resulting pattern of the 29 reactions was converted into a ten-digit profile number that was used as the basis for identification in the BBL Crystal ID System (Sneath 1957).

Table 3-2. Five *Listeria monocytogenes* strains used for inoculum.

Strain	Original Source
ATCC 7644	Human
ATCC 19112	Spinal fluid of man (Scotland)
ATCC 19115	Human
ATCC 19118	Chicken (England)
SLR 2249 Cornell University	Laboratory-developed strain where ActA gene was removed

[1]ATCC=American Type Culture Collection.

Media preparation

Tryptic soy broth was prepared by adding 30 g of the media to 1 L of distilled water, mixed thoroughly, and then heated in a microwave for 2 min. Then, TSB was transferred to a 1 L bottle and sterilized at 121°C for 15 min using an Encore™ Series sterilizer (Continental Equipment Inc., Tonganoxie, KS).

Modified oxford agar (MOX; Difco BD and Company, Sparks, MD) was prepared by adding 57.5 g of the media to 1 L of distilled water, mixing, and then boiling for 1 min or until turbidity was lost. Next, MOX was transferred to a 1 L bottle and sterilized at 121°C for 15 min. Media was then cooled down at room temperature to about 45°C. Hydrolyzed oxford antimicrobic supplement (10 mL) (Difco BD and Company, Sparks, MD) dissolved in 10 mL distilled water was immediately added into cooled media. This supplement contains cycloheximide, colistin sulfate, acriflavine, cefotetan and fosfomycin, which make MOX selective for *L. monocytogenes*. The supplemented media was then poured into disposable agar plates and allowed to set for at least 2 h at ambient temperature.

Peptone water (0.1%) was prepared by adding 1 g of Bacto™ Peptone (Difco BD and Company, Sparks, MD) to 1 L of distilled water, mixed thoroughly, and sterilized at 121°C for 15 min. For preparing peptone blanks for serial dilution, 9 mL of 0.1% peptone was dispensed into 15.0 mL screw-capped tubes and sterilized at 121°C for 15 min.

Packaging material and oxygen scavenger

The packaging material used in this study was an oxygen impermeable resealable, 5-mil-thick, low-linear-density polyethylene (LLDPE) clear pouch (oxygen transmission rate: 17 ml/100 in^2 [~645 cm^2] in 24 h, moisture vapor transmission rate of 0.084 g/100 in^2 [~645 cm^2] in

24 h) (19 cm x 36 cm; TPG Co., LTD, Gyeonggi-do, Korea). This was sized to a final dimension of 19 cm x 18 cm. In addition, oxygen scavengers (O_2-Zero BJ100; TPG Co., LTD, Gyeonggi-do, Korea) were used for HSOS and NFOS treatments.

Sample inoculation and packaging

Prior to sample inoculation, a 1 mL portion of the *L. monocytogenes* cocktail was transferred to 9 mL 0.1 % peptone blank to determine the initial inoculum level. Two samples per treatment uncured or cured jerky pieces for each replicate, measuring 4.0 cm x 4.0 cm, were obtained aseptically and dipped, two at a time, in 1 L *L. monocytogenes* cocktail for 1 min. Samples were immediately hung using sterile binder clips and air dried for 1-2 h (Figure 3-1) or until the a_w was ≤ 0.80 (measured using procedure in a_w determination under chemical and proximate analyses). Surviving *L. monocytogenes* was immediately enumerated on two samples to determine the *L. monocytogenes* initial attachment to the uncured or cured jerky sample. Samples were then assigned to one of the four packaging treatments and storage times. Samples were prepared in duplicate for each packaging treatment and holding time for a total of 40 packages per replicate. Samples assigned to NFOS were flushed for 10 s using food grade 100% N_2 and a vacuum packager (Multivac C100, Gepufte Sicherheit, Germany). The VAC samples were packaged using the vacuum packer with 600 mm Hg. The HS and HSOS packages were sealed using the same equipment by turning off the gas flush and setting the gauge to zero mm Hg. An oxygen scavenger was added to each sample packaged in NFOS or HSOS prior to heat sealing or N_2 flushing.
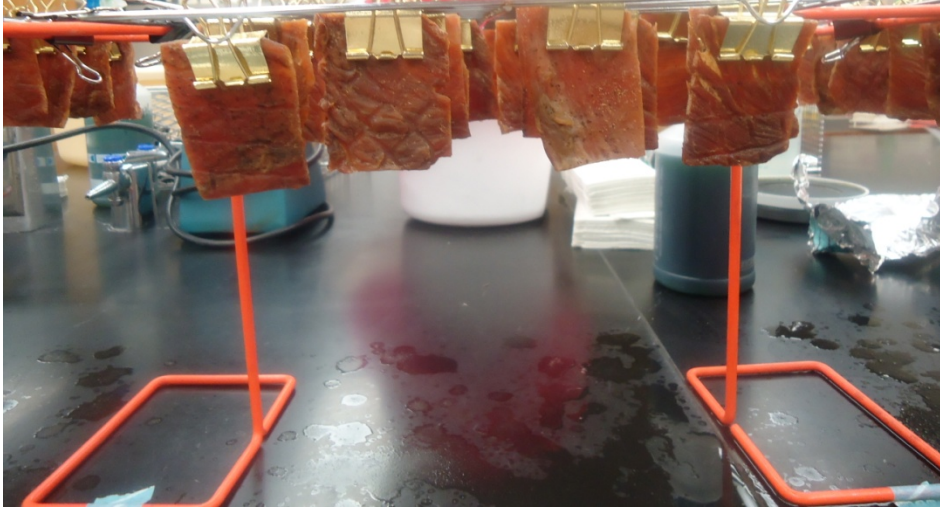
Figure 3-1. Drying arrangement for turkey jerky after *Listeria monocytogenes* inoculation.

Chemical and proximate analyses

Two packaged samples per treatment of uncured or cured turkey jerky pieces for each replicate, measuring 4.0 cm x 4.0 cm, were used for determination of a_w and pH. Four composite samples per treatment were used to determine moisture and protein content. This was done to check whether there were significant changes in chemical properties of the jerky pieces throughout storage as a result of the dipping procedure. Since it was not possible to measure the chemical characteristics of inoculated samples, a simulation of the inoculation procedure was done by dipping turkey jerky pieces into sterile TSB media without the *L. monocytogenes* inoculum. The a_w, pH, and moisture and protein content were measured initially before dipping and immediately after drying, and after 24, 48, and 72 h, 14 and 30 d of ambient temperature storage. After drying, the non-inoculated TSB dipped jerky pieces were packaged in HSOS.

The a_w was determined using an a_w meter (AQUALAB CX2 series 3TE; Decagon, Pullman, WA). The a_w meter was calibrated using a 0.760 NaCl (6.0 molal in water; Decagon, Pullman, WA) verification standard and distilled water at 25.3°C. Turkey jerky pieces were cut into a hexagonal shape with a diameter of approximately 3.2 cm and were placed in sample container following the procedure described by Harper and others (2010) for a_w measurement. Two samples per treatment were used for measurement and duplicate readings were taken for each sample at an average temperature of 25.3°C.

The determination of pH was done by preparing a meat slurry containing 10 g of non-inoculated uncured or cured turkey jerky with 90 mL distilled water in a double-chamber filter

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bag (Fisherbrand, Ste-Julie, Quebec, Canada) and stomached (Seward Stomacher 400; Tekmar, Diversified Equipment Company Inc., Lorton, VA) for 1 min. The pH was measured with a calibrated pH meter (Acumet AB15, Fischer Scientific, Kent City, MI) fitted with a flat-surface combination electrode (13-620-530A; Fisherbrand, Vernon Hills, IL) inserted into the filtered portion of the bag. The pH meter was calibrated using pH 4.0 and pH 7.0 buffer solutions (Fisher Scientific, Fair Lawn, NJ) and a pH reading was obtained on duplicate samples per treatment.

The moisture and protein content of four composite samples packaged in HSOS was determined using the SMART system 5 (CEM Corp., NC) procedure for moisture analysis (AOAC Official Methods 985.14, 977.11, 985.26 and 2008.6) and LECO FP-2000 Protein/Nitrogen Analyzer (model 602-600; LECO Corp., MI) procedure was used to determine nitrogen/protein content (AOAC Official Method 990.03). These values were used to calculate MPR for turkey jerky.

The oxygen content of the packaged inoculated samples was measured after 0 (2 non-inoculated dummy samples), 24, 48, 72 h, 14 and 30 d of ambient temperature storage using an oxygen analyzer (Checkpoint-O$_2$; PBI Dansensor, DK-400 Kingsted, Denmark). The oxygen concentration in each package type except VAC was measured prior to *L. monocytogenes* enumeration. This was done by piercing the package, 1.0 cm away from the seal with the oxygen detector needle inserted at a 45° angle.

Enumeration of *Listeria monocytogenes*

Two samples per treatment were obtained for *L. monocytogenes* enumeration. Surviving *L. monocytogenes* population in a packaged sample was enumerated using the spread-plate technique on MOX (with hydrolyzed oxford antimicrobic supplement) plates. This was done by aseptically removing one inoculated turkey jerky sample from a package and transferring it to double-chamber stomacher bag (Fisherbrand, Ste-Julie, Quebec, Canada). Next, 50 mL of 0.1% peptone diluent was added, and contents were stomached (Seward Stomacher 400; Tekmar, Diversified Equipment Company Inc., Lorton, VA) for 1 min. Three serial dilutions were prepared by transferring a 1.0 mL aliquot of the stomached treatment to 9.0 mL of 0.1% peptone blanks. To determine the initial inoculum level, dilutions 0-3 were plated in duplicate on MOX. For treatments that were stored for 24, 48, and 72 h, dilutions 0-2 were plated, while dilutions 0 and 1 were plated in duplicate on MOX for treatments that were stored for 14 and 30 d. MOX plates were incubated at 35 $\pm$ 2°C for 48 $\pm$ 2 h and counts were reported as log CFU/cm^2. Log

reduction was calculated by subtracting *L. monocytogenes* population enumerated at each storage time from initial *L. monocytogenes* population enumerated at day 0.

RESULTS AND DISCUSSION

The processing characteristics of turkey jerky manufactured at Kansas State University meat lab are shown in Table 3-3. Prior to inoculation, the initial mean water activity (a_w), pH, and MPR of uncured turkey jerky were 0.69, 5.96, and 0.59, respectively. Cured turkey jerky initial mean a_w, pH, and MPR prior to inoculation were 0.71, 6.00, and 0.65 respectively. Cured jerky had higher percent pickup and MPR than uncured turkey jerky. Sodium in the modern cure in cured turkey jerky could be contributing to a higher percent pickup and MPR in the cured formulation through binding and retention of moisture.

Table 3-3. Processing characteristics of uncured and cured turkey jerky.

Processing parameters	Uncured	SEM[1]	Cured	SEM[1]
pH[2] (raw turkey breast)	6.24[a]	0.02	6.26[a]	0.03
pH[2] (finished product)	5.96[a]	0.02	6.00[a]	0.01
Incoming nitrite[3] (ppm)	-	-	147.70	1.36
% Pickup[3]	28.87[a]	0.45	31.78[b]	0.29
% Shrink[3]	49.68[a]	0.48	50.07[a]	0.15
a_w[4]	0.69[a]	0.01	0.71[a]	0.01
MPR[5]	0.59[a]	0.01	0.65[b]	0.01

[a-b]Within the same row, means with different letters are significantly different ($P<0.05$).

[1]Standard error of the mean.

[2]n=6.

[3]n=3.

[4]Water activity (n=6).

[5]Moisture-to-protein ratio (n=6).

At a given storage time post inoculation, a_w, pH, MPR, and package oxygen concentration of uncured and cured turkey jerky were measured (Table 3-4). No two-way interaction ($P>0.05$) was observed for formulation type and storage time (Table A-1 in Appendix A). Presence of sodium nitrite did not affect ($P>0.05$) a_w, pH, and MPR values, regardless of

storage time (Table A-1 in Appendix A). Neither time nor formulation treatment affected ($P>0.05$) a_w. The a_w remained constant at 0.73-0.75 ($P>0.05$) throughout 30 d of ambient temperature storage (Table 3-4). A similar observation was reported by Lobaton-Sulabo (2011), where a_w of turkey jerky remained constant at 0.73-0.74 ($P>0.05$) throughout 30 d storage at 25.5°C. Storage time affected ($P<0.05$) pH and MPR, thus, each storage time treatment mean was pooled from formulation treatment means (Table 3-4). By 24 h storage, pH and MPR decreased ($P<0.05$) from 5.95 to 5.83 and 0.58 to 0.53, respectively. There was no change ($P>0.05$) in pH and MPR between 24 h and 30 d of storage; however, the pH of packaged turkey jerky sample at day 30 was slightly more acidic compared to the sample packaged at 72 h.

The package oxygen concentration of turkey jerky packaged in HS, HSOS, and NFOS was also measured and is shown in Table 3-4. The oxygen concentration in HS and NFOS remained constant at 18.7-18.8% and <0.1-0.1%, respectively, throughout storage. As expected, using an oxygen scavenger resulted in a dramatic decrease in oxygen concentration in HSOS between 0 h and 24 h of ambient storage, and then the oxygen concentration remained constant at 0.1% for the remainder of the 30 d storage. The addition of oxygen scavenger in HSOS was effective in decreasing package oxygen concentration. Similar findings were observed for oxygen concentration in HS, HSOS, and NFOS packaged turkey jerky and beef jerky (Lobaton-Sulabo 2009; Lobaton-Sulabo and others 2011). It was reported that oxygen concentration remained constant at an average of 18.9 and <0.2% for HS and NFOS, respectively (Lobaton-Sulabo 2009).

Table 3-4. Mean[1] ± standard error for physical and chemical characteristics of turkey jerky packaged in HSOS and package oxygen concentration of turkey jerky packaged in three packaging systems[2,3] and stored at 21.5°C for up to 30 d.

Time	a_w[4]	pH[5]	MPR[6]	Package Oxygen Concentration[7] (%)		
				HS[2]	HSOS[2]	NFOS[2]
0 h[8]	0.73 ± 0.01^a	5.95 ± 0.03^c	0.58 ± 0.01^c	18.7	18.8	0.1
24 h	0.74 ± 0.01^a	5.83 ± 0.05^{ab}	0.53 ± 0.02^{ab}	18.8	0.2	< 0.1
48 h	0.73 ± 0.01^a	5.83 ± 0.06^{ab}	0.57 ± 0.02^{bc}	18.7	0.1	< 0.1
72 h	0.72 ± 0.01^a	5.91 ± 0.04^{bc}	0.56 ± 0.02^{bc}	18.7	0.1	< 0.1
14 d	0.73 ± 0.01^a	5.76 ± 0.02^a	0.52 ± 0.02^a	18.7	0.1	< 0.1
30 d	0.75 ± 0.01^a	5.80 ± 0.04^{ab}	0.52 ± 0.02^a	18.8	0.1	< 0.1

[a-c]Within the same column, means with different letters are significantly different ($P<0.05$).

[1]Formulation treatment (uncured and cured) means were pooled for each storage time.

[2]HS= Heat sealed; HSOS= Heat sealed with oxygen scavenger; NFOS= Nitrogen flushed with oxygen scavenger.

[3]Oxygen concentration of vacuum packaged treatment was not determined.

[4]a_w=Water activity (n=12).

[5]n=12.

[6]MPR=Moisture-to-protein ratio (n=6).

[7]n=12.

[8]Initial a_w after inoculation and before packaging.

The initial *L. monocytogenes* populations on uncured and cured turkey jerky were 5.93 and 5.94 log CFU/cm^2, respectively. No three-way or formulation by time interactions ($P>0.05$) were observed (Table A-2 in Appendix A). An average of 147.7 ppm ingoing nitrite (Table 3-3) in turkey jerky did not affect ($P>0.05$) *L. monocytogenes* log reductions regardless of packaging type and storage time (Table A-2 in Appendix A). It was expected that incorporation of nitrite in the turkey jerky formulation would increase log reductions since previous reports have indicated that it decreases survival times and slows down growth (Buchanan and Phillips and others 1990; McClure and Kelly 1991; Schlyter and others 1993; Buchanan and others 1994; Duffy and others

1994; Roenbaugh 2011). Contrary to these reports, nitrite addition did not affect survival of *L. monocytogenes* in a simulated uncooked-fermented meat system (Whiting and Masana 1994). This might be due to rapidly declining nitrite level after contacting meat and subsequent heat treatment (Nordin 1969; Dethmers and others 1975; Tompkin 1983; Kilic and others 2001). Junttila and others (1989) concluded that addition of nitrite and nitrate to meat products at officially approved levels may not cause elimination of *L. monocytogenes*. In addition, Kilic and others (2001) indicated that turkey meat has an influence on residual nitrite level in cured meat products. An increased amount of turkey meat in a wiener formula decreased residual nitrite level of the finished product.

A two-way interaction ($P<0.05$) was observed for packaging by time for *L. monocytogenes* reductions (Table A-2 in Appendix A), thus, formulation type treatment means were pooled for each packaging type and storage time. Figure 3-2 shows the mean log reduction (log CFU/cm^2) of *L. monocytogenes* on turkey jerky packaged in the four packaging environments and held at ambient temperature up to 30 d. A mean log reduction of <1.0 log CFU/cm^2 of *L. monocytogenes* was observed in turkey jerky packaged in HSOS, NFOS, and VAC, held for 24, 48, and 72 h at ambient temperature. For these packaging treatments, a mean log reduction above 1.0 log CFU/cm^2 was achieved after 14 d of storage at ambient temperature. For turkey jerky packaged in HS, *L. monocytogenes* was reduced by >1.0 log CFU/cm^2 after 48 or 72 h of storage. Lobaton-Sulabo (2009) performed a similar study using commercially produced uncured turkey jerky and found that mean log reductions of *L. monocytogenes* for all packaging treatments (HS, HSOS, NFOS, and VAC) were <1.0 log CFU/cm^2 after 72 h of ambient temperature.

In addition, Lobaton-Sulabo (2009) also found that the greatest ($P<0.05$) log reduction of *L. monocytogenes* was achieved by packaging turkey jerky in HS and storing it for 30 d, followed by VAC and 30 d storage at ambient temperature. Results of the current study show that the most effective ($P<0.05$) combinations in reducing *L. monocytogenes* were packaging turkey jerky in HS held for 14 and 30 d (2.23 and 2.51 log CFU/cm^2, respectively) and VAC held for 30 d of storage (2.19 log CFU/cm^2) (Figure 3-2). The mean log reduction of *L. monocytogenes* achieved by VAC and 30 d storage combination in the current study was 0.50 log less than the value reported by Ingham and others (2004). In that study, a mean log reduction of

2.7 log CFU/sample was observed on beef jerky packaged in vacuum and stored for 5 weeks at 21°C (Ingham and others 2004).
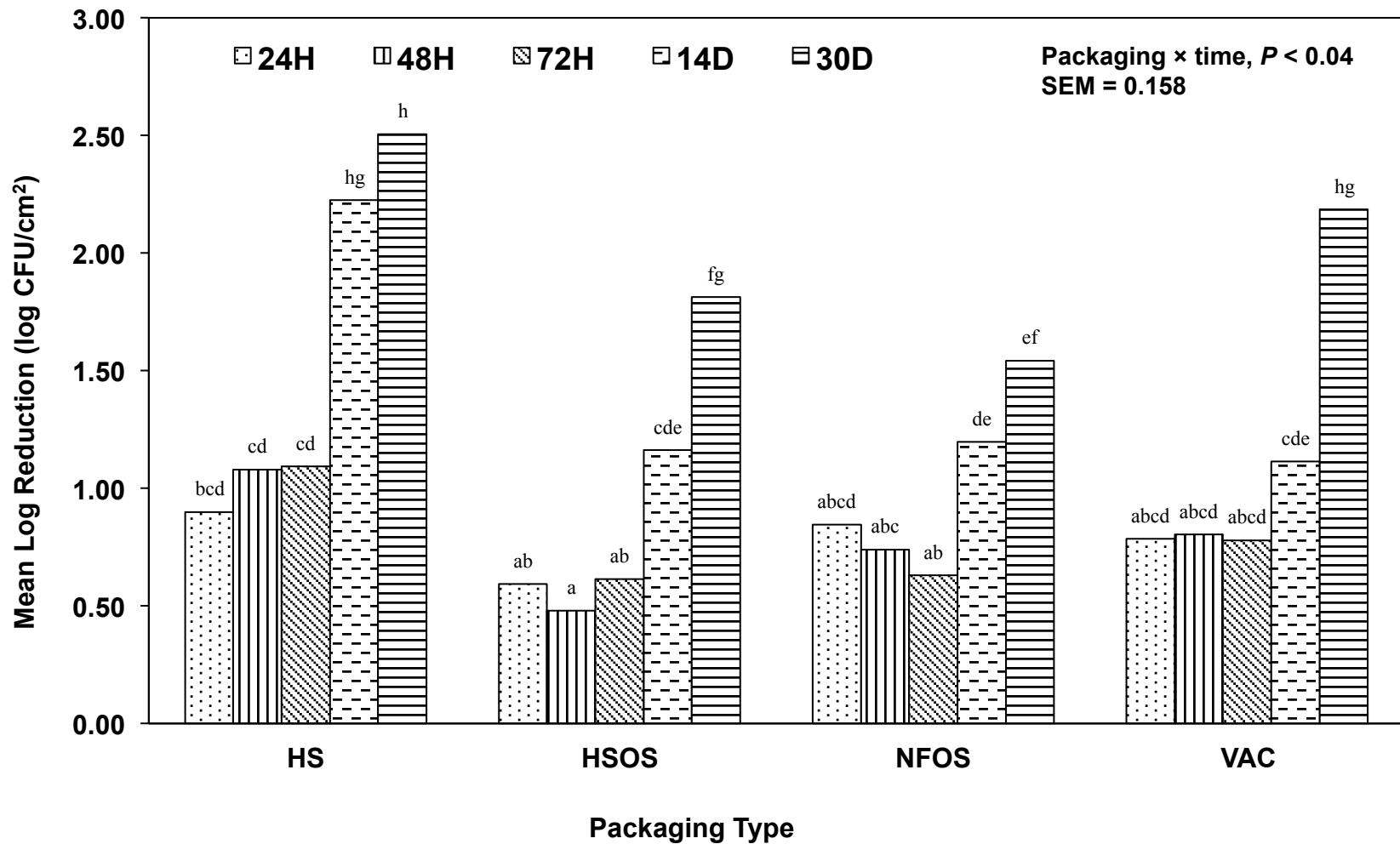
Figure 3-2. Pooled mean (n=12) log reduction (log CFU/cm^2) of *Listeria monocytogenes* on turkey jerky packaged in four packaging treatments and stored at ambient temperature for up to 30 d. HS: heat sealed; HSOS: heat sealed with oxygen scavenger; NFOS: nitrogen flushed with oxygen scavenger; VAC: vacuum packaged. [abcde] Means

Listeria monocytogenes grows well in the presence or absence of oxygen because it is a facultative anaerobic organism (Lungu and others 2009). Results from the current study shows that the aerobic (HS) condition had similar *L. monocytogenes* reduction ($P>0.05$) compared to anaerobic (VAC) conditions during the first 72 h of storage and after 30 d extended storage. The aerobic condition appeared to be more effective ($P<0.05$) in *L. monocytogenes* reduction than reduced oxygen conditions found in HSOS and NFOS, especially after 72 h, 14 and 30 d of storage. Buchanan and Klawitter (1990) reported enhanced growth of *L. monocytogenes* under oxygen restriction in tryptose phosphate broth with a pH 4.5 at 19°C. *Listeria monocytogenes* appears to better tolerate reduced oxygen conditions compared to aerobic conditions. This observation supports the findings of Buchanan and Klawitter (1990) who found that oxygen limitation appeared to enhance growth and survival of *L. monocytogenes* in tryptose phosphate broth (TPB). They observed that *L. monocytogenes* under reduced oxygen condition (N_2 flushed cultures in TPB) had decreased lag phase duration and generation times as compared to *L. monocytogenes* under aerobic conditions at 19°C. Lungu and others (2009) noted that enzymes involved in the tricarboxylic acid cycle and respiration were not found to be highly expressed among low G+C gram positive bacteria like *L. monocytogenes*. They suggested that *L. monocytogenes* had probably adapted to grow rapidly in anaerobic or limited oxygen environments. This is possibly one reason why a higher reduction of *L. monocytogenes* was observed in aerobic packaging (HS) compared to reduced oxygen packaging systems in the current study.

Results show that a_w of 0.73-0.75, as an additional hurdle, was inhibitory against *L. monocytogenes* throughout 30 d storage. Previous studies have shown that RTE meat and poultry products with a ≥24 h storage time at ambient temperature resulted in >1.0 log reductions for RTE meat and poultry products with $a_w<0.85$ (Ingham and others 2004; Ingham and others 2006; Lobaton-Sulabo and others 2011; Uppal and others 2012). Beef jerky with an a_w of <0.79 and packaged in vacuum or heat-sealed with oxygen scavenger after 24 h storage at 25.5°C resulted in a >1.0 log decrease in *L. monocytogenes* population (Lobaton-Sulabo and others 2011). In a similar study, *L. monocytogenes* was reduced by ≥1.0 log CFU/cm^2 on kippered beef and turkey tenders with an average a_w of 0.81 that were stored in vacuum, nitrogen flushed, heat-sealed, or heat-sealed with oxygen scavenger for at least 24 h storage at 23°C (Uppal and others 2012). *Listeria monocytogenes* populations on beef jerky with an a_w of 0.75 decreased by 2.4 log CFU

per sample after being vacuum packaged for 1 week and stored at 21°C; after 4 weeks, no surviving cells were recovered (Ingham and others 2006). Failure of *L. monocytogenes* growth under these conditions supports the theory that no microorganism can grow below the minimum a_w requirement for growth. The minimum a_w for *L. monocytogenes* is 0.92 (ICMSF 1996; Fontana 2008), thus an a_w below this value did not support *L. monocytogenes* growth as evident in the current study. Buchanan and others (1997) noted that *L. monocytogenes* population decreased over time when the microorganism was placed in an environment that does not support growth.

Current results show that a mean reduction of <1.0 log CFU/cm^2 of *L. monocytogenes* was observed in uncured or cured turkey jerky packaged in HS held for 24 h, and in HSOS, NFOS, and VAC held for 24, 48, and 72 h at ambient storage. On the contrary, Lobaton-Sulabo and others (2011) reported a mean log decrease of >1 log CFU/cm^2 on beef jerky packaged in HS after 24 h, and in HSOS, NFOS, and VAC after 48 h of ambient storage. The contrasting results between the two studies could be attributed to the difference in the meat type used. Harper and Getty (2012) reported that addition of NaCl, KCl, CaCl$_2$, and MgCl$_2$, or replacement salt at 2% level in ground turkey formulation did not decrease ($P>0.05$) growth of *L. monocytogenes* during 5 d storage at 4°C. In contrast, a 0.41 log decrease ($P<0.05$) in *L. monocytogenes* population was observed in ground beef formulated with similar salt ingredients and concentration (Harper and Getty 2012).

CONCLUSION

Addition of sodium nitrite did not enhance *L. monocytogenes* reduction regardless of packaging system and storage time. Current data show that 14 d holding time period for HSOS, NFOS, or VAC, and 48 or 72 h holding time for HS packaging achieved >1 log CFU/cm^2 of *L. monocytogenes* on cured or uncured turkey jerky. Processors could use a 48 h holding time for HS packaging and be in compliance with the current *Listeria* Rule of post-lethality treatment in achieving at least 1 log reduction of *L. monocytogenes*.

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Chapter 4 - Investigating *Listeria monocytogenes* Attachment on Uncured or Cured Turkey jerky

ABSTRACT

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) were used to investigate attachment of *L. monocytogenes* to uncured and cured turkey jerky. Laboratory produced uncured or cured turkey jerky was aseptically cut into 4.0 x 4.0 cm pieces, dipped into a five-strain *L. monocytogenes* cocktail, and then air dried at 24.8°C until an approximate $\leq$0.80 water activity was achieved (1-2 h). Samples were then assigned to no packaging (NP) and 14 d packaging treatments, including heat sealed (HS), heat sealed with oxygen scavenger (HSOS), nitrogen flushed with oxygen scavenger (NFOS), and vacuum (VAC). A 7.5 mm diameter sample core was obtained from each treatment and immersion fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium Cacodylate buffer for 16-18 h. SEM analysis showed that *L. monocytogenes* is capable of adhering to turkey jerky surfaces. No difference (P>0.05) was observed in cell attachment between packaging treatments or formulation type due to large variation in cell counts. Results showed that no element was unique to or elevated at sites of *L. monocytogenes* attachment. Based on elemental composition, presence of elemental sulfur might be an indication of sulfur-containing amino acids present in uncured or cured turkey jerky. *Listeria monocytogenes* attachment on turkey jerky could potentially pose a serious health risk in the absence of antilisterial intervention since turkey jerky is consumed without heating.

INTRODUCTION

Listeria monocytogenes is a foodborne pathogen that was responsible for major listeriosis outbreaks associated in ready-to-eat (RTE) turkey products in the U.S. (CDC, 1998; CDC, 1999; CDC, 2002). In spite of its heat sensitivity, its ubiquitous nature enables it to re-contaminate any type of RTE products such as paté, deli meats, and frankfurters (Farber 1991) after heat treatment. It is an opportunistic and a multisystemic (colonizes in multiple host tissues) invasive pathogen that causes severe infections characterized by septicemia and meningoencephalitis (Kuhn and others 2008; EFSA and ECDC 2011). Through blood-borne colonization of the placenta in pregnant women, *L. monocytogenes* could invade the fetus that could eventually lead to abortion. Thus, *L. monocytogenes* post-processing contamination of RTE meats and poultry products potentially poses a serious health risk as these products are consumed without cooking.

In 2011, The CDC published a population-based surveillance report for foodborne illnesses acquired in the U.S. caused by 31 major pathogens, including *L. monocytogenes* (Scallan and others 2011). According to this report, within 2000-2008, an estimated 1,662 illnesses, 1,520 hospitalizations, and 16% case fatality rate were caused by *L. monocytogenes* annually (Scallan and others 2011). All value estimates used in this report were based on the U.S. population in 2006, which was 299 million (Scallan and others 2011). To date, turkey jerky products have not been associated with listeriosis outbreaks. However, jerky products have been subject to recalls due to *L. monocytogenes* contamination detected through routine sampling conducted by processors or regulators in the U.S. (SPCNETWORK 2000, McClure et al 1997, USDA FSIS 2004, USDA FSIS 1996, USDA FSIS 2000).

Previous studies have shown that *L. monocytogenes* was capable of attaching, colonizing, and forming biofilms both on food and abiotic materials commonly used as food-contact surfaces such as radishes, potatoes, RTE meat products, stainless steel, glass, polymers, rubber, granite, polystyrene, marble, and polypropylene (Mafu and others 1990; Hood and Zottola 1997; Smoot 1998; Kalmokoff and others 2001; Chae and others 2006; Silva and others 2008; Caly and others 2009). The association of *L. monocytogenes* with biotic and abiotic surfaces is believed to be a complex process that is governed by physiochemical properties (i.e. charge, hydrophobicity, hydrophilicity, stearic hindrance, roughness, structures and flagella) of the bacterial cell and solid surfaces, components of conditioning layer, protein availability (Chavant and others 2002;

Cunliffe and others 1999; Dickson Koomarie and others 1989; Mafu and others 1991; Smoot 1998), and chemotaxis (Galsworthy and other 1990; Katsikogianni and Missirlis 2004; Baker 2006). Results from these studies are of great value especially in understanding the concern for *L. monocytogenes* contamination of RTE meat products in the absence of antimicrobial interventions to control this organism. This is due to the fact that once *L. monocytogenes* is immobilized on the surface, interactions with its environment, as affected by pH, a_w, temperature, salt content, concentration of salts of organic acids, and package atmospheres, could influence its survival and growth (Glass and others 2002; Ingham and others 2004; Membré and others 2004; Ingham and others 2006; Glass and others 2007; Zuliani and others 2007; Lobaton-Sulabo and others 2011; Uppal and others 2012).

Studying substrate or substratum composition in attachment studies is important because certain elements such as Na, Cl, Fe, Mg, and Ca present in the substrate can influence bacterial growth, stress response, as well as surface chemistry and reactivity (Barnes and others 1999; Jensen and others 2007; Andrews and others 2003; Kerchove and Elimelech 2008; McLaughlin and others 2011). It was reported that NaCl and salts of divalent cations such as Mg^{2+} and Ca^{2+} in alginate film and micro titer plate, enhanced attachment and aggregation of *L. monocytogenes* (Barnes et al. 1999; Jensen and others 2007; Kerchove and Elimech 2008). It was proposed that enhancements in bacterial adhesion by divalent cations rely on screening and neutralization of surface charge (Simoni et al. 2000, Chen and Walker 2007) and on the formation of cationic bridges between specific negatively charged functional groups (Davis et al 2003). Furthermore, Fe is known to be a cofactor in numerous cellular enzymes and its uptake by *L. monocytogenes* supports growth and survival in many diverse environments (McLaughlin and others 2011).

The objective of this study was to determine elemental composition of turkey jerky that could be correlated to attachment. Also, this experiment investigated whether different packaging systems and formulation type affected attachment of *L. monocytogenes* on turkey jerky.

MATERIALS AND METHODS

Description of treatments and design of the experiment

The experiment was a two-factorial design, with formulation type (cured and uncured) and packaging as the factors, with no true replications of experimental units. Analysis of variance of means for cell attachment (log cells/mm^2) was performed using the PROC MIXED procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). Least square means were calculated for each independent variable. Statistical significance and tendencies were set at $P<0.05$ level of significance. The controls used in this experiment were the unfixed uncured and cured turkey jerky. Treated samples included inoculated sample at 0 d and 14 d. Samples at 0 d are those that were analyzed immediately after inoculation (see inoculation procedure) and assigned as no packaging (NP) treatment. Samples at 14 d were packaged samples in different packaging systems: heat seal (HS), heat seal with oxygen scavenger (HSOS), nitrogen flush with oxygen scavenger (NFOS) and vacuum (VAC). One uncured or cured sample (see sample preparation) was assigned to one of the packaging treatments at 14 d ambient storage and all these treatments were stored at 24.8°C.

Sample preparation

Sample preparation, bacterial culture preparation, media preparation, sample inoculation, packaging, and enumeration of *L. monocytogenes* were all conducted in a BL2 laboratory facility at Kansas State University, under IBC #291.

Uncured and cured turkey jerky samples used in this study were obtained from products produced in study 1 (see sample preparation in Chapter 3). Jerky samples were assigned to control and treatment groups. Control samples were immediately processed for SEM imaging (see sample processing) and were not subjected to fixation procedure. Samples in treatment groups were immediately cut into 4 cm x 4 cm pieces using sterile scissors and were placed on a sterile metal tray for inoculation.

Bacterial culture preparation

Lyophilized reference stock cultures of five *L. monocytogenes* strains (Table 4-1) were obtained (Kwik-Stik, Microbiologics, Inc., Grenobel Cedex, France). A pure subculture of each strain was prepared by aseptically transferring a loopful of stock culture to 10 mL of sterile

tryptic soy broth (TSB; Difco BD and Company, Sparks, MD) and incubated at 35 $\pm$ 2°C for 24 h. One liter of the five-strain *L. monocytogenes* cocktail was prepared by aseptically transferring 0.5 mL of each pure culture isolate to 200 mL sterile TSB, and then incubating at 35 $\pm$ 2°C for 24 h. Next, each 200 mL inoculum was transferred into a sterile 1 L beaker to obtain a liter of the five-strain cocktail. A total of 2.0 L of *L. monocytogenes* cocktail per replicate was prepared. One liter was used for uncured turkey jerky and the other liter was used for cured turkey jerky inoculation. Each liter of the five-strain *L. monocytogenes* cocktail contained 8.0 CFU/mL inoculum level. The inoculum level of the five-strain cocktail was determined by spread-plate enumeration technique of *L. monocytogenes* populations (MOX; see media preparation and enumeration of *Listeria monocytogenes* procedure in Chapter 3).

Table 4-1. Five *Listeria monocytogenes* strains used for inoculum.

Strain	Original Source
ATCC 7644	Human
ATCC 19112	Human
ATCC 19115	Chicken (England)
ATCC 19118	Spinal fluid of man (Scotland)
SLR 2249 Cornell University	Laboratory-developed strain where ActA gene was removed

[1]ATCC=American Type Culture Collection.

For confirmation of *L. monocytogenes* cultures, a BD BBL Crystal™ Gram-Positive Identification system (Difco BD and Company, Sparks, MD) was used and performed at least 1 week before inoculation. The kit contained 29 dried biochemical and enzymatic substrates that were used to identify *L. monocytogenes*. The kit also contained a fluorescence negative control in addition to the 29 dehydrated substrates. Color changes or presence of fluorescence, as a result of *L. monocytogenes* metabolic activities, were examined using a BBL panel viewer. The resulting pattern of the 29 reactions was converted into a ten-digit profile number that was used as the basis for identification in the BBL Crystal ID System (Sneath 1957).

Media preparation

Tryptic soy broth was prepared by adding 30 g of the media to 1 L of distilled water, mixed thoroughly, and then microwaved for 2 min until dissolved. Then, TSB was transferred to a 1 L bottle and sterilized at 121°C for 15 min using an Encore[TM] Series sterilizer (Continental Equipment Inc., Tonganoxie, KS).

Packaging and oxygen scavengers

The packaging material used in this study was an oxygen impermeable resealable, 5-mil-thick, low-linear-density polyethylene (LLDPE) clear pouch (oxygen transmission rate: 17 ml/100 in^2 [~645 cm^2] in 24 h, moisture vapor transmission rate of 0.084 g/100 in^2 [~645 cm^2] in 24 h) (19 cm x 36 cm; TPG Co., LTD, Gyeonggi-do, Korea). This was sized to a final dimension of 19 cm x 18 cm and oxygen scavengers (O$_2$-Zero BJ100; TPG Co., LTD, Gyeonggi-do, Korea) were used for HSOS and NFOS treatments.

Sample inoculation and packaging

Inoculation was done at room temperature at 24.8°C. Prior to sample inoculation (control), uncured and cured jerky pieces were obtained and were processed for SEM imaging (see sample processing procedure). A total of 5 uncured and 5 cured jerky pieces, measuring 4.0 cm x 4.0 cm, were obtained aseptically and dipped two at a time in 1 L of *L. monocytogenes* cocktail for 1 min. Samples were immediately hung using sterile binder clips and air dried for 1-2 h. Samples assigned to NP were immediately processed for SEM imaging. The remaining samples were assigned to one of the four packaging treatments. An oxygen scavenger was added to each sample packaged in NFOS or HSOS prior flushing or sealing. Samples assigned to NFOS were flushed with food grade 100% N$_2$ for 10 s and vacuum packaged (Multivac C100, Gepufte Sicherheit, Germany). VAC samples were packaged using the vacuum packer with 600 mm Hg. HS and HSOS packages were sealed using the same equipment by turning off the gas flush and setting the gauge to zero mm Hg. After packaging, all samples were stored for 14 d at ambient temperature at 24.8°C prior to sample processing for SEM analysis.

Sample processing and fixation

A small jerky sample core was aseptically obtained from each sample by punching a circular piece from a packaged sample using a 7.5 mm corneal trephine blade (Ambler Surgical; Exton, PA) against a vinyl chopping board. Using forceps, adhered packaging film in each

sample core was aseptically removed. Each sample core was then removed from the trephine blade. Then, the sample was immediately immersed in 0.50 mL of a fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium Cacodylate buffer (pH= 7.4) in a 2.0 mL clear Wheaton-33® sample vial (Electron Microscopy Sciences; Hatfield, PA) for approximately 16-18 h at 24.8°C with constant agitation in a 60 rpm orbit shaker (RotoMix 48215; Thermolyne, Dubuque, IA). The sample was then rinsed with 0.1 M sodium Cacodylate buffer. After rinsing, the sample was immersed in Cacodylate buffer for 5 min at constant rotation to ensure fixative removal. This rinsing process was done three times. After rinsing, samples were placed under a vacuum of $\sim10^{-3}$ torr overnight in a vacuum oven (Model 5831; National Appliance Co., Portland, OR). Samples were then placed on 12 mm standard carbon adhesive tabs (Electron Microscopy Sciences; Hatfield, PA) attached to 12.7 mm aluminum mounts (Electron Microscopy Sciences; Hatfield, PA) and were stored at 24.8°C in a desiccator prior to analysis.

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS)

A field emission gun scanning electron microscope (FESEM) (Nova NanoSEM 430; FEI® Company, Hillsboro, Oregon, USA) equipped with a low voltage high contrast detector (vCD) at 5kV operating voltage and beam deceleration equal to a landing energy of 1kV was used to examine attachment of *L. monocytogenes* to uncured or cured turkey jerky surfaces. For each sample, there were 10-13 consecutive fields in the x or y directions to eliminate bias and generate a representative overview of *L. monocytogenes* attachment. A total of 10-13 micrographs representing different field of views were examined for direct cell counting. The total square area of each field is shown in Table A-3 in Appendix A. Cell counter plugin in Image J (National Institutes of Health; downloaded from: http://rsbweb.nih.gov/ij/) was used for enumeration and results were reported as log (cells/mm^2). The log (cells/mm^2) was calculated by taking the natural logarithm of the normalized value of direct cell counts per square area in cells/mm^2. Normalized cells/mm^2 was calculated by obtaining the ratio of direct cell counts to the unit area of field of view.

Energy dispersive X-ray spectroscopy (EDS) was performed using a field emission gun scanning electron microscope (FESEM) (Nova NanoSEM 430; FEI Company, Hillsboro, Oregon, USA) equipped with an X-Max Large Area Analytical electron EDS silicon drift detector (SDD) 80 mm^2 (Oxford Instruments; Abingdon, Oxfordshire, UK) at accelerating

voltage of 18 kV and spot size of 3.0. Electron dispersive X-ray spectroscopy was used for spot analysis for identification and quantification of elemental components of control and samples inoculated with *L. monocytogenes*. The element used for optimization was silicon and the standards used are summarized in Table 4-2. Three types of reports were generated: (1) EDS spectra; (2) quantitative report showing concentration in weight % (Wt. %) of each element detected in the sample; and (3) elemental maps showing spatial arrangements and distribution of each element. Concentration of a given element relates to the pixel intensity which corresponds to the number of X-ray counts that enter the X-ray detector at a given energy range, thus, the higher the pixel intensity, the higher the concentration of a given element.

Table 4-2. Elements and standards used for energy dispersive X-ray spectroscopy analysis.

Element	Standard
C	$CaCO_3$
O	SiO_2
N	Not specified
Na	Albite
P	GaP
S	FeS_2
Cl	KCl
K	MAD-10 Feldspar
Ca	Wollastonite
Mg	MgO
Fe	Fe
Si	SiO_2
As	InAs

RESULTS AND DISCUSION

Attachment of *L. monocytogenes* to turkey jerky surfaces was examined under SEM. It was observed that *L. monocytogenes* was capable of attachment on the surface of a dried turkey jerky product. Sample micrographs in Figure 4-1 clearly show that *L. monocytogenes* attached (single to clustered in arrangement) on turkey jerky before and after packaging treatments. Micrographs A and B in Figure 4-1 show initial *L. monocytogenes* attachment on uncured and cured turkey jerky, respectively. This suggests that *L. monocytogenes* was able to attach to uncured and cured turkey jerky after 1 min of contact-time between the sample and the five-strain *L. monocytogenes* inoculum and after 2 h drying. Micrographs also show attachment of *L. monocytogenes* after 14 d packaging (Figure 4-1C to 4-1J), but there is no indication that biofilms were formed. This suggests that conditions in uncured or cured turkey jerky were not sufficient for biofilm formation.

Previous studies have shown both rapid and long contact time permits attachment of *L. monocytogenes* to inert surfaces commonly used as food contact surfaces, such as plastic, rubber, stainless steel, and glass (Mafu and others 1990; Chae and others 2006). Chae and others (2006) demonstrated attachment of *L. monocytogenes* to glass at 37°C after 3 h. An earlier study showed more rapid attachment of *L. monocytogenes*, as short as 20 min to 1 h, onto stainless steel, glass, polypropylene, and rubber (Mafu and others 1990). In vegetable tissues and RTE meat products, *L. monocytogenes* attached more rapidly. Within 5 min of contact time, *L. monocytogenes* was able to attach onto radish slices, potato disks, frankfurters, bologna, ham, and roast beef (Gorski and others 2003; Foong and Dickson 2004; Garrood and others 2004). Although the current experiment used a shorter contact time compared to these studies, the drying time in the procedure provided extra contact time between the product and the five-strain *L. monocytogenes* inoculum.

Attachment of *L. monocytogenes* to turkey jerky samples before and after 14 d packaging are summarized in Table 4-3. Results show no difference ($P>0.05$) in cell attachment between two formulation types or between packaging treatments. No difference in cell attachment observed between treatments was due to large variation in cell counts in each treatment.
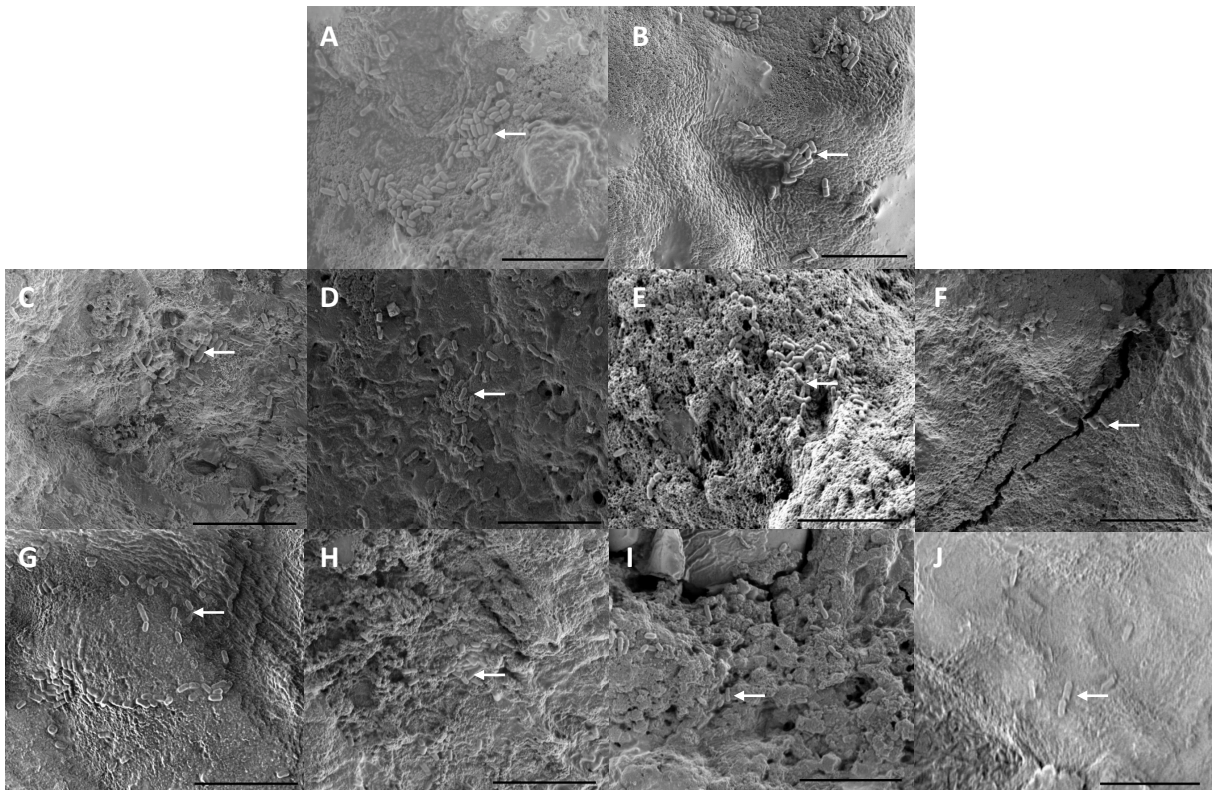
Figure 4-1. Scanning electron microscope (SEM) micrographs showing *Listeria monocytogenes* (pointed by arrows) attachment on turkey jerky (TJ) surfaces. Images were obtained using SEM equipped with vCD detector at 5kV accelerating voltage/landing energy 1kV and 5000x magnification. A=uncured TJ at 0 d; B=cured TJ at 0 d; C=uncured TJ after 14 d HS; D=cured TJ after 14 d HS; E=uncured TJ after 14 d HSOS packaging; F=cured TJ after 14 d HSOS packaging; G=uncured TJ after 14 d NFOS packaging; H=cured TJ after 14 d NFOS packaging; I=uncured after 14 d VAC packaging; J=cured TJ after 14 d VAC packaging. Scale bars equivalent to 10 μm.

Table 4-3. Pooled means[1,2] for *Listeria monocytogenes* attachment on cured or uncured turkey jerky surfaces before and after 14 d packaging.

Formulation	Packaging treatment	Number of fields counted	Total area analyzed (mm^2)	Log (cells/ mm^2)
Uncured		58	0.033590	4.11 ± 0.18^a
Cured		55	0.033048	3.40 ± 0.28^a
	NP[3]	23	0.013719	3.79 ± 0.43^a
	HS 14 d[4]	21	0.010896	3.60 ± 0.46^a
	HSOS 14 d[5]	23	0.011282	4.24 ± 0.30^a
	NFOS 14 d[6]	25	0.013949	4.29 ± 0.27^a
	VAC 14 d[7]	23	0.016790	2.86 ± 0.43^a

[a]Within the same formulation type and packaging treatment, means with different superscripts are significantly different ($P<0.05$).

[1]All packaging treatment means were pooled for each formulation type.

[2]All formulation treatment means were pooled for each packaging treatment.

[3]NP= No packaging; sample analyzed at day 0 or before packaging.

[4]HS= Heat sealed packaging for 14 d.

[5]HSOS= Heat sealed packaging with oxygen scavenger for 14 d.

[6]NFOS= Nitrogen flushed with oxygen scavenger for 14 d.

[7]VAC= Vacuum packaging for 14 d.

The EDS analysis was performed to determine whether specific elements present on turkey jerky were associated with *L. monocytogenes* attachment. It generated three types of data. The first was an X-ray spectrum of elemental components presented as a histogram with horizontal axis labeled in energy units, keV and the vertical axis in x-ray counts (maximum 9566 counts). Second were the measured X-ray intensities of each element in terms of concentration in Wt. %. Lastly, EDS analysis generated elemental maps that showed spatial distribution of each element. Figure 4-2 shows samples of EDS spectra collected from an 830.5 um^2 area on unfixed and fixed uncured or cured turkey jerky samples. X-ray spectra of unfixed samples showed peaks for C, O, N, P, S, Cl, Ca, Fe, K, Na, Mg, and Si (Figure 4-2A and 4-2B). Similar spectral components were obtained from fixed turkey jerky samples except for an additional peak for As (Figure 4-2C and 4-2D). Some elements show multiple peaks such as Fe, K, Ca, and As in the

generated X-ray spectra. Multiple peaks correlate to multiple electron shell interactions. Shell interactions occur when X-rays produced from incident electrons collide with electrons orbiting the K or L shells producing specific element energies related to Kα,β or Lα,β spectral peaks in each element. Presence of an As peak in the X-ray spectra generated from fixed samples was a result of fixation and washing procedures. Sodium Cacodylate buffer used as the washing solution and component of fixative contains As (Hayat, MA 1981). Thus, As peak was removed from all raw X-ray spectra of fixed samples and was not quantified.
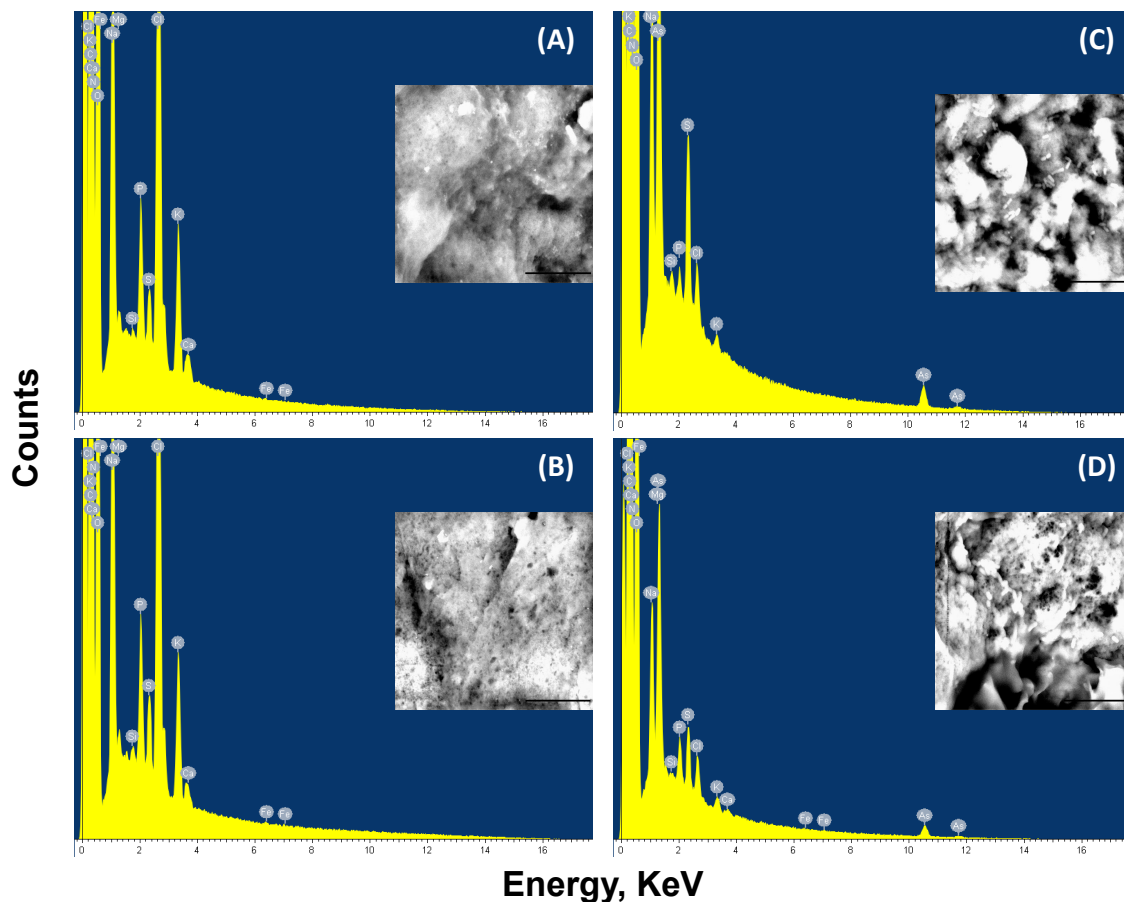
Figure 4-2. Energy dispersive x-ray (EDS) spectrum of unfixed uncured (A) and cured (B) turkey jerky; fixed inoculated uncured (C) and cured (D) turkey jerky with corresponding SEM images of analyzed surfaces below. Scale bar equivalent to 10 µm and surface area analyzed equivalent to 830.5 µm². EDS spectra were generated by X-Max Large Area Analytical EDS equipped with silicon drift detector using 18 kV accelerating voltage at 5000X magnification.

The estimated elemental composition of control and treated samples were determined and estimated Wt. % of elements is summarized in Table 4-4. It was expected that major elements detected in the samples were C, O, and N and these elements approximately comprised more than 50, 15, and 10 Wt. %, respectively. These elements are known to occur in biological specimens as elemental components of amino acids in proteins, fat, and carbohydrates. Other non-metallic components such as P and S consistently showed <1.0 Wt. %. Mineral components such as Na, K, and halide Cl clearly dropped in concentration after inoculation and 14 d packaging. The drop in concentration was possibly a result of the dip-inoculation procedure. The concentration of Mg ranged from 0.11-0.58 Wt. %, while Ca was <0.20 Wt. %. Traces of Si and Fe were also present with <0.10 Wt. %, but Fe was below detection in cured samples without packaging and VAC packaging treatments.

Presence of <1.0 Wt. % of elemental sulfur could be an indication that this might be one of the factors for the slower reduction of *L. monocytogenes* on turkey jerky compared to previous *L. monocytogenes* reduction on beef jerky and kippered beef (Lobaton-Sulabo and others 2011; Uppal and others 2012). Among the non-metallic elements present in turkey jerky, elemental sulfur is of particular concern because *L. monocytogenes* lacks sulphate reductase, thus, it will depend on reduced sulfur sources, such as sulfur-containing amino acids like cysteine and methionine (Slaghuis and others 2007). These two amino acids were confirmed to be present in higher amounts in turkey meat protein compared to beef protein (Brandsch and others 2006) and were reported to be essential amino acids for *L. monocytogenes* growth (Tsai and Hodgson 2003). Tsai and Hodgson (2003) observed that only methionine and cysteine are essential among the 20 amino acids tested on *L. monocytogenes* strain 10403 growth on Hsiang-Ning Tsai medium. This supported the conclusion of Slaghuis and others (2007) that extracellular replicating *L. monocytogenes* will depend on cysteine or methionine (in the absence of cysteine) supply from the host due to its inability to use oxidized sulfur and nitrogen sources. However, the current study was limited to evaluating the elemental composition of turkey jerky. Further studies could focus on comparing elemental or amino acid composition between species and RTE product types.

Table 4-4. Estimated elemental composition of control and inoculated uncured and cured turkey jerky by x-ray analysis.

	Estimated Weight % $\pm$ Standard Deviation[a]											
	Negative Control		0 d after inoculation		HS[b]		HSOS[c]		NFOS[d]		VAC[e]	
Element	UC[f]	C[g]	UC	C	UC	C	UC	C	UC	C	UC	C
Nonmetals												
C	51.88 $\pm$ 5.37	55.90 $\pm$ 0.63	59.36 $\pm$ 4.83	59.14 $\pm$ 2.64	62.21 $\pm$ 0.63	59.76 $\pm$ 0.16	65.72 $\pm$ 1.89	61.82 $\pm$ 1.72	57.33 $\pm$ 0.91	59.14 $\pm$ 2.64	63.09 $\pm$ 4.99	61.11 $\pm$ 0.31
O	28.34 $\pm$ 3.80	22.75 $\pm$ 0.36	25.93 $\pm$ 78	23.37 $\pm$ 2.15	20.42 $\pm$ 0.72	22.94 $\pm$ 0.18	18.29 $\pm$ 1.41	22.60 $\pm$ 1.71	26.92 $\pm$ 1.82	23.37 $\pm$ 2.15	16.94 $\pm$ 2.46	23.37 $\pm$ 0.40
N	10.83 $\pm$ 0.90	12.65 $\pm$ 0.62	12.58 $\pm$ 1.09	15.31 $\pm$ 0.77	14.50 $\pm$ 0.65	15.09 $\pm$ 0.12	12.86 $\pm$ 0.17	13.56 $\pm$ 0.10	13.32 $\pm$ 0.87	15.31 $\pm$ 0.77	18.15 $\pm$ 6.81	13.94 $\pm$ 0.35
P	0.62 $\pm$ 0.14	0.69 $\pm$ 0.06	0.15 $\pm$ 0.03	0.09 $\pm$ 0.01	0.14 $\pm$ 0.03	0.08 $\pm$ 0.02	0.10 $\pm$ 0.01	0.13 $\pm$ 0.01	0.15 $\pm$ 0.02	0.09 $\pm$ 0.01	0.09 $\pm$ 0.06	0.10 $\pm$ 0.01
S	0.35 $\pm$ 0.09	0.40 $\pm$ 0.01	0.23 $\pm$ 0.01	0.41 $\pm$ 0.03	0.55 $\pm$ 0.18	0.36 $\pm$ 0.02	0.35 $\pm$ 0.04	0.36 $\pm$ 0.03	0.68 $\pm$ 0.15	0.41 $\pm$ 0.02	0.32 $\pm$ 0.24	0.36 $\pm$ 0.02
Cl	4.16 $\pm$ 1.99	3.44 $\pm$ 0.13	0.19 $\pm$ 0.01	0.19 $\pm$ 0.03	0.33 $\pm$ 0.07	0.28 $\pm$ 0.01	0.11 $\pm$ 0.02	0.41 $\pm$ 0.02	0.29 $\pm$ 0.06	0.57 $\pm$ 0.11	0.26 $\pm$ 0.25	0.17 $\pm$ 0.01
Metals												
Ca	0.19 $\pm$ 0.09	0.07 $\pm$ 0.02	0.03 $\pm$ 0.01	0.01 $\pm$ 0.00	0.04 $\pm$ 0.02	0.13 $\pm$ 0.01	0.04 $\pm$ 0.01	0.08 $\pm$ 0.06	0.12 $\pm$ 0.03	0.03 $\pm$ 0.01	0.03 $\pm$ 0.02	0.05 $\pm$ 0.03
Fe	0.01 $\pm$ 0.01	0.03 $\pm$ 0.01	0.01 $\pm$ 0.01	<0.01	0.03 $\pm$ 0.02	0.03 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.06 $\pm$ 0.03	0.02 $\pm$ 0.01	0.01 $\pm$ 0.02	<0.01
K	1.26 $\pm$ 0.81	0.88 $\pm$ 0.06	0.06 $\pm$ 0.01	0.05 $\pm$ 0.01	0.04 $\pm$ 0.02	0.05 $\pm$ 0.01	0.04 $\pm$ 0.01	0.08 $\pm$ 0.01	0.14 $\pm$ 0.01	0.10 $\pm$ 0.02	0.04 $\pm$ 0.03	0.04 $\pm$ 0.00
Na	2.07 $\pm$ 0.62	2.95 $\pm$ 0.14	0.88 $\pm$ 0.01	0.97 $\pm$ 0.05	0.63 $\pm$ 0.04	0.74 $\pm$ 0.01	0.52 $\pm$ 0.02	0.60 $\pm$ 0.04	0.52 $\pm$ 0.04	0.95 $\pm$ 0.07	0.39 $\pm$ 0.12	0.53 $\pm$ 0.02
Mg	0.11 $\pm$ 0.02	0.15 $\pm$ 0.02	0.57 $\pm$ 0.03	0.42 $\pm$ 0.37	0.43 $\pm$ 0.06	0.51 $\pm$ 0.00	0.41 $\pm$ 0.02	0.17 $\pm$ 0.13	0.43 $\pm$ 0.06	0.58 $\pm$ 0.02	0.27 $\pm$ 0.12	0.31 $\pm$ 0.01
Si	0.04 $\pm$ 0.03	0.07 $\pm$ 0.01	0.03 $\pm$ 0.01	0.03 $\pm$ 0.03	0.04 $\pm$ 0.03	0.04 $\pm$ 0.01	0.03 $\pm$ 0.01	0.06 $\pm$ 0.00	0.05 $\pm$ 0.00	0.04 $\pm$ 0.01	0.04 $\pm$ 0.04	0.02 $\pm$ 0.01

[a] n= 3 sites.
[b] Heat sealed .
[c] Heat sealed with oxygen scavenger.
[d] Nitrogen flushed with oxygen scavenger.
[e] Vacuum.
[f] Uncured turkey jerky.
[g] Cured turkey jerky.

Elemental maps were obtained to determine whether spatial distribution of elements over a selected spot in each sample is associated to attachment location of *L. monocytogenes*. Sample elemental dot maps are shown in Figure 4-3. It was expected that there would be concentration of certain elements such as Na, Cl, Ca, K, Mg, and Fe on sites of attachment. These elements were reported to enhance adhesion and play a significant role in the growth and homeostasis of *L. monocytogenes* (Barnes and others 1999; Geesya and others 2000; Andrews and others 2003; Jensen and others 2007; Kerchove and Elimelech 2008; McLaughlin and others 2011). In addition, Hochella and others (1989) noted that composition of the substrate could control the reactivity of the surface, which could influence the binding capabilities of the substrate. However, dot maps show that distribution of each element was random (Figure 4-3), thus, indicating that in terms of location, none of the elements were directly associated with attachment of *L. monocytogenes* on turkey jerky.
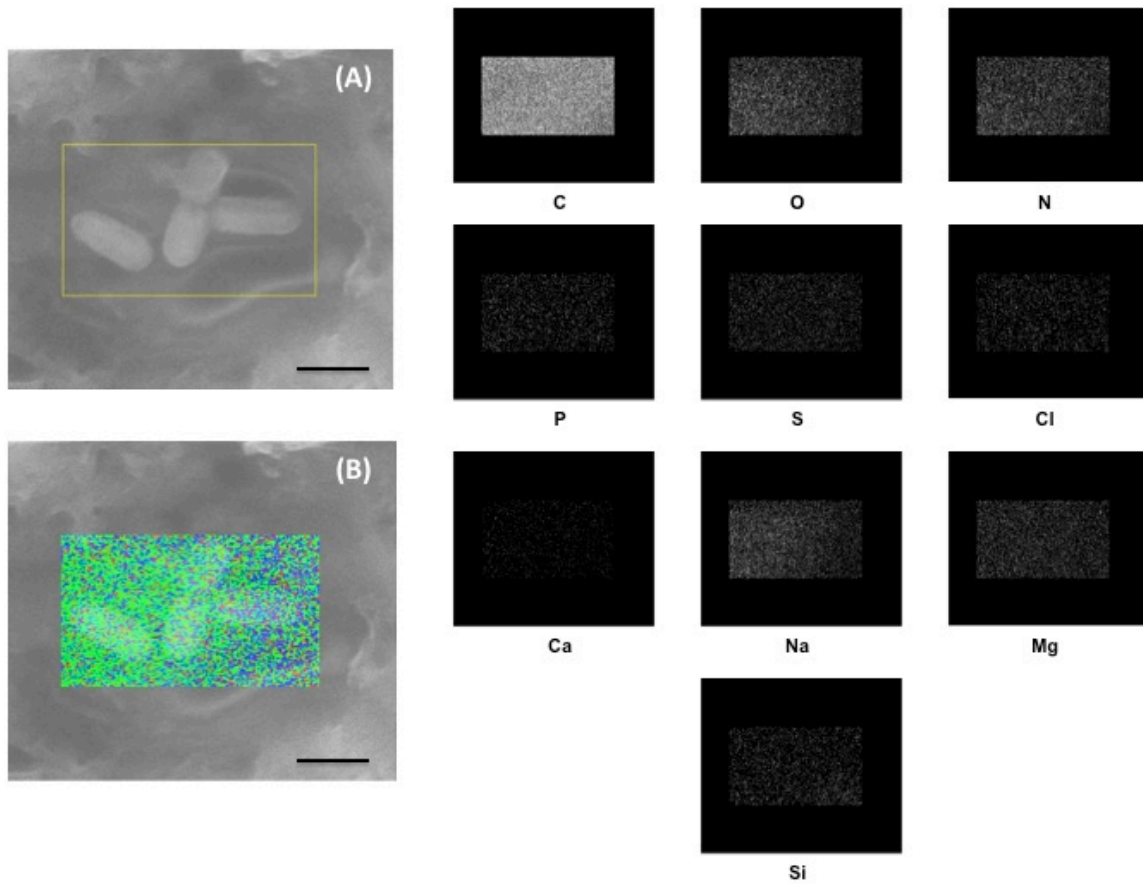
Figure 4-3. Energy dispersive X-ray elemental maps (C, O, N, P, S, Cl, Ca, Na, Mg, and Si) on site of *L. monocytogenes* attachment on cured turkey jerky generated by X-Max Large Area Analytical EDS SDD using 18 kV accelerating voltage at 20000X magnification. Scale bar equivalent to 2 µm. [(A) reference image with *L. monocytogenes* enclosed by yellow box; (B) mixed maps of Ca in red, Na in green, and P in blue with *L. monocytogenes*]. Fe was below detection so no Fe map is shown.

CONCLUSION

Overall, results of SEM analysis suggest that *L. monocytogenes* is capable of adhering to the dried surface of turkey jerky. Results showed that no element was unique to or elevated at sites of *L. monocytogenes* attachment. Based on elemental composition, presence of elemental sulfur might be an indication of sulfur-containing amino acids present in uncured or cured turkey jerky. Further research is needed to determine whether sulfur-containing compounds in RTE products, such as sulfur-containing amino acids, might have an impact on *L. monocytogenes* attachment on turkey jerky products. Since turkey jerky is consumed without heating, *L. monocytogenes* attachment on this product could potentially pose a serious health risk.

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Chapter 5 - Effect of Packaging and Storage Times on Reduction of *Listeria monocytogenes* on RTE Meats and Poultry Snack Products

ABSTRACT

To determine the effect of packaging system and storage time on *Listeria monocytogenes* reduction on five commercial ready-to-eat (RTE) meat and poultry snack products, two packaging types [heat sealed with oxygen scavenger (HSOS) and nitrogen flushed with oxygen scavenger (NFOS)] and four storage times (24, 48, and 72 h, and 14 d) were evaluated. Three batches each of five RTE meat and poultry snack products, namely, whole muscle beef jerky, beef tenders, beef sausage sticks, pork jerky, and turkey sausage sticks, were obtained from a local retail establishment. Samples were dipped into a five-strain *L. monocytogenes* cocktail, air dried at 24.0°C for 1-2 hours, packaged, and then enumerated for *L. monocytogenes* at 0 (for initial count), 24, 48, and 72 h, and 14 d after packaging. Based on measured product characteristics, whole muscle and restructured jerky products had an a_w of ≤0.80, pH of ≤6.35, MPR of 0.71-0.75:1, and 5-26% fat content. Sausage sticks had an a_w of ≤0.91, pH of ≤5.10, MPR of 1.07-1.45:1, and 22-39% fat content. Initial populations following inoculation of *L. monocytogenes* on beef jerky, beef tenders, pork jerky, beef sausage sticks, and turkey sausage sticks were 5.1, 4.7, 4.8, 3.8, and 3.7 log CFU/cm^2, respectively. No product type by packaging by time interaction or packaging by time interaction ($P>0.05$) was observed for mean log CFU/cm^2 reduction of *L. monocytogenes*. Mean log CFU/cm^2 reduction was independently affected by time and product type ($P<0.05$). Results showed that regardless of packaging type and ambient storage time, a mean *L. monocytogenes* reduction of >1.0 log was achieved on all five RTE meat and poultry snack products evaluated. Correlation analysis provided some indication that increasing fat content seemed to increase log CFU/cm^2 reduction; however, the strength of the linear correlation was not sufficient to account for differences in mean log CFU/cm^2 reduction of *L. monocytogenes* on five RTE products. Under the *Listeria* Rule, processors producing beef tenders, beef jerky, beef sausage sticks, pork jerky, and turkey sausage sticks could use HSOS or NFOS in conjunction with 24 h storage time as a post-lethality treatment. Further research is needed to evaluate the effect of fat content on each product type.

INTRODUCTION

Listeria monocytogenes is considered to be one of the major foodborne pathogens in the U.S. accounting for 1,662 illnesses and 1,552 hospitalizations annually (Scallan and others 2011). The organism is ubiquitous throughout nature (Fenlon 1999), thus, it is considered as an environmental contaminant. Several reports have shown that this organism has been frequently isolated from food processing environments (Jaquet and others 1993; Rørvick and others 1995; Hood and Zottola 1995; Destro and others 1996; Gravani 1999; Johansson and others 1999; Miettinen and others 1999). Tompkin and others (1999) noted that common sites for *L. monocytogenes* contamination in food processing plants included filling or packaging equipment, conveyors, chilling solutions, slicers, dicers, shredders, blenders, racks for transporting products, hand tools, gloves, aprons, spiral or blast freezers, product containers, and other equipment and tools used post-thermal processing and before packaging. It was noted that most of the risk of *L. monocytogenes* contamination occurred post-lethality or post-cooking as a result of recontamination (Holley 1997; Tompkin and others 1999).

Ready-to-eat (RTE) meat and poultry products have been associated with major listeriosis outbreaks in the U.S. that caused a total of 177 cases, 37 deaths, and 15 miscarriages from 1998 to 2002 (CDC 1999; CDC 2000; CDC 2002). Semidry sausages and jerky products have yet to be linked to listeriosis; however, these products were subject to recalls due to *L. monocytogenes* contamination detected through routine sampling by processors or regulators (USDA FSIS 1998; USDA FSIS 1999; USDA FSIS 2000; SPCNETWORK 2000; Just-food.com 2002; USDA FSIS 2004; USDA FSIS 2005; Parker Waichman LLP 2008). The United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) has maintained a "zero tolerance" policy for *L. monocytogenes* under the *Listeria* Rule (USDA FSIS 1999; USDA FSIS 2003) due to the severity of the foodborne illness associated with this organism. Under the *Listeria* Rule, the agency mandates establishments producing post-lethality exposed RTE meat and poultry products to employ one of three alternatives to control *L. monocytogenes* (USDA FSIS 2003; USDA FSIS 2012). An establishment operating under alternative 1 must use a post-lethality treatment that reduces or eliminates *L. monocytogenes* on finished products and an antimicrobial agent or process that suppresses or limits *L. monocytogenes* growth throughout the product shelf life. Under alternative 2, an establishment must use either a post-lethality or an antimicrobial agent or process, while under alternative 3, an establishment must use sanitation

measures to control *L. monocytogenes* contamination on the product and in the processing environment.

To increase the margin of safety of RTE meat and poultry products for *L. monocytogenes* control, antilisterial interventions must be able to inhibit growth or inactivate the organism throughout the products shelf life. The USDA FSIS expects post-lethality treatments and antimicrobial agents or processes to achieve at least 1-log lethality and no more than 2-logs of *L. monocytogenes* growth throughout shelf life, respectively (USDA FSIS 2003; USDA FSIS 2012). The USDA FSIS also requires that post-lethality treatments or antimicrobial processes be scientifically validated (USDA FSIS 2003; USDA FSIS 2012). Common antilisterial interventions that have been evaluated and used in RTE meat and poultry products include incorporation of organic acids, nitrite, salt, sulfites, sorbates, lactates and diacetates in the formulation, as well as drying, fermentation, freezing, and packaging (Schlyter and others 1993; Williams and Golden 2001; Mbandi and Shelef 2002; Seman and others 2002; Gill and Holley 2003; Zhu and others 2005; Lobaton-Sulabo and others 2011; Uppal and others 2012).

Packaging and storage time combinations previously evaluated found that heat sealed, heat sealed with oxygen scavenger, nitrogen flushed with oxygen scavenger, or vacuum packaging in conjunction with 24 and 48 h ambient storage can be used as a post-lethality treatment or antimicrobial process in RTE meat and poultry products (Lobaton-Sulabo and others 2011; Uppal and others 2012). Using reduced oxygen packaging such as vacuum, nitrogen flushing and heat sealing with addition of oxygen scavenger for beef jerky, kippered beef, turkey tenders, pork and beef sausage sticks and subsequent storage at ambient temperature for 24 h to four weeks can generate at least 1 log *L. monocytogenes* reduction and growth of this organism was suppressed throughout product shelf life (Ingham and others 2004; Ingham and others 2006b; Lobaton-Sulabo and others 2011; Uppal and others 2012). Lonnecker and others (2010) reported that 78%, 32%, and 14% of the respondents from a survey of Midwestern meat processors used vacuum, no vacuum, and gas flush, respectively, to package shelf stable meat products. This suggests that in spite of the benefits of vacuum packaging, 46% of the shelf stable products were packaged using alternative packaging strategies.

There is evidence that chemical properties such as fat content could decrease or enhance resistance of *L. monocytogenes* in RTE meat system models (Hu and Shelef 1996; Barmpalia-Davis and others 2009; Hack-Youn 2012; Perumalla and others 2013). There is lack of

information whether different product characteristics such as fat and protein content could influence the antilisterial capacity of packaging methods and short-term ambient temperature storage. Thus, the objective of this study was to evaluate the efficacy of different packaging methods and storage times on reducing *L. monocytogenes* on five RTE meat and poultry snack products with different fat and protein contents.

MATERIALS AND METHODS

Treatments, experimental design, and statistical analysis

Five RTE product types were used in this study, including beef jerky, beef tenders, beef sausage sticks, pork jerky, and turkey sausage sticks. Two packaging treatments were used including heat seal with oxygen scavenger (HSOS) and nitrogen flushed with oxygen scavenger (NFOS). All treatments were subjected to four different storage times that included 24, 48, and 72 h, and 14 d. The experimental design used was a completely randomized design (CRD) using a 5 x 2 x 4 factorial treatment structure with sample as the experimental unit. The fixed effects for the statistical analysis were product type, packaging treatment, and storage time, and the interactions of product type x packaging treatment x storage time, product type x storage time, and packaging treatment x storage time. Replication was the random effect. Analysis of variance for comparing treatment mean log reduction was performed using the PROC MIXED procedure of SAS version 9.3 (SAS Inst. Inc., Cary, NC). Least square means were calculated for each independent variable. Statistical significance and tendencies were set at $P<0.05$ level of significance. The experiment was done in triplicate and each replication consisted of duplicate samples in each treatment. Two samples were enumerated for initial *L. monocytogenes* population on day 0, and two samples served as a negative control.

To determine whether water activity (a_w), pH, moisture, protein, fat content, and MPR were affected by storage time (0, 24, 48, and 72 h, and 14 d) and product type after inoculation, a two-way ANOVA with a 5 x 5 factorial treatment structure was used. Analysis of variance was performed using the PROC MIXED procedure of SAS version 9.3 (SAS Inst. Inc., Cary, NC). Least square means were calculated for each independent variable. Statistical significance and tendencies were set at $P<0.05$ level of significance. The experiment was also done in triplicate and each replication consisted of duplicate samples in each treatment.

Strength of linear correlation, Pearson correlation coefficient (r), between log CFU/cm^2 and product characteristics (fat, moisture, and protein content, a_w, and pH) measured at 24, 48, and 72 h, and 14 d were determined using PROC CORR procedure in SAS version 9.3 (SAS Inst. Inc., Cary, NC).

Sample description and preparation

Sample preparation, bacterial culture preparation, media preparation, sample inoculation, packaging, and enumeration of *L. monocytogenes* were all conducted in a BL2 laboratory facility at Kansas State University, under IBC #291. Five commercially available RTE meat and poultry products were purchased from local retail establishments in Manhattan and Junction City, Kansas. A total of three lots per product, representing three production dates, were used (Table 5-1). All samples were aseptically cut into smaller pieces with dimensions described in Table 5-2, and placed in metal trays lined with sterile aluminum foil.

Table 5-1. Ingredients of ready-to-eat meat products evaluated for *Listeria monocytogenes* log reduction during storage.

Product	Quantity	Ingredients
Whole Muscle Beef jerky	Five 280-g bags per lot	Beef, water, sugar, less than 2% salt, corn syrup solids, hydrolyzed corn and soy protein, dried soy sauce (wheat, soybean, salt), monosodium glutamate, flavoring, maltodextrin, spice, sodium erythorbate, and sodium nitrite
Restructured Beef tender strips	Three 340-g bags per lot	Beef, water, soy protein concentrate, dextrose, less than 2% spices, salt, brown sugar, dried beef stock, hydrolyzed corn/soy/wheat proteins, maltodextrins, monosodium glutamate, flavoring, smoke flavor, sodium erythorbate, and sodium nitrite, treated with a solution of sodium sorbate to ensure freshness
Beef sausage sticks	Three 3266-g bags per lot	Beef, salt, corn syrup, spices, contains 2% or less of spices, dextrose, sugar, corn syrup, monosodium glutamate, water, sodium erythorbate, lactic acid starter culture, flavorings, BHA, BHT, citric acid, soybean oil, potassium sorbate
Whole Muscle Pork jerky	Fifteen 1474-g bags per lot	Pork, brown sugar, water, salt, tomato paste, spices and natural flavorings, pineapple juice concentrate, vinegar, and natural smoke flavor
Turkey sausage sticks	Eighteen 680-g sticks per lot	Turkey, mechanically separated turkey, $\leq$ 2% of: salt, corn syrup solids, natural spices, dextrose, lactic acid starter culture, carrageenan, sodium erythorbate (made from sugar), garlic powder, sodium nitrite in collagen casing

Table 5-2. Dimensions and surface area of samples used.

Product	Dimensions (cm)				Surface area (cm^2)
	Length	Width	Height	Radius	
Beef jerky	4.0	4.0	0.1	-	33.6
Beef tenders	8.0	2.0	0.1	-	34.0
Beef sticks	-	-	8.0	0.5	26.7
Pork jerky	4.0	4.0	0.1	-	33.6
Turkey sticks	-	-	8.0	0.5	26.7

Bacterial culture preparation

Lyophilized reference stock cultures of five *L. monocytogenes* strains (Table 5-3) were obtained (Kwik-Stik, Microbiologics, Inc., Grenobel Cedex, France). A pure subculture of each strain was prepared by transferring a loopful of stock culture to 10 mL of pre-sterilized tryptic soy broth (TSB; Difco BD and Company, Sparks, MD) and incubated at $35 \pm 2°C$ for 24 h. One liter of the five-strain *L. monocytogenes* cocktail was prepared aseptically by first transferring 0.5 mL of each pure culture isolate to 200 mL pre-sterilized TSB, and then incubating at $35 \pm 2°C$ for 24 h. Next, each 200 mL inoculum was transferred into a sterile 1 L beaker to obtain one liter of the five-strain cocktail. A total of 5.0 L of *L. monocytogenes* cocktail per replicate was prepared. Each liter of the five-strain *L. monocytogenes* cocktail contained 8-9 log CFU/mL inoculum level. Inoculum level of the five-strain cocktail was determined by spread-plate enumeration technique of *L. monocytogenes* populations (MOX; see media preparation and enumeration of *Listeria monocytogenes* procedure).

For confirmation of *L. monocytogenes* cultures, a BD BBL Crystal ™ Gram-Positive Identification system (Difco BD and Company, Sparks, MD) was used and performed at least 1 week before inoculation. The kit contained 29 dried biochemical and enzymatic substrates that were used to identify *L. monocytogenes*. The kit also contained a fluorescence negative control in addition to the 29 dehydrated substrates. Color changes or presence of fluorescence, as a result of *L. monocytogenes* metabolic activities, were examined using a BBL panel viewer. The resulting pattern of the 29 reactions was converted into a ten-digit profile number that was used as the basis for identification in the BBL Crystal ID System (Sneath 1957).

Table 5-3. Five *Listeria monocytogenes* strains used for inoculum.

Strain	Original Source
ATCC[1] 13932	Spinal fluid of child with meningitis (Germany)
ATCC 19112	Human spinal fluid (Scotland)
ATCC 19115	Human
ATCC 19118	Chicken (England)
SLR 2249 Cornell University	Laboratory-developed strain where ActA gene was removed

[1]ATCC=American Type Culture Collection.

Media preparation

Tryptic soy broth was prepared by adding 30 g of the media to 1 L of distilled water, mixed thoroughly, and then heated in a microwave for 2 min. Then, TSB was transferred to a 1 L bottle and sterilized at 121°C for 15 min using an Encore[TM] Series sterilizer (Continental Equipment Inc., Tonganoxie, KS).

Modified oxford agar (Difco BD and Company, Sparks, MD) was prepared by adding 57.5 g of the media to 1 L of distilled water, mixing, and then boiling for 1 min or until turbidity was lost. Next, MOX was transferred to a 1 L bottle and sterilized at 121°C for 15 min. Media was then cooled down at room temperature to about 45°C. Hydrolyzed oxford antimicrobic supplement (10 mL) (Difco BD and Company, Sparks, MD) dissolved in 10 mL distilled water was immediately added into cooled media. This supplement makes the media selective for *L. monocytogenes*. The supplemented media was then poured into disposable agar plates and allowed to set for at least 2 h at ambient temperature.

Peptone water (0.1%) was prepared by adding 1 g of Bacto[TM] Peptone (Difco BD and Company, Sparks, MD) to 1 L of distilled water, mixed thoroughly, and sterilized at 121°C for 15 min. For preparing peptone blanks for serial dilution, 9 mL of 0.1% peptone was dispensed into 15.0 mL screw-capped tubes and sterilized at 121°C for 15 min.

Packaging and oxygen scavengers

The packaging material used in this study was an oxygen impermeable resealable, 5-mil-thick, low-linear-density polyethylene (LLDPE) clear pouch (oxygen transmission rate: 17 ml/100 in^2 [~645 cm^2] in 24 h, moisture vapor transmission rate of 0.084 g/100 in^2 [~645 cm^2] in

24 h) (19 cm x 36 cm; TPG Co., LTD, Gyeonggi-do, Korea). This was sized to a final dimension of 19 cm x 18 cm. In addition, oxygen scavengers (O_2-Zero BJ100; TPG Co., LTD, Gyeonggi-do, Korea) were used for HSOS and NFOS treatments.

Sample inoculation and packaging

Prior to sample inoculation, a 1 mL portion of the *L. monocytogenes* cocktail was transferred to 9 mL 0.1 % peptone blank to determine the initial inoculum level. Inoculation was performed under a safety cabinet (Purifer Class II Safety Cabinet; LABCONCO, Kansas City, MO). In each product, two samples per treatment for each replicate were obtained aseptically and dipped, two at a time, in 1 L *L. monocytogenes* cocktail for 1 min. Samples were immediately hung using sterile binder clips and air dried for 1-2 h. Surviving *L. monocytogenes* was immediately enumerated on two samples to determine the initial attachment of *L. monocytogenes* to the samples. Samples were then assigned to one of the two packaging treatments and four storage times. Samples assigned to NFOS were flushed for 10 s using food grade 100% N_2 and a vacuum packager (Multivac C100, Gepufte Sicherheit, Germany). The HSOS packages were sealed using the same equipment by turning off the gas flush and setting the gauge to zero mm Hg. One oxygen scavenger was aseptically added to each sample packaged in NFOS or HSOS prior to heat sealing or N_2 flushing. After packaging, all samples were stored at room temperature of 24.0°C for 24, 48, and 72 h, and 14 d storage times.

Chemical and proximate analyses

Two packaged samples per treatment of each product for each replicate were used for determination of a_w and pH. Four composite samples per treatment of each product were used to determine moisture, fat, and protein content. This was done to determine whether there were significant changes in chemical properties of each product throughout storage as a result of the dipping procedure. Since it was not possible to measure the chemical characteristics of inoculated samples, a simulation of the inoculation procedure was done by dipping samples into sterile TSB media without the *L. monocytogenes* inoculum. The a_w, pH, and moisture, fat, and protein content were measured initially before dipping, immediately after drying, and after 24, 48, and 72 h, and 14 d of ambient temperature storage. After drying, the non-inoculated TSB dipped sample pieces were packaged in HSOS.

The a_w of two samples per treatment were determined using an a_w meter (AQUALAB CX2 series 3TE; Decagon, Pullman, WA). The a_w meter was calibrated using a 0.760 NaCl (6.0 molal in water; Decagon, Pullman, WA) verification standard and distilled water at 24.5°C. Each whole muscle pork jerky or beef jerky sample piece was cut into a hexagonal shape with a diameter of approximately 3.2 cm and was placed in sample container following the procedure described by Harper and others (2010) for a_w measurement. Beef tenders were cut using the sample cup as a template, to ensure that sample pieces completely covered the bottom of the sample cup. Beef sausage sticks and turkey sausage sticks were thinly sliced (crosswise) and 7 slices were placed into a sample cup. Duplicate readings were taken for each sample at an average temperature of 24.7°C.

Determination of pH was done by preparing a meat slurry containing 10 g non-inoculated sample with 90 mL distilled water in a double-chamber filter bag (Fisherbrand, Ste-Julie, Quebec, Canada) and stomached (Seward Stomacher 400; Tekmar, Diversified Equipment Company Inc., Lorton, VA) for 1 min. The pH was measured using a calibrated pH meter (Acumet AB15, Fischer Scientific, Kent City, MI) fitted with a flat-surface combination electrode (13-620-530A; Fisherbrand, Vernon Hills, IL) inserted into the filtered portion of the bag. The pH meter was calibrated using pH 4.0 and pH 7.0 buffer solutions (Fisher Scientific, Fair Lawn, NJ) and a pH reading was obtained on two samples per treatment.

The moisture and fat content of four composite samples per treatment packaged in HSOS was determined using the SMART system 5 (CEM Corp., NC) and SMART Trac (CEM Corp., NC) procedure for moisture and fat analysis (AOAC Official 2008.06), respectively. A LECO FP-2000 Protein/Nitrogen Analyzer (model 602-600; LECO Corp., MI) was used to determine nitrogen/protein content (AOAC Official Method 990.03). Measurements were done in duplicate (total of 8 samples).

The oxygen content of the packaged samples was measured after 0, 24, 48, 72 h, and 14 d of ambient temperature storage using an oxygen analyzer (Checkpoint-O_2; PBI Dansensor, DK-400 Kingsted, Denmark). This was done by piercing the package, 1.0 cm away from the seal, with the oxygen detector needle inserted at a 45° angle.

Enumeration of *Listeria monocytogenes*

Two samples per treatment were obtained for *L. monocytogenes* enumeration. Surviving *L. monocytogenes* population in a packaged sample was enumerated using the spread-plate

technique on MOX (with hydrolyzed antimicrobic supplement) plates. This was done by aseptically removing one inoculated sample from a package and transferring it to double-chamber stomacher bag (Fisherbrand, Ste-Julie, Quebec, Canada). Next, 50 mL of 0.1% peptone diluent was added, and contents were stomached (Seward Stomacher 400; Tekmar, Diversified Equipment Company Inc., Lorton, VA) for 1 min. Three serial dilutions were prepared by transferring a 1.0 mL aliquot of the stomached treatment to 9.0 mL of 0.1% peptone blanks. To determine the initial inoculum level, dilutions 1-3 were plated in duplicate on MOX. For treatments that were stored for 24, 48, and 72 h, dilutions 0-3 were plated, while dilutions 0-2 were plated in duplicate on MOX for treatments that were stored for 14 d. MOX plates were incubated at $35 \pm 2°C$ for 48 ± 2 h and counts were reported as log CFU/cm^2. Log reduction was calculated by subtracting *L. monocytogenes* population enumerated at each storage time from the initial *L. monocytogenes* population enumerated at day 0.

RESULTS AND DISCUSSION

The pooled means for chemical and proximate analyses for the five RTE products packaged in HSOS over 14 d of storage are shown in Table 5-4. Water activity (a_w), moisture, protein, and fat content, and MPR of all products were not influenced ($P>0.05$) by storage time after inoculation. Therefore, all time treatment means were pooled for each product type. There were differences ($P<0.05$) in pooled means of a_w, moisture, protein, and content, and MPR between product types. Turkey sausage sticks exhibited the highest ($P<0.05$) a_w of 0.91 followed by beef sausage sticks, beef tenders, and whole muscle pork and beef jerky, which exhibited a_w values of ≤ 0.85. Turkey sausage sticks also contained approximately 11-14% more moisture ($P<0.05$) than beef jerky, beef sausage sticks, pork jerky, and turkey sausage sticks. Beef and pork jerky, and beef tenders exhibited higher ($P<0.05$) protein content (>34%) than beef and turkey sausage sticks. Beef jerky had approximately 6 to 7% more protein content ($P<0.05$) than pork jerky and beef tenders, and at least 15% more protein content than beef and turkey sausage sticks. Among the five RTE products, beef sausage sticks had the highest fat content, being almost 5 and 8 times higher than the fat content of whole muscle pork jerky and beef jerky, respectively. Beef tenders, and whole muscle pork and beef jerky had similar MPR ($P<0.05$) of <0.75, while beef sausage and turkey sausage sticks exhibited an MPR of >1.0.

Table 5-4. Pooled mean[1] $\pm$ standard error for chemical and proximate analyses of five ready-to-eat (RTE) meat and poultry products packaged in HSOS[2] and stored for up to 14 d at 24°C.

RTE Products	a_w[3]	% Moisture	% Protein	% Fat	MPR[4]
Beef jerky	0.73 ± 0.00^a	29.18 ± 0.38^b	41.34 ± 0.45^d	5.14 ± 0.16^a	0.70 ± 0.01^a
Beef tenders	0.80 ± 0.00^c	25.83 ± 0.19^a	34.78 ± 0.19^c	25.70 ± 0.11^d	0.74 ± 0.01^a
Beef sausage sticks	0.85 ± 0.00^d	26.12 ± 0.40^a	24.50 ± 0.05^a	38.63 ± 0.31^e	1.07 ± 0.02^b
Pork jerky	0.77 ± 0.01^b	26.26 ± 0.26^a	35.31 ± 0.22^c	8.18 ± 0.15^b	0.74 ± 0.01^a
Turkey sausage sticks	0.91 ± 0.00^e	39.69 ± 0.49^c	26.47 ± 0.27^b	21.37 ± 0.25^d	1.51 ± 0.03^c

[a-e]Within the same column, means with different letters are significantly different ($P<0.05$).

[1]All storage time treatment means (n=30) were pooled for each product type.

[2]HSOS=Heat sealed with oxygen scavenger.

[3]a_w=Water activity.

[4]MPR=Moisture-to-protein ratio.

There was a product type and storage time interactions ($P<0.05$) for pH of the five RTE meat and poultry products. The measured pH of all RTE products packaged in HSOS for up to 14 d are shown in Table 5-5. Except for beef jerky, pH values of beef sausage and turkey sausage sticks, beef tenders, and pork jerky remained constant throughout 14 d ambient storage. The pH value of beef jerky declined ($P<0.05$) by approximately 0.50 units after 48 h of storage and remained constant 0 ($P>0.05$) throughout the remainder of 14 d storage. This fluctuation in pH value might be due to product stabilization inside the packaging after inoculation. Beef sausage sticks and pork jerky had the lowest and highest pH ($P<0.05$), respectively, throughout 14 d ambient storage.

Table 5-5. Pooled mean[1] ± standard error for pH of five ready-to-eat (RTE) products packaged in heat sealed with oxygen scavenger and stored for up to 14 d at 24°C.

RTE products	Time				
	0 h	24 h	48 h	72 h	14 d
Beef jerky	6.18 ± 0.04^d	6.22 ± 0.04^{de}	5.75 ± 0.04^c	5.69 ± 0.04^c	5.73 ± 0.04^c
Beef tenders	6.21 ± 0.08^d	6.12 ± 0.04^{de}	6.28 ± 0.04^{def}	6.14 ± 0.05^d	6.13 ± 0.04^d
Beef sausage sticks	4.74 ± 0.02^a	4.75 ± 0.02^a	4.75 ± 0.02^a	4.73 ± 0.05^a	4.73 ± 0.01^a
Pork jerky	6.30 ± 0.08^{ef}	6.35 ± 0.04^f	6.33 ± 0.04^f	6.34 ± 0.04^f	6.41 ± 0.03^f
Turkey sausage sticks	5.10 ± 0.02^b	4.98 ± 0.01^b	5.13 ± 0.01^b	5.07 ± 0.03^b	5.05 ± 0.02^b

[abcdef]Means having different superscripts differ within and across ready-to-eat product type ($P<0.05$).

[1]n=6.

The measured package oxygen concentration of all RTE products packaged in HSOS and NFOS initially and during storage time are shown in Table 5-6. The oxygen concentration in HSOS for all products decreased from an initial mean oxygen concentration of 18.7% to 0.10%. The oxygen of NFOS decreased slightly from a mean initial oxygen concentration of 0.20% to <0.10% throughout storage. Nitrogen flushing and addition of oxygen scavenger were effective in decreasing the oxygen concentration in these packaging systems.

Table 5-6. Mean package oxygen concentration for five ready-to-eat meat and poultry products packaged using two packaging systems and stored for up to 14 d at 24.0°C.

| | Mean oxygen concentration (%) | | | |
| | HSOS[1] | | NFOS[2] | |
RTE product	Initial[3]	Storage[4]	Initial[3]	Storage[4]
Beef jerky	18.7	0.10	0.20	<0.10
Beef tenders	18.7	0.10	0.20	<0.10
Beef sticks	18.8	0.10	0.20	<0.10
Pork jerky	18.7	0.10	0.20	<0.10
Turkey sticks	18.8	0.10	0.20	<0.10

[1]HSOS= Heat sealed with oxygen scavenger.

[2]NFOS= Nitrogen flushed with oxygen scavenger.

[3]Initial oxygen concentration (n=6) measured immediately after packaging.

[4]Mean oxygen concentration (n=24) during storage for up to 14 d.

There was no two-way or three-way interactions ($P>0.05$) observed for mean *L. monocytogenes* reductions. Packaging type did not affect ($P>0.05$) mean log CFU/cm^2 reduction of *L. monocytogenes* on five RTE products. Product type and storage time individually affected ($P<0.05$) mean log CFU/cm^2 reduction of *L. monocytogenes* on all products regardless of packaging type. Figure 5-1 shows the effect of time on mean log CFU/cm^2 reduction of *L. monocytogenes*. Each storage time mean was pooled from product type and packaging treatment means. Regardless of product and packaging type, a mean *L. monocytogenes* reduction of >1.0 log (CFU/cm^2) was achieved throughout 72 h storage time. Mean log reduction increased ($P< 0.05$) to 2.0 log CFU/cm^2 after 14 d ambient storage. The mean log CFU/cm^2 reduction of *L. monocytogenes* increased ($P<0.05$) by 0.45 log CFU/cm^2 after 48 h ambient storage and slightly decreased by 0.27 log CFU/cm^2 after 72 h storage.
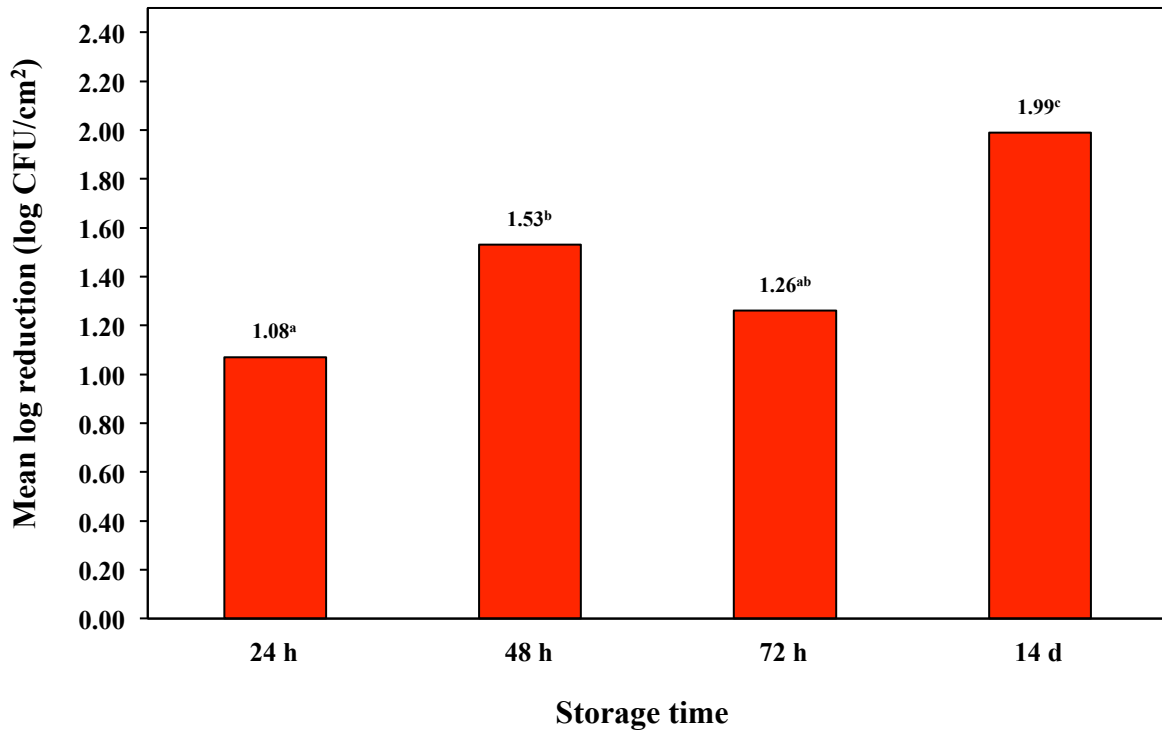
Figure 5-1. Pooled mean (n=60) log CFU/cm^2 reduction of *Listeria monocytogenes* on five packaged ready-to-eat products stored for up to 14 d at 24°C, regardless of packaging type and product type. Reduction was based on an initial *L. monocytogenes* inoculation level (log CFU/cm^2) of 5.1 for beef jerky, 4.7 for beef tenders, 3.8 for beef sausage sticks, 4.8 for pork jerky, and 3.7 for turkey sausage sticks enumerated at 0 d. [a-b]Means having different superscripts differ (*P*<0.05).

Figure 5-2 shows pooled mean *L. monocytogenes* log reductions on packaged RTE products. Each product type mean was pooled from packaging type and storage time treatment means. All products achieved >1.0 pooled mean log CFU/cm^2 reduction of *L. monocytogenes* regardless of packaging and storage time. It is also shown that pork jerky had a lower ($P<0.05$) mean log CFU/cm^2 reduction of *L. monocytogenes* compared to the beef sausage sticks and beef tenders. Beef jerky exhibited 0.50 log reduction less ($P<0.05$) than the mean log reduction of *L. monocytogenes* on beef tenders, but had a similar ($P>0.05$) mean reduction with pork jerky, turkey sausage sticks, and beef sausage sticks.
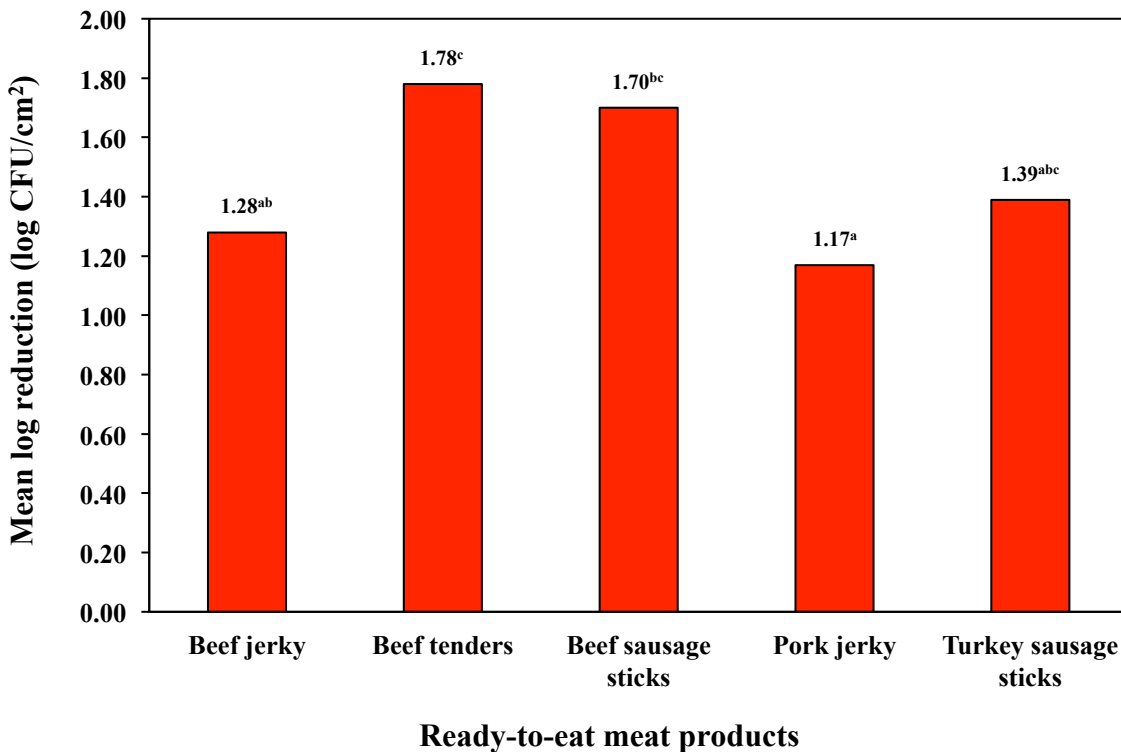
Figure 5-2. Pooled mean (n=48) log CFU/cm^2 reduction of *Listeria monocytogenes* on five ready-to-eat meat and poultry snack products stored at 24°C, regardless of packaging type and storage time. Storage time and packaging treatment means were pooled for each product type. Reduction was based on an initial *L. monocytogenes* inoculation level (log CFU/cm^2) of 5.1 for beef jerky, 4.7 for beef tenders, 3.8 for beef sausage sticks, 4.8 for pork jerky, and 3.7 for turkey sausage sticks enumerated at 0 d. [a-b]Means having different superscripts differ ($P<0.05$).

Except for fat content, protein and moisture content, pH, and a_w had no linear correlation with increasing or decreasing mean log CFU/cm^2 of *L. monocytogenes* (Table 5-7). Barmpalia-Davis and others (2009) found that there was increased resistance of *L. monocytogenes* against gastric conditions in frankfurters containing 32.5% fat versus frankfurters with 4.5%. Conversely, current results suggest that a positive but weak linear correlation ($P</r/$) existed between log CFU/cm^2 reduction of *L. monocytogenes* and fat content (Table 5-7). This suggests that log reduction increases with fat content. The current study shows that mean log CFU/cm^2 reduction of *L. monocytogenes* was higher ($P<0.05$) on higher fat products, such as beef sausage sticks and beef tenders (Figure 5-2) compared to pork jerky. However, there was an approximately 13% difference in fat content between beef sausage sticks and beef tenders although the pooled mean log CFU/cm^2 reduction of *L. monocytogenes* was similar in these two products (Figure 5-3). Increasing fat content from 8 to 26% and 39%, respectively, achieved changes of 0.53 and 0.61 log CFU/cm^2 reduction of *L. monocytogenes*, respectively. Hu and Shelef (1996) reported a decrease of <1.5 CFU/g of *L. monocytogenes* growth when fat content in pork liver sausage batters was increased from 22% to 67%.

The weak correlation between fat content and log reduction of *L. monocytogenes* indicates that knowing the fat content of the product does not provide enough information to predict log reductions of *L. monocytogenes*. It also suggests that fat content is not sufficient to account for differences in mean log CFU/cm^2 reductions of *L. monocytogenes* on the five RTE meat products evaluated. First, storage time affected mean log CFU/cm^2 reduction while fat content remained constant throughout 14 d storage time. In addition, other underlying factors could be contributing to mean log CFU/cm^2 reduction differences in *L. monocytogenes*. One underlying factor could be meat composition that can affect *L. monocytogenes* growth. For instance, sulfur-containing amino acids in meat proteins, such as cysteine and methionine, were reported to be stimulatory and essential amino acids for *L. monocytogenes* growth (Siddiqi and Khan, 1989; Tsai and Hodgson 2003; Slaghuis and others (2007). These two amino acids were reported to be two of the unstable amino acids during extrusion cooking (Ilo and Berghofer 2003). In addition, the type and level of unsaturated fatty acids present in the products might be contributing to differences in mean log CFU/reduction. Unsaturated fatty acids were previously reported to inhibit gram-positive pathogens such as *L. monocytogenes* (Kabarra 1978; Mbandi and others 2004). Furthermore, absence of antimicrobial ingredients in the formulation might

also be attributed on reduced mean log reduction of *L. monocytogenes* on pork jerky compared to other products.

Table 5-7. Correlation coefficients of the relationship between log CFU/cm^2 reduction and chemical characteristics of five ready-to-eat (RTE) meat and poultry products[1].

	a$_w$^{2}	% Fat	% Protein	% Moisture	pH
Pearson correlation coefficients, *r*	0.0664	0.2569	-0.1100	-0.1276	-0.1280
P-value	0.4713	0.0046	0.2319	0.1648	0.1646

[1]Beef jerky, beef tenders, beef sausage sticks, pork sausage, and turkey sausage sticks.

[2]a$_w$= Water activity.

CONCLUSION

Regardless of packaging type and ambient storage time, a mean *L. monocytogenes* reduction of >1.0 log was achieved on five RTE meat and poultry products evaluated. Hence, under the *Listeria* Rule, processors producing beef tenders, beef jerky, beef sausage sticks, pork jerky, and turkey sausage sticks could use HSOS or NFOS in conjunction with 24 h ambient temperature storage time as a post-lethality treatment. Correlation analysis provide some indication that increasing fat content seemed to increase log CFU/cm^2 reduction; however, the strength of the linear correlation was not sufficient to account for differences in mean log CFU/cm^2 reduction of *L. monocytogenes* on five RTE products. Further research is needed to evaluate the effect of fat content on each product type.

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Chapter 6 - Conclusions and Implications

- Inclusion of sodium nitrite in the turkey jerky meat formulation did not affect *L. monocytogenes* reduction. Thus, using sodium nitrite in the meat turkey jerky formulation does not contribute to *L. monocytogenes* control.

- Packaging uncured or cured turkey jerky in HSOS, NFOS, or VAC in conjunction with 24, 48, or 72 h ambient storage time was not effective in reducing *L. monocytogenes* population by at least 1 log. Hence, under the *Listeria* Rule, turkey jerky processors could not use these packaging and storage time combinations as post-lethality treatments.

- Packaging uncured or cured turkey jerky in HS in conjunction with 48 h generated >1 log reduction in *L. monocytogenes* populations, hence, under the *Listeria* Rule, turkey jerky processors could use this packaging and storage time combination as post-lethality treatment.

- Scanning electron microscopy analysis showed that *L. monocytogenes* was capable of attachment and adherence to turkey jerky. Energy dispersive X-ray spectroscopy elemental maps indicate that no element was unique to or elevated at sites of *L. monocytogenes* attachment. Presence of elemental sulfur on turkey jerky might be an indication of sulfur-containing amino acids, such as cysteine and methionine. These two amino acids were previously reported to be essential amino acids for *L. monocytogenes* growth.

- Either HSOS or NFOS in conjunction with 24, 48, or 72 h was effective in reducing *L. monocytogenes* populations by at least 1 log on beef jerky, beef tenders, beef sausage sticks, pork jerky, and turkey sausage sticks. Thus, under the *Listeria* Rule, processors producing these products could use any of these packaging and storage time combinations as post-lethality treatment.

- Storing uncured or cured turkey jerky in HSOS or NFOS for 24, 48, or 72 h was not effective in reducing *L. monocytogenes* population by at least 1 log. In contrast, beef tenders, beef jerky, beef sausage sticks, pork jerky, and turkey sausage sticks packaged in

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HSOS or NFOS for at least 24 h was enough to achieve at least 1 log reduction of *L. monocytogenes*. Specific components present in turkey jerky, such as sulfur-containing amino acids could be contributing to contrasting results. The EDS analysis provide some indication that elemental sulfur is present in turkey jerky, but further research is needed in this area.

- Fat content seemed to increase reduction of *L. monocytogenes* populations on five RTE meat and poultry products. However, correlation analysis revealed that the correlation between mean log reduction and fat content was weak. This indicates that knowing the fat content of the product did not provide enough information to predict log reductions of *L. monocytogenes*. Further research is needed to determine the effect of increasing level of fat content on each product type.

- Overall, nitrite was not an effective ingredient to control *L. monocytogenes* in turkey jerky. However, packaging such as HS, HSOS, NFOS or VAC and at least 24 h holding time were effective hurdles for controlling *L. monocytogenes* at post-lethality.

Appendix A - Supplemental Tables

Table A-1. Probability values (*P*-value) for fixed effects for chemical analyses of uncured and cured turkey jerky.

	P-value		
Fixed effects	a_w[1]	pH	MPR[2]
Time	0.1278	0.0230	0.0375
Formulation type	0.8799	0.8597	0.6400
Time x nitrite	0.1978	0.2304	0.4659

[1]Water activity.

[2]Moisture-to-protein ratio.

Table A-2. Probability values (*P*-value) for fixed effects for mean log CFU/cm^2 reduction of *L. monocytogenes* on uncured and cured turkey jerky.

Fixed effects	*P*-value
Packaging	<0.0001
Time	<0.0001
Formulation type	0.9173
Packaging x Time	0.0353
Packaging x Formulation type	0.5039
Time x Formulation type	0.3428
Packaging x Time x Formulation type	0.2740

Table A-3. Total number of fields and total area analyzed for direct cell count in each uncured and cured turkey jerky samples.

Formulation	Packaging treatment	Number of fields counted	Total area analyzed (μm^2)
Uncured	NP 0h[1]	11	7411.74
Cured	NP 0h	12	6307.85
Uncured	HS 14 d[2]	11	4404.20
Cured	HS 14 d	10	6491.87
Uncured	HSOS 14 d[3]	12	6307.78
Cured	HSOS 14 d	11	4974.38
Uncured	NFOS 14 d[4]	13	7071.08
Cured	NFOS 14 d	12	6878.04
Uncured	VAC 14 d[5]	11	8395.45
Cured	VAC 14 d	10	8395.45

[1]NP= No packaging.

[2]HS= Heat sealed packaging for 14 d.

[3]HSOS= Heat sealed packaging with oxygen scavenger for 14 d.

[4]NFOS= Nitrogen flushed with oxygen scavenger for 14 d.

[5]VAC= Vacuum packaging for 14 d.

Table A-4. Chemical and proximate analyses of five ready-to-eat (RTE) meat and poultry products before inoculation.

RTE product	pH	a_w[1]	% Moisture	% Protein	% Fat
Beef jerky	6.28	0.74	29.79	42.32	5.25
Beef tenders	6.24	0.82	26.43	35.73	25.83
Beef sticks	4.76	0.84	26.21	24.37	39.07
Pork jerky	6.34	0.77	24.98	34.12	8.60
Turkey sticks	5.14	0.91	41.11	25.37	21.15

[1]a_w= Water activity.

Table A-5. Probability values (*P*-value) for fixed effects for mean log CFU/cm^2 reduction of *L. monocytogenes* on five ready-to-eat meat and poultry product.

Fixed effects	*P*-value
Product	0.0342
Packaging	0.9221
Product x Packaging	0.9611
Time	0.0002
Product x Time	0.9213
Packaging x Time	0.9834
Product x Packaging x Time	0.9999

Appendix B - SAS Codes

SAS codes for *L. monocytogenes* Log reductions on uncured and cured turkey jerky in four packaging systems and five storage times in Chapter 3

```
dm 'log;clear;output;clear;';
options ps=85 ls=85;
Title 'Listeria expt 4 x 5 x 2 factorial';
data log;
input packaging $ rep time $ nitrate $ log;
cards;
;
PROC MIXED data=log;
CLASS packaging rep time nitrate;
MODEL log = packaging|time|nitrate;
random rep;
lsmeans packaging|time|nitrate/pdiff;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;
%include 'L:\UIUC\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);
RUN;
```

SAS codes for physicochemical analysis for uncured and cured turkey jerky in HSOS packaging at 0, 24, 48, and 72 h, 14 and 30 d storage times in Chapter 3

SAS codes for a$_w$

```
dm 'Aw;clear;output;clear;';
options ps=85 ls=85;
Title 'Listeria expt 6 x 2 factorial';
data Aw;
input rep time $ nitrite $ Aw;
cards;
Rep    time    nitrite  Aw
;
PROC MIXED data=Aw;
CLASS rep time nitrite;
MODEL Aw = time|nitrite;
random rep;
lsmeans time|nitrite/pdiff;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;
```

```
%include 'L:\UIUC\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);
RUN;
```

SAS codes for pH

```
dm 'pH;clear;output;clear;';
options ps=85 ls=85;
Title 'Listeria expt 6 x 2 factorial';
data pH;
input rep time $ nitrite $ pH;
cards;
;
PROC MIXED data=pH;
CLASS rep time nitrite;
MODEL pH = time|nitrite;
random rep;
lsmeans time|nitrite/pdiff;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;
%include 'L:\UIUC\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);
RUN;
```

SAS codes for MPR

```
dm 'MPR;clear;output;clear;';
options ps=85 ls=85;
Title 'Listeria expt 6 x 2 factorial';
data MPR;
input rep time $ nitrite $ MPR;
cards;
;
PROC MIXED data=MPR;
CLASS rep time nitrite;
MODEL MPR = time|nitrite;
random rep;
lsmeans time|nitrite/pdiff;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;
%include 'L:\UIUC\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);
RUN;
```

SAS Codes for *L. monocytogenes* attachment on turkey jerky in Chapter 4

```
proc import
 datafile="E:\\Dropbox\\Xin Sun\\STAT 945\\ShayneCellDensityData.xls"
 DBMS=xls
 out=work.bacteria;
 getnames=yes;
run;
proc print data=bacteria;
Run;
Proc mixed data=bacteria;
 Class Formulation Treatment;
 Model log_cells_mm2=Formulation Treatment/outp=check;
 Random meat (Formulation Treatment);
 Lsmeans Formulation Treatment/pdiff adjust=tukey;
Run;
*********Check model assumption (variance of the errors should be constant)******;
Proc gplot data=check;
   plot resid*pred/vref=0;
Run;
Quit;
********Check normality of errors assumption*********;
Proc univariate data=check normal plot;
   var resid;
        histogram/normal;
Run;
```

SAS codes in Chapter 5

SAS codes for mean log reductions of *L. monocytogenes* on five ready-to-eat products in two packaging systems and four storage times

```
Title 'Listeria expt 5 x 2 x 4 factorial';
data log;
input product packaging $ rep time $ log;
cards;
;
PROC MIXED data=log;
CLASS product packaging rep time;
MODEL log = product|packaging|time;
random rep rep*product*packaging*time;
lsmeans packaging|time/pdiff;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;
```

SAS codes for chemical analysis of five ready-to-eat meat and poultry products

```
data chem;
input product rep time $ fat;
datalines;
;
proc mixed data=chem;
class product rep time;
model fat moisture protein MPR pH aw= product|time;
random rep;
lsmeans product|time/pdiff stderr;
run;
```

SAS codes for correlation analysis

```
ods graphics on;
data logAwfatpro;
input log Aw fat protein moisture pH MPR @@;
label log='log reduction in logCFU/cm2.'
    fat='fat content in %.'
    protein='protein content in %.'
    moisture='moisture content in %.';
cards;
PROC CORR data=logAwfatpro sscp plots=matrix;
var Aw fat protein moisture pH MPR;
with log;
run;
ods graphics off;
```

Appendix C - Definition of Terms

Antimicrobial process: "An operation applied to a ready-to-eat product that has the effect of suppressing or limiting the growth of a microorganism, such as *Listeria monocytogenes*, in the product throughout the product shelf life." Under the *Listeria* Rule, an antimicrobial process must demonstrate no >2 log increase in *L. monocytogenes* throughout the product shelf life."

Lethality: "A process, including the application of an antimicrobial agent, that eliminates or reduces the number of pathogenic microorganisms on or in a product to make the product safe for human consumption. Examples of lethality treatments are cooking or the application of an antimicrobial agent or process that eliminates or reduces pathogenic microorganisms."

Post-lethality exposed product: "An RTE product that comes into direct contact with a food contact surface after lethality treatment in post-lethality environment."

Post-lethality treatment: "A lethality treatment that is applied or is effective after post-lethality exposure. It is applied to the final product or sealed package of product in order to reduce or eliminate the level of pathogens resulting from contamination from post-lethality exposure." Under the *Listeria* Rule, a post-lethality treatment must demonstrate at least 1 log decrease in *L. monocytogenes* population.

Post-lethality environment: "The area of an establishment into which product is routed after having been subjected to an initial lethality treatment. The product may be exposed to the environment in this area as a result of slicing, peeling, re-bagging, cooling semipermeable encased product with a brine solution, or other procedures."

Ready-to-eat: "A meat or poultry product that is in the form that is edible without additional preparation to achieve food safety and may receive additional preparation for palatability or aesthetic epicurean, gastronomic, or culinary purposes."

References:
1. American Association of Meat Processors. Available at:
http://www.aamp.com/foodsafety/documents/Definitions.pdf

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