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Investigating the control of Listeria monocytogenes on uncured, no-nitrate-or-nitrite-added ready-to-eat meat products using natural antimicrobial ingredients and post-lethality interventions

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Investigating the control of *Listeria monocytogenes* on uncured, no-nitrate-or-nitrite-added ready-to-eat meat products using natural antimicrobial ingredients and post-lethality interventions

by

Nicolas Armando Lavieri

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Meat Science

Program of Study Committee:
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Iowa State University
Ames, Iowa
2013

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DEDICATION

This dissertation is dedicated to my beloved mother, father, and wife. Without you I simply could not have gotten this far in life. I deeply thank you for your support and encouragement throughout this entire process. Without the solid base that you each have represented, none of this would have been possible. I love you all.
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CHAPTER 1. GENERAL INTRODUCTION

The demand for minimally processed natural and organic meat products has grown dramatically over the years and, based on the latest estimates, that trend is likely to continue into the immediate future. Such products are generally viewed as safer and healthier than their conventionally produced counterparts and, as a result, consumers are willing to pay the higher prices associated with these categories of products. Nitrite (NO₂) is an ingredient commonly used in the manufacture of cured processed meat and poultry products and it is known to not only be responsible for the typical color and flavor associated with cured meats, but also to enhance the safety of said products by inhibiting the growth of significant foodborne pathogens such as Clostridium botulinum and, to a lesser degree, Listeria monocytogenes. Alternatively, nitrate (NO₃) can be used to generate nitrite in the manufacture of cured processed meat and poultry products. However, given their classification as chemical preservatives, the direct addition of nitrite and nitrate to natural or organic meat and poultry products is not permitted. It is for this reason that meat processors have started using the indirect addition of nitrite and nitrate obtained from natural sources, most often plant sources, as a means to manufacture meat and poultry products that resemble conventionally cured products in their characteristics but that meet natural or organic strict production requirements.

The use of nitrite and nitrate in the production of meat and poultry products is closely regulated and monitored by the United States Department of Agriculture Food Safety and Inspection Services (USDA FSIS). Additionally, the use of antimicrobial ingredients such as lactate and diacetate is a common practice in the manufacture of conventional processed meat and poultry products as these ingredients have been shown to represent
additional “hurdles” for foodborne pathogens that may be present on these products. All of these hurdles are part of many hurdle technologies used in the meat industry to produce safe and wholesome meat and poultry products for consumers. The importance of using or implementing multiple hurdles becomes even greater when dealing with meat and poultry products that are considered ready-to-eat (RTE) such as deli meats and frankfurters, as these products will typically not be reheated prior to consumption.

“Naturally” cured meat products exhibit cured properties similar to those exhibited by products manufactured using conventional procedures. However, the cured properties of naturally cured meat and poultry products vary in consistency due to factors such as nitrite and nitrate plant source, processing procedures and parameters used, and product characteristics. Additionally, the use of lactate and diacetate, along with that of many other preservatives and antimicrobials commonly used in the manufacture of conventional processed meat and poultry products, is not permitted. These restrictions, combined with the restricted use of nitrite and nitrate, have generated concerns over the perceived risk for foodborne illness associated with natural and organic RTE meat and poultry products.

RTE meat and poultry products receive special regulatory oversight by USDA FSIS due to the increased potential for the presence of *L. monocytogenes* in these products. *L. monocytogenes* is a pathogenic bacterium commonly found in the environment and the causative agent of the foodborne illness known as listeriosis. Due to the relatively high mortality rate associated with listeriosis in at-risk populations and the ubiquitous nature of *L. monocytogenes*, a “zero-tolerance” policy for the presence of this pathogen in RTE meat and poultry products has been instituted by USDA FSIS. This policy is enforced
through a set of strict regulatory guidelines aimed at reducing the incidence of this microorganism in RTE meat and poultry products collectively known as the “Listeria Rule.”

Post-lethality as well as natural antimicrobial interventions have been shown to be effective means to eliminate, reduce, or prevent the growth of *L. monocytogenes* in several food products. Technologies such as high hydrostatic pressure processing, post-packaging thermal treatment, organic acid sprays, among others, represent promising avenues that could lead to increased safety of RTE meat and poultry products in terms of *L. monocytogenes* control. Additionally, natural antimicrobials derived from plants or fruits could add another hurdle that could further hinder the survival and/or recovery of *L. monocytogenes* in RTE meat and poultry products. The use of these post-lethality and natural antimicrobial interventions, if used within guidelines and limits set forth by USDA FSIS, is allowed in natural and organic meat and poultry products. However, the literature on the application of these technologies to natural and organic processed meat products is very limited. Thus, the first objective of this research was to investigate how different post-lethality and natural antimicrobial interventions affect the growth and recovery of *L. monocytogenes* in RTE processed meat products. Furthermore, the second objective of this research was to evaluate the combination of post-lethality and natural antimicrobial interventions in their effectiveness at inhibiting the growth and recovery of *L. monocytogenes* in RTE processed meat products.
CHAPTER 2. LITERATURE REVIEW

History of Nitrite Usage in Processed Meats

Before refrigeration and modern food preservation techniques became widely available, salting, pickling, or marinating were the main means of food preservation, especially for fish, meat, and meat products. The addition of salt (sodium chloride) to such foodstuffs led to a decrease in water activity and, as a result, protected them from microbial spoilage and other negative changes that would otherwise take place over the life of these products (2, 58, 121). The process of using rock salt in the salting of fish, meat, and meat products was what ultimately led to modern curing practices (117, 121).

In ancient Greece, salt that came from “salt gardens” was the salt of choice for preserving fish. The Romans took notice of this practice and extended its application to the preservation of meat and various meat products such as liver sausage, pork sausage, and round sausage. The latter, according to preparation instructions, would be stuffed into a casing and smoked until it turned pink (2, 66). However, as salting became a widely used meat preservation technique, it was determined that using high concentrations of salt could lead to the development of an unattractive grey color in the lean portion of muscles. Thus, those “certain” salts that resulted in a pink color and a desirable flavor would be preferred over those that did not (117). As the art of meat curing was developed and became more widely implemented, it was understood as the addition of salt, sugar, spices, saltpeter (potassium nitrate) or nitrite to meat so as to prevent its spoilage or enhance its flavor (162).
The realization that the contamination of salt with nitrate was responsible for curing was the basis upon which scientists and meat processors around the world developed what we today know about curing reactions (58). Although when exactly nitrite per se was first used to cure meat remains undetermined, it was not until the later part of the nineteenth century that studies showed that nitrite, rather than nitrate, was the key ingredient in curing processes (117, 121). In 1901, Haldane (50) demonstrated by adding nitrite to hemoglobin, thereby forming nitrosylhemoglobin, and heating this mixture that the pigment responsible for the characteristic color of cooked cured meats was nitrosylhemochromogen. A later study conducted by Hoagland (56) concluded that reduction of nitrate in saltpeter to nitrite, nitrous acid (HNO₂), and nitric oxide (NO) thanks to the action of bacteria or enzymes, or a combination of both, was necessary for nitrosylhemoglobin to form. This robust scientific knowledge of the science behind meat curing reactions led to the more widespread use of nitrite, rather than nitrate, in the production of cured meats. That is not to say, however, that nitrate is not currently used in the curing of meat products. The use of nitrate is mostly limited to products that will require relatively long processing times (*i.e.* aging, fermentation) such as fermented sausages and dry-cured hams (2, 117, 139).

A limit of nitrite content for finished meat products was not established until the 1920s by the United States Department of Agriculture (USDA) (165). This limit was originally established at 200 parts per million (ppm or mg/kg) in all finished meat products. Over the next decade, the discovery that reducing compounds such as ascorbic acid could yield NO from nitrite represented another major development in the production of cured meat products. However, it was not until the 1950s that the USDA
allowed for the use of ascorbic acid and ascorbate, as well as their isomers erythorobic acid and erythorbate, in cures (57). We now know these compounds serve as reducing agents that accelerate the conversion of HNO₂ to NO, the latter being the compound that will react with myoglobin in curing reactions (58, 117). In the 1970s, increased demand for finished products resulted in the implementation of acidulating agents that speed up curing reaction rates such as citric acid, acidic phosphates, and glucono-δ-lactone (GDL) (117).

The meat industry has derived unquantifiable benefits from the use of nitrite. Increased food safety, improved flavor and lipid stability, and an overall increased shelf-life of cured meat products are a few of the advantages we have come to expect from cured meat products (138, 147). Thus, it is safe to say that the use of nitrite in cured meat and poultry production has led to the existence of products whose specific flavors, colors, and textures cannot be reproduced by using any other ingredient (121, 138, 147).

**Role of Nitrite in Processed Meats Production**

**Color**

Purchasing decisions made by consumers at the point of purchase are primarily based on the color of the product, regardless of whether it is raw or cooked (120, 121). This is due to the fact that consumers rely heavily on the color of meat and meat products as an indicator of freshness and wholesomeness (93). Annual losses due to surface discoloration in beef products have been estimated to be as high as US $1 billion and as much as 15% of retail beef is discounted due to that very reason (150). The color of meat can range from the deep purple observed in freshly cut beef to the light pink, almost grey
Color associated with faded cured pork, passing through the greenish-brown color seen in overwrapped packages of ground beef that are sometimes found at retail stores. This wide variation in color can stem from several different factors such as age and gender, species, pre- and post-slaughter handling conditions, anatomical location, and many others.

Color, as perceived by the human eye, is the end result of a combination of three key factors; hue, chroma, and value. Hue describes the wavelength of light radiation and, as a result, what we would normally describe as color (blue, red, or yellow). These are often referred to as fundamental colors. Chroma, on the other hand, refers to the purity or saturation of a fundamental color and, therefore, describes its intensity with respect to the amount of white light that is mixed with it. Lastly, value estimates the amount of light reflected by the color, or brightness (2). These three factors can be influenced by many variables such as viewing conditions and an individual’s color perception.

**Fresh Meat Color**

Skeletal muscles are composed of approximately 65-80% water, 16-22% proteins, 1.5-13% lipids, 0.5-1.3% carbohydrates, and trace amounts of minerals (1). Of all of the proteins found in meat, myoglobin is the principal protein responsible for meat color. In fact, in well-bled muscle tissue, as much as 80-90% of the total pigment content is represented by myoglobin (1). However, other proteins such as Cytochrome C and hemoglobin may also play a role in meat color (93, 121). Given that myoglobin is the principal protein responsible for governing meat color, special attention must be paid to it and a thorough understanding of its structure is necessary in order to fully comprehend the chemistry of meat color (2, 93).
Myoglobin is classified as a sarcoplasmic monomeric heme protein and its role in muscle biology is to store oxygen that is to be used by muscles as part of normal biochemical processes (3, 70, 121). Myoglobin is made up two portions; a globular protein portion (globin) and a nonprotein or prosthetic portion referred to as a heme ring (3). Put simply, the globin portion of myoglobin is wrapped around the heme ring and the overall structure of the protein is stabilized by bonding between the two (121). Furthermore, the globin portion of myoglobin is composed of a single polypeptide chain made up of 153 amino acids arranged in eight α-helical structures whose lengths vary from 7 to 24 amino acids. These eight α-helical structures make up approximately 80% of the molecule and are separated by nonhelical regions (83, 120, 121).

Differences in oxygen demands based on anatomical location represent one of the main reasons why marked differences in the myoglobin content of muscles exist. For example, muscles used for support (i.e., longissimus dorsi) have lower oxygen demands than muscles used for locomotion (i.e., semitendinosus) and, as a result, the latter group tends to have a darker red color (3, 93, 120). Species (e.g., the muscles of a bovine are richer in myoglobin content than those of a porcine), age (e.g., the muscles of a cow have higher myoglobin levels than those of a heifer), sex (e.g., the muscles of a bull contain more myoglobin than those of a heifer), training and exercise (e.g., muscles of game animals tend to present greater levels of myoglobin when contrasted to domesticated animals), among others, are a few of the factors that influence the myoglobin content of muscles (3, 120).

Of the two previously mentioned portions that make up myoglobin, the heme ring and, more specifically, the oxidation state of the iron within it have been the focus of
scientists and researchers around the world due to their influence on the ultimate color of meat (3). The heme ring holds a centrally located iron atom that can form up to six bonds with donor ligands. Of these six bonds, four are formed between the iron atom and pyrrole nitrogens while the fifth is formed with the proximal histidine-93. The remaining (sixth) site is free to reversibly bind ligands. The ligand found at that sixth site and the valence of the iron within the heme ring determine muscle color. As a result, four major chemical forms of myoglobin exist and these are considered to be the ones primarily responsible for fresh meat color; deoxymyoglobin, oxymyoglobin, metmyoglobin, and carboxymyoglobin (93, 121).

Deoxymyoglobin results when no ligand is found at the sixth coordination site and the heme iron is found in its ferrous (Fe$^{2+}$) state. These conditions give rise to the purplish-red color associated with freshly cut and vacuum packaged fresh meats (93, 120). Exposure of deoxymyoglobin to oxygen that has diffused through the aqueous environment of meat leads to the development of a bright cherry-red color, a process commonly referred to as blooming. During this process, which typically occurs within 30-45 minutes after exposure to air (3), oxygen becomes bound at the sixth coordination site on the iron atom but no change in the latter’s valence is registered (93, 120). Over time and depending on factors such as oxygen partial pressure, temperature, pH of the meat, and competition for oxygen by bacterial growth and/or other respiratory processes, depth of oxygen penetration will lead to the development of oxymyoglobin below the meat’s surface (3, 93). The stability of the oxymyoglobin complex is strengthened by the presence of a distal histidine-64 (120).
The discoloration of meat stems from the oxidation of both Fe\(^{2+}\) myoglobin derivatives, deoxy and oxymyoglobin, to ferric (Fe\(^{3+}\)) iron (87, 93). Once the iron within the heme ring is oxidized to its Fe\(^{3+}\) state, it cannot combine with other ligands such as oxygen and the pigment is referred to as metmyoglobin (3). Oxygen partial pressure, temperature, pH, the meat’s reducing activity and microbial activity are some of the factors that influence the rate and degree of metmyoglobin formation in fresh meats (93). The formation of metmyoglobin, which is brown in color, represents a major defect in the eyes of consumers given that color, as previously mentioned, is the attribute most commonly judged so as to ascertain the freshness of meat.

Following the oxidation of either deoxy or oxymyoglobin to metmyoglobin, the reduction of the latter pigment can occur. Oxygen scavenging enzymes naturally present in muscles, the nicotinamide adenine dinucleotide (NADH) pool as well as reducing enzyme systems all play an important role in the reduction of metmyoglobin. The reduction of metmyoglobin represents a crucial factor that relates to meat color life (93). The equilibrium that exists between deoxy, oxy and metmyoglobin in the presence of oxygen, an equilibrium that exists thanks to the interconversion of the three, ultimately determines the color of fresh meat (120).

The use of carbon monoxide at levels no greater than 0.4% in modified atmosphere packaging systems (MAP) represents a relatively new packaging technology that was first approved by the United States Food and Drug Administration (USFDA) in 2002 (171). The premise behind the use of carbon monoxide in MAP systems is that the formation of the meat pigment carboxymyoglobin, a pigment that is more stable to oxidation than oxymyoglobin but very similar in color, will take place (15, 93). This
increased stability and relative similarity in appeal observed in carboxymyoglobin has led to several studies aimed at investigating the potential use of carbon monoxide in retail MAP systems (90, 91, 93). However, fears over the potential that the color of fresh meats packaged in carbon monoxide MAP systems may outlast their microbial life and, in the process, compromise the safety of the product have limited the industry wide adoption of such systems (35, 68).

Nitrosylmyoglobin is a fifth chemical form of myoglobin that has recently received increased attention from processors and scientists alike. This pigment is responsible for the red color of raw cured meats that have not been cooked and has been described as a ferrous mononitrosylheme complex wherein a NO group is bound to the sixth coordinate position of the iron atom located within the heme ring of myoglobin (121). The formation of nitrosylmyoglobin in raw meats can stem from direct addition of nitrite (121) or from bacterial action (84). Innovative packaging systems that utilize the coating of packaging films with tightly controlled amounts of sodium nitrite are a relatively new technology that has garnered the attention of the meat industry. This packaging technology recently gained USDA and USFDA recognition as generally recognized as safe (GRAS) after a petition was made by Bemis Company, Inc. Current regulations limit the amount of nitrite to be extruded onto the film to 113 mg per m² of film (172). Furthermore, fresh meats packaged using this system must have a “use by” or “freeze by” date stated on the product label and a statement comparable to “color maintained with sodium nitrite from packaging” must be placed contiguous to the product name (166, 172).
Cured Meat Color

The chemical reactions that take place during curing of meat products are varied and complex. However, they all begin with the generation of a nitrosating species and end with the formation of nitrosylmyochromogen, a pigment that has an attractive pink color and that we commonly associate with cooked cured meat products (120, 121). It is important to understand how this pigment is formed in meat systems and the factors that affect not only how much of it is formed, but also the rate at which it develops.

Nitrite, the conjugate base of the weak acid HNO₂, is not the main nitrosating species but rather one of its derivatives. Given that the pKa of HNO₂ is 3.36 and the pH of most meats will fall in the 5.5-6.5 range, its concentration in cured meat is expected to be low (0.1-1.0%). Instead, it is believed that the principal reactive species found in meat systems is its anhydride, dinitrogen trioxide (N₂O₃). Endogenous or exogenous reductants (HRd) such as NADH or ascorbic acid, respectively, react with N₂O₃ to generate NO, a paramagnetic molecule that is known to form very stable complexes with transition metals such as iron (115, 120, 121). The reactions described above are summarized below and were adapted from Pegg and Shahidi (120):

\[
\text{HNO}_2 \leftrightarrow \text{H}^+ + \text{NO}_2^- \\
2\text{HNO}_2 \leftrightarrow \text{N}_2\text{O}_3 + \text{H}_2\text{O} \\
\text{N}_2\text{O}_3 + \text{HRd} \leftrightarrow \text{RdNO} + \text{HNO}_2 \\
\text{RdNO} \leftrightarrow \text{Rd} + \text{NO}
\]
Shortly after the addition of nitrite to fresh meats, the meat will develop a brown color due to the strong heme oxidant effect of nitrite. Both deoxy and oxymyoglobin are readily oxidized to metmyoglobin by nitrite. Furthermore, upon reduction of nitrite to NO by the action of endogenous or exogenous reductants, as previously described, an intermediate pigment referred to as nitrosylmetmyoglobin is formed. The latter is a relatively unstable pigment that readily autoreduces to its ferrous form, nitrosylmyoglobin, over time and in the presence of endogenous and exogenous reductants. Nitrosylmyoglobin, as previously mentioned, is responsible for the bright red color associated with cured meats that have not been cooked.

Thermal processing brings about the denaturation of the globin portion of nitrosylmyoglobin and its separation from the iron atom. As a result, the pink color characteristic to cooked cured meats will form. The pigment responsible for this color, as previously mentioned, is nitrosylmyochromogen, but it has also been referred to as nitrosylprotoheme and cooked cured-meat pigment. Although additional cooking will not lead to additional changes in nitrosylmyochromogen, this pigment is rather susceptible to photoxidation or photodissociation if presented with the right conditions. This process, commonly referred to as “cured color fading,” has been described as a two-step reaction that takes place when cooked cured meats are stored under aerobic conditions and exposed to light. The first of the two steps is the dissociation of NO from the heme as caused by light. The second step, on the other hand, consists of the oxidation of NO by oxygen. The end result of these two reactions is a brownish-gray color on the exposed meat surface. Thus, the packaging system used and display conditions become essential considerations when it comes to protecting the color life of cured meats.
cooked cured meat products. Enhanced protection of the color life of cooked cured meat products is commonly achieved nowadays by vacuum packaging such products using oxygen impermeable films or by using light impermeable packaging materials (2, 115, 120, 121).

**Flavor**

A flavor that is particular to meat products to which nitrite has been added was first described in 1940 (16, 147) after ham and bacon prepared using brines that contained varying levels of nitrate and nitrate were prepared. Since then, it has been proposed that the impact of nitrite on flavor is a result of the complex chemistry of cured color reactions and that the cured flavor itself is a rather complex stimulus that involves the aroma, odor, texture, temperature, and taste properties of the product (49, 147). However, any specific chemical compounds that may be either directly or indirectly responsible for the characteristic flavor associated with cured meats remain unknown (147).

One of the most important advantages gained by curing meat products is the flavor stability observed in these products during storage (2). The suppression of oxidation compounds, such as lipid oxidation products, due to the antioxidant effect of nitrite has been taunted as a potential explanation for why flavor differences exist between products containing nitrites and those that do not (142, 184). In fact, a reduction in thiobarbituric acid reactive substances (TBARS) as well as in cholesterol oxidation products (COPs) has been observed in products that contain nitrate or nitrite as part of their formulation (38, 107, 184).
The formation of nitrosylmyochromogen and the accompanying immobilization of the heme iron in the complex have been proposed as the main antioxidant effects of nitrite. The immobilization of the iron prevents it from catalyzing the oxidation of unsaturated fatty acids that are present in the product and, as a result, the formation of volatile compounds responsible for warmed-over flavor is reduced. Furthermore, NO can terminate oxidative rancidity reactions that may have already started, demonstrating a second antioxidant effect of nitrite in cured meats (2). Overall, four different mechanisms to explain the antioxidant effects of nitrite in meat systems have been proposed (119):

1. Prevention of the release of iron from the porphyrin molecule due to the formation of a stable complex between heme pigments and nitrite

2. Suppression of the initiation of lipid oxidation through stabilization of unsaturated lipids within membranes

3. Chelation of trace metals, including any non-heme iron from denatured heme pigments

4. Scavenging of free radicals by nitroso and nitrosyl compounds which possess antioxidant properties

**Antimicrobial Properties**

As previously mentioned, one of the main reasons for the development of curing meats throughout the history of mankind was the fact that meats that were cured exhibited an increased microbial shelf-life over those that were not. The use of salt, which contained saltpeter, brought about a decrease in water activity and, as a result, a
reduction in the rate of microbial growth. However, it was not until the 1920s that investigations into the antimicrobial properties or effects of nitrate and nitrite began (118).

Bacterial activity under acidic conditions, when compared to neutral or alkaline conditions, is greatly reduced by the addition of nitrate (92). This phenomenon, these authors concluded, was not due to salt concentration but it was rather a result of the production of small amounts of nitrous and nitric acid from nitrate in mixtures containing reducing substances (92). After investigating the effects of sodium nitrite added at a concentration of 200 mg per kg of fish muscle, Tarr (158, 159) concluded that it exerted a bacteriostatic effect on bacterial species such as *Escherichia*, *Pseudomonas*, *Micrococcus*, *Aerobacter*, and *Achromobacter*. These experiments further cemented the relationship between pH and the efficacy of nitrite after showing that the inhibition of these organisms was greater at pH 5.7 and 6.0 than at pH 7.0.

Nitrite has generally been considered to be more effective at preventing or controlling the growth of Gram-positive bacteria than that of Gram-negative bacteria (147). For example, Buchanan and Solberg (20) concluded that a nitrite concentration of 200 mg/kg had a bacteriostatic effect on *Staphylococcus aureus*, a known foodborne pathogen. On the other hand, Tompkin (161) concluded that nitrite is generally considered to be ineffective at controlling the growth of Gram-negative pathogens such as *Escherichia coli* and *Salmonella*. It is likely that several factors such as residual nitrite level, salt concentration, pH, and presence of exogenous reductants, among other factors, influence the antimicrobial or bacteriostatic properties of nitrite (161).
Nitrite is perhaps better known for preventing the growth of spore forming microorganisms, especially *Clostridium botulinum*. However, the growth of other members of the *Clostridium* genus (i.e., *C. butyricum*, *C. tyrobutyricum*, *C. sporogenes*, and *C. perfringens*) is known to be also affected by nitrite (65). Nonetheless, *C. botulinum* remains one of the deadliest pathogenic foodborne microorganisms and it is for that reason that special attention is paid to the mechanisms involved in its inhibition by nitrite.

Although the color and flavor stability benefits derived from using nitrite are clear, of greater significance are its antibotulinal properties. *C. botulinum* is a Gram positive, anaerobic, spore forming, rod shaped bacterium that is commonly found in soils and waters (65). It is due to its ability to produce a potent neurotoxin (BoNT) which causes a condition known as botulism that this microorganism is of great concern to the food industry. So potent is BoNT that as little as 0.1-1.0 µg can be lethal to humans. Upon ingestion of foods wherein *C. botulinum* has grown and produced BoNT, the neurotoxin is absorbed through the walls of the stomach and intestines and, ultimately, enters the bloodstream. BoNT will then enter the central nervous system and block the release of the important neurotransmitter acetylcholine at nerve-muscle junctions. This neurotransmitter is responsible for the regulation of muscle contraction. Thus, a direct effect of BoNT is a characteristic flaccid paralysis that can lead to muscle weakness, difficulty speaking and swallowing, gastrointestinal disturbances and, in about 30-65% of botulism cases, respiratory failure and death (65). Interestingly, a rather innovative therapeutic use of BoNT type A is its intramuscular injection into patients seeking facial rejuvenation treatment (26). Such treatments have been shown to lead to decreases in
frown lines due to the localized reduction of muscle activity. Fatal incidents in which botulism was involved have decreased mainly due to improvements in the medical care associated with the disease and prompt administration of the BoNT antitoxin (26, 65).

Given its requirement for anaerobic conditions to exist for growth to occur, *C. botulinum* is of particular concern when dealing with products packaged in anaerobic environments (*i.e.*, vacuum packaged). However, concerns over the growth of this microorganism in vacuum packaged meat products are virtually nonexistent so long as nitrite is used as an ingredient. The potent antibotulinal properties of nitrite are poorly understood but speculated to arise from the production of HNO₂ that occurs in the acidic environment of meat systems. Although the antibotulinal effect of nitrite is dependent upon many different factors (*i.e.*, spore level, pH, ingoing nitrite level, residual nitrite level, iron content, salt concentration, temperature, and presence of reducing agents, among others), this effect has been proposed to occur in two stages. The first stage involves the inhibition of the generation of vegetative cells from spores. The second stage involves the control or inhibition of cell division in any vegetative cells that do result from surviving spores (124). Furthermore, the level of ingoing nitrite seems to be more relevant when it comes to inhibiting the growth of *C. botulinum* (63). These same authors suggested that the antibotulinal effect of nitrite arises from nitrite-related reactions that take place during curing and that, when used at levels ≥ 50 mg/kg on an ingoing basis, inhibition of spore germination is achieved while the color and flavor quality of the product are not compromised (63).
Nitrite and Human Health

The use of nitrates and nitrites in the manufacture of food products has been the subject of heated debates and polarizing opinions for many years. Although their use in the curing of meat products, for example, cannot be denied, many consumers still prefer products that are free of nitrates and nitrites. A closer look at the history of the use of nitrates and nitrites reveals that several key events took place during the twentieth century have dictated the course of regulations and beliefs that are still in effect to this very day.

Nitrosamines and Cancer

Of particular importance and relevance to the “nitrite debate” is a report published in 1970 in *Nature* titled “Nitrosamines as Environmental Carcinogens,” by Lijinsky and Epstein (85). This report concluded that nitrosamines were significant carcinogenic compounds and that the best means to address the potential exposure to them was to either eliminate nitrites or to eliminate secondary amines, the two essential precursors needed for any nitrosamine to form. The authors concluded that exposure to both essential precursors resulting from the consumption of cured meat products was likely and, as a result, the consumption of cured meats was viewed as a potential health hazard (85).

Then, in 1979, a study published in *Science* by Dr. Paul Newberne (105) concluded that nitrite itself could lead to the formation of carcinogenic tumors in rats. This study evaluated the effects of supplementing the feed, water, or a semi-purified feed (agar gel) of rats with nitrite concentrations ranging anywhere from 0-2,000 mg/kg nitrite and
concluded that the incidence of malignant lymphomas was increased in all of the treatment groups fed nitrite (10.2% combined incidence) compared to those that were not (5.4% combined incidence). The rats subjected to this study were killed 6, 12, 18, 24 or 56 months into the study and evaluated for the presence of malignant tumors (105). These findings, needless to say, gave rise to debates between regulatory agencies, industry organizations, scientists and, of course, media outlets over the use of nitrite in the manufacture of cured meat and poultry products.

Due to increasing concerns over the use of nitrites in foods, a special National Academy of Sciences (NAS) committee was created in the early 1980s and was tasked with reviewing the available literature on the safety, or lack thereof, of nitrites. After extensive review of the available literature, the NAS committee published two reports titled “The Health Effects of Nitrate, Nitrite, and N-Nitroso Compounds” and “Alternatives to the Current use of Nitrite in Foods” in 1981 and 1982, respectively (101, 102). These reports concluded that “nitrosamines formed endogenously from nitrite in cured meats provide only a small proportion of the total exposure of the general population to nitrosamines from all sources.” It was also concluded by the NAS committee that “…the degree of protection against botulism is likely to decrease if the essential preservative uses of nitrite are substantially reduced without introducing an efficacious, but safer alternative.” A direct result of these two reports was an avoidance of a total ban on the use of nitrite as a food additive in the United States.

Another recommendation made by the NAS committee upon the conclusion of their reports was that nitrite be more thoroughly evaluated in cancer bioassays. It was for that very reason that the FDA suggested nitrite be studied as part of the National
Toxicology Program (NTP) (103). Upon completion of a two-year study, the NTP concluded that there was no evidence of carcinogenic activity of sodium nitrite in male or female F344/N rats and in male B6C3F mice exposed to 750, 1,500 or 3,000 mg/kg in drinking water. Furthermore, the NTP concluded that there was only equivocal evidence (e.g., evidence showing a marginal increase) of carcinogenic activity of sodium nitrite in female B6C3F mice (103). The results obtained by the NTP were viewed as a resounding statement supporting the safety of nitrite as a food additive.

The California Safe Drinking Water and Toxic Enforcement Act of 1986, better known as Proposition 65, sought to classify nitrite as a developmental and reproductive toxicant (DART) (25). A direct consequence of DART classification would have required meat products that contained nitrite to bear a warning on their labels. However, the state’s review committee of independent technical experts voted eight to one against classifying nitrite as a DART.

Despite the fact that the NAS reports, the NTP report, and the Proposition 65 review board all concluded that, based on the evidence available to them, nitrite was not a carcinogenic food additive, the relationship between the consumption of nitrite and cured meats and cancer will undoubtedly continued to be studied and headlined in the media. The relationship, or lack thereof, between red meat consumption and cancer will surely continue to be a subject of studies, reports, and polarizing views for years to come.

**Toxicity**

Nitrate and nitrite are chemical compounds that, when used inappropriately, can be toxic and even deadly to humans. A condition known as methemoglobinemia is caused
by the ingestion of high levels of nitrate or nitrite. The most common symptom of this condition is the development of blue color in the skin. This color develops due to the high amounts of unoxygenated hemoglobin in the blood (176). Upon ingestion, nitrite will oxidize the iron atom within the heme ring of hemoglobin from its Fe\(^{2+}\) to its Fe\(^{3+}\) state, effectively preventing hemoglobin from binding oxygen and delivering it to muscles and other body tissues (124, 176). It has been reported that doses \(\geq 300\) mg of nitrite per kg of body weight are hazardous.

Young children and infants are particularly susceptible to methemoglobinemia due to a lack or underdevelopment of enzyme systems designed to combat oxidizing agents that may be present in the blood. It is for this reason that the USDA currently bans the use of nitrates and nitrites in baby, junior, or toddler foods (169). The term “blue baby syndrome” has been used to describe cases of methemoglobinemia in children and young infants in the past (78). The most common etiology of methemoglobinemia is exposure to oxidizing agents such as drugs. However, contaminated well water and accidental exposure have also been implicated with cases of the disorder (78, 124, 176). The consumption of meat products that contain the legal limits of nitrate and nitrite do not present a methemoglobinemia hazard (124). Nonetheless, prompt recognition of the condition and treatment is of utmost importance in the therapy of methemoglobinemia (176).

**Nitrate, Nitrite, Nitric Oxide, and Human Health**

While nitrate and nitrite represent an integral part of meat curing systems and technologies, they are not only found in cured meat products but also in several other
foodstuffs. In fact, other dietary sources of nitrate and nitrite include, but are not limited to, potatoes, broccoli, beets, celery, spinach, lettuce, fruits, breads, and even our own saliva (174). The fact that vegetables contain such high levels of nitrate should come as no surprise given that nitrate and nitrite are part of the nitrogen cycle of plants and are also byproducts of photosynthesis (13). Furthermore, as much as four fifths of the dietary source of nitrate is represented by vegetables and only one fifth is represented by cured meat products (174). As far as nitrite is concerned, approximately two thirds of the dietary intake comes from saliva and less than one third from cured meats (174).

NO can be synthesized in the human body through the reduction of nitrate by NO synthases, which use the amino acid L-arginine as a substrate, and also by bacteria present in a specialized area on the posterior part of the tongue (7, 22, 46, 47). In fact, it has been estimated that as much as 93% of the total ingested nitrite comes from the salivary reduction of nitrate to nitrite (7). Thus, it is logical to conclude that humans derive the majority of their dietary nitrite from salivary sources and vegetables and that, given the fact that most of it is synthesized in vivo, it may have health benefits associated with it.

NO is a molecule with many different functions within the human body. It has been shown to play a role in the control of smooth muscle relaxation, immune response and the healing of wounds, and neurotransmission, among others. So important is this molecule that the 1998 Nobel Prize for Physiology or Medicine was awarded jointly to Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad “for their discoveries concerning NO as a signaling molecule in the cardiovascular system” (151). Emerging evidence suggests that nitrite itself may play an important role as a significant signaling molecule and even as a
regulator of gene expression independent of NO (18). Furthermore, the action of nitrite as a vasodilator and, therefore, an important regulator of blood flow upon its reduction to NO by hemoglobin has recently been elucidated (36).

After reviewing the available literature on the subject, Suschek and others (156) concluded that NO derived from nitrite plays an integral role in human health. The authors concluded that NO can provide protection via compensatory vasodilation during hypoxia, that in some tissues it can act as an antimicrobial agent, that it may contribute to skin tanning, and that it may also protect against ultra violet- and ROS-induced cell damage. The authors suggested that NO may be such an active molecule due to its relatively rapid diffusion and its ability to penetrate cell membranes. In fact, when compared to molecules such as oxygen and carbon monoxide, the diffusion coefficient of NO has been calculated to be as much as 1.4 times higher than that of oxygen and carbon monoxide at 37°C and, therefore, its diffusion distance in body tissues has been estimated to be of many cell diameters (80). The fact that nitrite is much more stable than NO under physiological conditions and that it can be converted to nitrate, which is even more stable, allows nitrite/nitrate to travel longer distances within the body than NO does. These facts, according to Suschek and others (156), warrant that nitrite be considered a NO “prodrug.”

Upon completion of a two-day symposium at the US National Institutes of Health focused on recent advances in the understanding of nitrite biochemistry, physiology and therapeutics, Gladwin and others (44) concluded that the following are areas wherein more research should be conducted:
The mechanisms of cytoprotection afforded by nitrite after ischemia-reperfusion, and the role of endogenous nitrite and diet in modulating these events

The contribution of NO-dependent and NO-independent signaling in cellular processes regulated by nitrite

The potential role of nitrite-hemoglobin reaction in regulating vascular homeostasis, signaling, and hypoxic vasodilation, and the study of potential intermediates in these reactions and mechanisms of NO export from the red cell

The role of myoglobin and other heme proteins, xanthine oxidoreductase, and other enzyme systems in the ‘physiological’ reduction of nitrite to NO in different tissues at different pH or oxygen gradients”

It should come as no surprise that, given its relatively high biological reactivity, NO has been the target of drug companies seeking to find a therapeutic use for it. As a result, many drugs used in the treatment of different diseases or conditions focus on the targeted delivery of NO to specific tissues. One such example is the use NO gas as part of inhaled NO therapy, which is used as treatment for hypoxaemic respiratory failure and persistent pulmonary hypertension in newborn infants (77). Nitroglycerin, a cardiac drug utilized to dilate smooth muscles and improve oxygenation of heart tissue, uses NO as its active ingredient (32). Reperfusion injury caused by cardiac ischemia is alleviated by treatment with nitrite itself (17). Furthermore, some of the toothpastes designed to treat dentine hypersensitivity are formulated with up to 5% potassium nitrate (173).
Nitrate and Nitrite Regulations in the United States

During the early part of the twentieth century, many of the benefits of meats curing and some of the science associated with them began to become more recognized by the meat industry and researchers, as previously described. Thus, an increase in meat curing practices occurred. Recognizing that unwholesome products could result from the improper use of nitrates and nitrites as part of curing systems, the USDA Bureau of Animal Industry (BAI) commissioned a series of experiments designed to shed some light on the level/safety relationship associated with nitrates and nitrites in cured meats in the 1920s. The following conclusions were reached upon conduction of those studies (14, 147):

- “From 0.25-1 oz of sodium nitrite is sufficient to fix the color in 100 lb of meat, the exact quantity depending on the meat to be cured and process to be employed

- A shortening of the customary curing period may be obtained by the use of nitrite

- Meats cured with sodium nitrite need contain no more nitrites than meats cured with nitrates, and are free from the unconverted nitrates regularly present in nitrate-cured meats”

These findings resulted in the first regulatory limits for the use of nitrate and nitrite in the curing of meat products in 1925 (165). At that time, the established regulations dictated that \( \leq 200 \text{ mg/kg} \) nitrate, nitrite, or a combination of both on an ingoing basis was permitted.
Further research in nitrate and nitrite chemistry and, more importantly, the increased demand for finished products and shorter processing times led to further research into cure accelerators in the 1930s (117). The discovery that ascorbic acid would effectively reduce nitrate to nitrite was made in 1934 (75). However, the approval for use in curing systems of ascorbic acid, ascorbate and their isomers, erythorbic acid and erythorbate, by the USDA had to wait until the 1950s (57). These ingredients, as previously stated, serve as reductants and, as such, accelerate the conversion of HNO₂ to NO and the latter’s subsequent reaction with myoglobin. Additionally, these ingredients serve as oxygen scavengers and assist in the prevention of cured color fading (117).

An industry-wide shift towards the use of nitrite instead of nitrate was observed in the 1970s and it is thought to have occurred mainly due to several reasons such as an increased demand for cured products and the accompanying need for shorter curing times, an increased use of cure accelerators, and a growing concern over the potential formation of nitrosamines (60). This trend towards a more widespread use of nitrite is still seen today as the use of nitrate is rare and typically restricted to the manufacture of specialty products that require longer production times (i.e., dry and semi-dry sausages, fermented sausages, dry-cured hams) (169).

Current regulations limit nitrate and nitrite usage to the following levels (mg/kg) based on the curing method employed (169):
Curing Methods

<table>
<thead>
<tr>
<th>Curing Agent</th>
<th>Immersion Cured</th>
<th>Massaged or Pumped</th>
<th>Comminuted</th>
<th>Dry Cured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Nitrite</td>
<td>200</td>
<td>200</td>
<td>156</td>
<td>625</td>
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<tr>
<td>Potassium Nitrite</td>
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<td>2187</td>
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<tr>
<td>Potassium Nitrate</td>
<td>700</td>
<td>700</td>
<td>1718</td>
<td>2187</td>
</tr>
</tbody>
</table>

Table 2.1. Maximum ingoing nitrate and nitrite limits (in mg/kg) for meat and poultry products except for bacon, a product for which more stringent limits for curing agents exist to reduce the formation of nitrosamines (169).

All calculations associated with curing agents are based on the green (meat block) weight of the meat and/or poultry and/or meat/poultry byproduct. As per Table 2.1, maximum allowable levels of nitrate and nitrite vary based on the curing method employed. This is due to the fact the different curing methods are expected to vary in the efficiency with which the curing agent is brought into contact with the meat and/or poultry product. As a result, comminuted products wherein the chopping, emulsification, grinding, and/or mixing processes will augment surface area and distribution of the curing agents in contrast with dry curing, for example, require less of those curing agents for the development of the typical characteristics of a cured product. More than one of the curing methods listed in Table 2.1 may be used to manufacture a meat and/or poultry product. In such cases, however, nitrate and/or nitrite mg/kg must first be calculated independently for each method. Each curing agent must then be calculated as a percentage of the maximum permitted for the curing method employed and the total percentage of each curing agent cannot exceed 100% (169). Furthermore, nitrate and nitrite may be used together in a single curing method of a meat and/or poultry product. In such a case, nitrate and nitrite mg/kg must be calculated independently and each one is
allowed to be used at up to the permitted levels listed in Table 2.1. A combination of nitrate and nitrite must not result in more than 200 mg/kg nitrite, calculated as sodium nitrite, in the finished product as calculated by laboratory analysis (169).

Due to concerns associated with the potential formation of nitrosamines in bacon, the use of nitrate is no longer permitted and more stringent nitrite limits are in place. 120 mg/kg sodium nitrite or 148 mg/kg potassium nitrite must be added to massaged and/or pumped rind-off bacon. The potassium form of nitrite may be added at the higher rate to account for that element’s higher atomic weight. Additionally, 550 mg/kg sodium ascorbate or sodium erythorbate must be added to pumped and/or massaged bacon on an ingoing basis. In the case of dry cured, rind-off bacon, the maximum level permitted is 200 mg/kg sodium nitrite or 246 potassium nitrite. If bacon is to be manufactured with the rind (skin) on, regardless of the curing method to be employed, the maximum limits of sodium and potassium nitrite as well as those of cure accelerators must be adjusted due to the fact that the skin, which represents about 10% of the weight of the belly, retains practically no cure agent or cure solution. This means that the levels of sodium or potassium nitrite as well as those of the cure accelerators must be reduced by 10% each. Furthermore, USDA FSIS allows for a ± 20% allowance at the time of injecting or massaging due to “variables in pumping procedures, draining, purge, etc.” (169).

USDA FSIS require that all cured “Keep Refrigerated” meat and/or poultry products contain a minimum of 120 mg/kg ingoing nitrite unless the establishment can demonstrate that safety can be assured by some other preservation method, such as pH control, moisture control, or thermal processing. On the other hand, there is no regulatory requirement as far as a minimum nitrite concentration for those products that have been
processed to ensure their shelf stability. However, some canned products that have been thermally processed and are shelf-stable do have a minimum nitrite level that must be monitored because it is viewed as critical in the products’ process schedule. Finally, no nitrate or nitrite can be used in the manufacture of baby, junior or toddler foods, as previously mentioned (169).

Uncured, No-Nitrate-or-Nitrate-Added, Natural and Organic Meat and Poultry Products

Rationale

In many parts of the world, natural and organic foods have been experiencing noticeable market growth over the last few decades (139, 175). Processed meat products within both of those categories have accounted for a significant part of that growth. In fact, in 2011 in the United States, meat, fish and poultry represented the fastest growing of the eight categories of organic foods after experiencing a 13% increase in sales over 2010 (108). Sales growth predictions for the entire organic foods sector indicated that the ≥ 9% yearly growth observed over the last few years is expected to be maintained through 2013, at least (108). This growth is expected even though price premiums associated with organic products have been estimated to range between 10-40% (175) and those of organic meat and poultry products to sometimes reach 200% or higher (10).

The rapid growth seen in the organic foods sector has been attributed to many different factors. The most common ones of these factors seem to be increased consumer confidence in organic foods and concerns about potential health risks and environmental impacts of conventional food production methods (175). Relatively recent foodborne
illness outbreaks, food crises such as “mad cow” disease and foot-and-mouth disease, and concerns over the use of pesticides, antibiotics, and other chemicals in conventional production systems have also contributed to the overall sales growth seen in the organic foods sector (39, 143). However, a systematic review of peer-reviewed literature published from 1958-2010 conducted by Dangour and others concluded that there is no scientific evidence to prove that differences between the nutritional quality of organic and conventionally produced foods exist (37).

In the United States, natural and organic foods fall under the jurisdiction of the USDA and the regulations specified by it. Although many natural and organic products resemble their conventionally produced counterparts, the stringent regulations that apply to natural and organic foods may render the use of certain ingredients illegal. The use of nitrate and nitrite in the production of cured processed meat products such as ham and frankfurters, among others, is one such example as the use of either is not permitted when manufacturing natural and organic processed meat products. Given that there are no direct substitutes for nitrite, regardless of whether it is added to the product directly or derived from the addition of nitrate and its subsequent reduction, the production of natural and organic processed meat products whose quality and safety properties and characteristics resemble those of their conventionally cured counterparts has represented a challenge to the meat industry. Because the quality and safety benefits derived from meat curing are unquestionable, the indirect addition of nitrate or nitrite to natural and organic processed meat products, sometimes referred to as “natural curing,” represents a new technology that has garnered interested from processors, consumers, and scientists alike (139, 147).
Definitions of Uncured, Natural and Organic Meat and Poultry Products

Uncured

Meat and poultry products to which nitrate or nitrite can or is required to be added may be manufactured without either but must be labeled accordingly. Thus, the production of “uncured” versions of traditional cured meat and/or poultry products may be manufactured and labeled according to 9 CFR 317.17 (33). This regulation establishes that “any product, such as bacon and pepperoni, which is required to be labeled by a common or usual name or descriptive name in accordance with § 317.2(c)(1) and to which nitrate or nitrite is permitted or required to be added may be prepared without nitrate or nitrite and labeled with such common or usual name or descriptive name when immediately preceded with the term ‘Uncured’ as part of the product name in the same size and style of lettering as the product name, provided that the product is found by the Administrator to be similar in size, flavor, consistency, and general appearance to such product as commonly prepared with nitrate or nitrite, or both.” The same regulation also states the following regarding “uncured” meat and poultry products: “Products… which contain no nitrate or nitrite shall bear the statement ‘No Nitrate or Nitrite Added.’ This statement shall be adjacent to the product name in lettering of easily readable style and at least one-half the size of the product name. Products… shall bear, adjacent to the product name in lettering of easily readable style and at least one-half the size of the product name, the statement ‘Not Preserved—Keep Refrigerated Below 40 °F. At All Times’ unless they have been thermally processed to F₀ 3 or more; they have been fermented or pickled to pH of 4.6 or less; or they have been dried to a water activity of 0.92 or less.”
While “natural” and “organic” are two distinct categories of meat and/or poultry products in terms of USDA regulatory and labeling requirements, the direct addition of sodium or potassium nitrate or nitrite to products within both categories is not permitted. Processed meat and/or poultry products that are labeled “natural” must comply with the requirements established in the USDA Food Standards and Labeling Policy Book (168). These requirements establish that a “natural” meat and/or poultry product …“(1) does not contain any artificial flavor or flavoring, coloring ingredient, or chemical preservative (as defined in 21 CFR 101.22), or any other artificial or synthetic ingredient; and (2) the product and its ingredients are not more than minimally processed.” The term “minimally processed” applies to “(a) those traditional processes used to make food edible or to preserve it or to make it safe for human consumption, e.g., smoking, roasting, freezing, drying, and fermenting, or (b) those physical processes which do not fundamentally alter the raw product and/or which only separate a whole, intact food into component parts, e.g., grinding meat…”.

Even though the definition of a “natural” meat product exists, controversies surrounding this product category still arise. One such example was highlighted by USDA itself in the same August 2005 Food Standards and Labeling Policy Book that defines the term “natural” (168). As part of the definition, the agency added a note indicating that “sugar, sodium lactate (from corn), and natural flavorings from oleoresins or extractives are acceptable for ‘all natural’ claims.” This note would be amended by USDA to read “sugar and natural flavorings from oleoresins or extractives are acceptable for ‘all natural claims,’” effectively removing sodium lactate and prohibiting it from use
in natural meat and poultry products. Sodium lactate from a corn source was originally added to the definition of “natural” because it could be shown that it was from a natural source, no more than minimally processed, and provided a flavoring effect, not an antimicrobial effect, at levels consistent with those regulated for the purpose of flavoring (i.e., less than 2% of the formulation). The removal of sodium lactate, and also of its potassium and calcium equivalents, from the definition of “natural” was caused by a petition submitted to USDA wherein it was argued that, even if added at levels consistent with those regulated for the purpose of flavoring, lactate provided an antimicrobial effect. This antimicrobial effect would, therefore, be in direct violation of the “no chemical preservative” part of the definition of “natural” established by USDA (168). Nonetheless, USDA went on to establish that “the use of this ingredient (lactate) or any other ingredient known to have multiple technical effects needs to be judged on a case-by-case basis at the time of label approval to assess that the intended use, level of use, and technical function are consistent…” (168). Clearly, the definition of the term “natural,” when it comes to meat and poultry products, lends itself to controversies and new rulemaking on the subject should be expected in the near future (139).

Organic

Although the term “natural” may be confusing and even misleading, “organic” products have a specified set or rules and requirements they have to abide by and be produced under. The USDA introduced the Organic Foods Production Act (OFPA) as part of the 1990 Farm Bill and, by doing so it sought to assure consumers that organic products met a consistent standard, to facilitate the marketing of organically produced products, and to facilitate the interstate commerce of such products (164). With the
OFPA also came the creation of the National Organic Standards Board which developed a National List of Allowed and Prohibited Substances and also the National Organic Program Standards. These standards specify not only substances but also methods and practices that may be used for the production, processing, and handling of organic foods. As an example, “organic” meat must originate from a USDA-certified farm and the ingredients used for the manufacture of an “organic” processed meat product must be defined as permitted in the OFPA National List (163). Furthermore, an allowed or prohibited substance may not remain on the National List of Allowed and Prohibited Substances for longer than five years unless it is reviewed and recommended for renewal by the National Organic Standards Board (NOSB) and adopted by the Secretary of Agriculture (175).

There are three different classifications of “organic” and they are based on the percentage of organic ingredients present in a product. The first of the three is “100% organic,” which signifies that the product contains ingredients that were produced according to USDA organic standards. The second of the three classifications of organic is “organic,” which applies to products that contain at least 95% organically produced ingredients. The remaining 5% of ingredients, however, must be approved for use in organic products and, therefore, listed on the National List of Approved Substances. Lastly, the third classification of organic is “made with organic ingredients.” This classification applies to products that contain at least 70% organically produced ingredients and also allows such products to list up to three of those ingredients on the principal display panel of the product. Furthermore, products that contain less than 70% organic ingredients may only list which ingredients are organic on the information panel.
Products that are classified as “100% organic” and “organic” may use the USDA organic seal, whereas products that are “made with organic ingredients” may not (139, 175).

The National Organic Program Standards establish that a USDA-accredited inspector must certify all organic operations in order to provide a guarantee that a particular product was grown/raised, processed, and distributed in such a manner that it meets the official organic standards. The use of a USDA-accredited inspector allows for third-party certification that the official organic standards are being met and reduces the misuse of the USDA organic label. Furthermore, the certification process is clearly delineated in the regulations so as to ensure that all inspectors certify according to the same standards (163, 175).

**Natural Sources of Nitrate or Nitrite Currently Used**

In the United States, the requirements for processed meat and/or poultry products to qualify as natural or organic have presented processors and researchers with challenges that have resulted in unique approaches to the development of these products. As previously mentioned, nitrate or nitrite, given their classification as chemical preservatives, are prohibited from use in either natural or organic processed meat and/or poultry products. Thus, scientists and processors around the world have placed emphasis on finding so called “naturally occurring” sources of nitrate or nitrite in vegetables and other sources. The use of these natural sources of nitrate or nitrite would allow for the development of natural and organic processed meat and poultry products that exhibit the positive quality and safety characteristics associated with traditional cured meat products previously discussed.
Sea salt has been proposed as a source of nitrate and nitrate due to its long standing use in the production of cured meat products. Sea salt, according to Heinerman and Anderson (54), is manufactured via the evaporation of sea water and remains unrefined through the entire process. As a result, sea salt may contain trace minerals characteristic of the source, two of which may be nitrate and nitrite. However, analyses conducted by Herrador and others (55) showed that sea salt from the Mediterranean Sea contained only 1.1 mg/kg of nitrate and 1.2 mg/kg of nitrite. It is likely that a high degree of variation in nitrate and/or nitrite content exists as a result of the geographical location and source of the sea water.

As previously discussed, some fruits and vegetables are known to contain relatively high levels of nitrate. Potatoes, lettuce, melons, cabbage, celery, spinach, beets, carrots, cauliflower, and broccoli are only a few examples of such vegetables and fruits (174). However, due to concerns over the flavor and/or color compatibility or clash that may stem from using some of these as sources of nitrate or nitrite in the production of natural and organic processed meat products, more emphasis has been placed on celery (Apium graveolens var. dulce) than on any other vegetable or fruit.

Analysis conducted by Sindelar and others (149) showed that a commercially available celery juice powder contained 27,462 mg/kg, or approximately 2.75%, nitrate. Celery juice and celery powder appear to possess sensory profiles that render them compatible with processed meat products. They not only contain little vegetable pigment, compared to beets, for example, but their flavor is not intense to the point where it would take away from the flavor of processed meat products. A recent addition to the list of vegetables being used as natural sources of nitrate is spray-dried Swiss chard (Beta
vulgariis var. cicla) powder, which, according to the manufacturer, contains 3.0-3.5% nitrate and is recommended to be used at concentrations around 0.15-0.30% (137). However, no literature was found on the compatibility of Swiss chard powder with meat products and its influence on the sensory attributes and/or cured characteristics of meat products at the time this literature review was conducted.

The use of a nitrate source such a celery juice or celery juice powder represents only half of the equation, for the reduction of nitrate to nitrite must take place prior to the development of cured characteristics in the product. Thus, a starter culture capable of reducing nitrate to nitrite has traditionally been used in the manufacture of natural or organic processed meat products to which a natural source of nitrate was added (139). Examples of starter cultures that have been shown to possess the ability to reduce nitrate to nitrite include, but are not limited to, Kocuria varians, Staphylococcus carnosus, and Staphylococcus xylosus. Of these, it appears that coagulase negative (nonpathogenic) Staphylococci spp. have received the most attention from researchers and, as a result, a clearer understanding of their nitrate reduction activities exists. More specifically, Staphylococcus carnosus has been shown to be most commonly used in the production of processed meat products to which nitrate was added due to its higher nitrate reductase activity when compared to other members of the Staphylococci species (104, 157).

Although the nitrate reducing efficiency of a particular starter culture may vary depending on factors such as incubation temperature and time, salt concentration, and any other extrinsic and intrinsic factor that could affect bacterial growth, most of these cultures can achieve nitrate reduction at 15-20°C, with optimum reducing temperatures above 30°C (27). Recommendations dictate that a holding temperature of 38-42°C should
be used in order to minimize the amount of time required by the starter culture to reduce nitrate to nitrite and, as a result, achieve proper development of cured characteristics in the finished product (139). Furthermore, it appears that holding time at 38°C is more critical than the amount of vegetable juice added to a product for the development of cured meat properties (145, 146, 149). The diameter of the product may play a role in this, as smaller diameter products (e.g. frankfurters) may require more stringent control of holding time and temperature for proper reduction of nitrate than larger diameter products (e.g. ham) (145, 146). This is thought to be due in part to the fact that the internal temperature of a large diameter product is expected to rise at a slower rate than that of a small diameter product, therefore allowing the starter culture enough time to reduce nitrate to nitrite.

The use of natural sources of nitrate and nitrate-reducing starter cultures, and the ensuing need for an incubation step for the reduction of nitrate to nitrite, in the production of natural or organic processed meat products results in increased production times. This scenario is not very compatible with today’s high throughput production systems and consumers’ increased demand for these categories of products. Thus, manufacturers of celery powders have begun to add nitrate-reducing starter cultures such as \textit{Staphylococcus carnosus} directly to the celery purees before the drying step and, as a result, have started to market “pre-converted” nitrite versions of celery powders. Once dried or slightly condensed, pre-converted celery powders or juices will contain 10,000-15,000 mg/kg, or 1.0-1.5%, nitrite. Recommended usage levels are different depending on not only the product but also the manufacturer of the celery powder or juice and range from 0.2-1.0% based on green (raw) meat weight. Using a pre-converted celery powder
in which the active ingredient is nitrite instead of nitrate will effectively eliminate the need for a nitrate reduction step and, therefore, result in decreased production times.

**Concerns Associated with Uncured, Natural, and Organic Processed Meat and Poultry Products**

**Nitrite Concentration**

As previously mentioned, commercially available celery juice powders contain approximately 27,500 mg/kg, or 2.75%, nitrate. The recommended usage levels for these natural sources of nitrate are around 0.30% based on the green (raw) weight of the meat. Assuming that a 100% conversion of nitrate to nitrite is achieved and that they were added at concentrations around 0.30%, products to which these natural sources of nitrate were added would contain a maximum of 82.5 mg/kg nitrite. If, on the other hand, a pre-converted celery powder/juice that contained 10,000-15,000 mg/kg, or 1.0-1.5%, nitrite was used at the recommended usage levels 0.2-1.0%, nitrite levels could range from 20-150 mg/kg. Thus, a high degree of variability in the amount of nitrite in the cooked product is expected. As a result, some of the quality and safety attributes of uncured, natural and organic processed meat and poultry products could, potentially, be affected negatively.

**Quality Aspects**

In a study conducted by Sindelar and others (146), the effects of varying levels of celery powder (0.2% or 0.4%) and different incubations times (30 min or 120 min) at 37.8°C wet-bulb on several quality attributes of emulsified, frankfurter-style, cooked sausages over 90 days of vacuum-packaged, refrigerated storage were evaluated. These
authors concluded that the shorter incubation time resulted in less cured color/redness development as measured by Hunter a⁺ values when compared to a sodium nitrite-added control treatment, but that this difference was not always significant. Furthermore, the authors also concluded that the longer incubation time resulted in comparable cured color/redness independent of vegetable juice concentration. Additionally, upon trained sensory panel analysis, it was determined that the sodium nitrite-added control received the highest scores for all sensory attributes and that visual cured color was affected by vegetable juice powder concentration and incubation time. As a result, the authors determined that incubation time was more critical for cured color development than was vegetable juice concentration under the conditions of the study (146). Finally, after evaluating the effects of the described treatments on lipid oxidation as measured by TBARS, the authors concluded that no significant differences were observed between treatments and that TBARS values ranged between 0.208 and 0.285, which are values well below accepted oxidized odor and oxidized flavor threshold values (0.5-1.0 and 1.0-2.0, respectively) (146).

A similar experiment conducted by Sindelar and others (145) sought to evaluate the effects of varying levels of celery powder (0.2 or 0.35%) and incubation time (0 or 120 min) at 37.8°C on several quality attributes of RTE, uncured ham over 90 days of vacuum-packaged, refrigerated storage. The authors were able to determine that there were no differences in objective color measurements and cured pigment concentrations when all treatments were compared to a sodium nitrite-added control. Sensory analysis of visual color yielded similar results. Interestingly, trained sensory panelists indicated that the greater celery powder concentration treatments resulted in higher vegetable aroma
and flavor than the lower vegetable juice concentration and the sodium nitrite-added control whereas the lower vegetable juice concentration treatments were similar to the sodium nitrite-added control for all sensory properties evaluated. This conclusion indicated that hams may possess a flavor profile that is more susceptible to vegetable-like off-flavors and off-odors (145).

A study conducted by Sindelar and others (148) represented an attempt to develop a method to manufacture uncured, no-nitrate-or-nitrite added whole muscle jerky. In this study, the authors evaluated the effects of different processing procedures such as no vacuum packaging followed by incubation for 2 hrs at 40.6°C and vacuum packaging followed by incubation (2 hrs at 40.6°C or 48 hrs at 5°C) on color, total and cured pigments, and TBARS of treatments that contained 0.35% of a vegetable juice powder and a nitrate-reducing starter culture in comparison to a sodium nitrite-added control. Results obtained by the authors suggested that, even though no significant differences on any given day were observed between the “naturally cured” treatments and the control as far as objective redness as measured by CIE $a^*$ values, the sodium nitrite-added control treatment yielded the highest $a^*$ values at each day. Similarly, no significant differences between naturally-cured treatments and the control treatments in regards to cured color fading as measured by reflectance ratio were observed. However, the authors concluded that their results indicated that the sodium nitrite-added control treatment received “excellent cured color” ratings on all days whereas the naturally cured treatments received lower cured color intensity ratings when the cured color intensity rating system developed by Hunt and others (61) was applied. Furthermore, results also indicated that the control treatment exhibited significantly higher cured pigment concentrations than all
other treatments on days 0, 28 and 56 and that the no vacuum packaging followed by incubation for 2 hrs at 40.6°C treatment resulted in significantly higher cured pigment concentrations than either vacuum packaging treatment regardless of incubation parameters on days 0 and 28. When evaluating treatment effects on TBARS, the authors concluded that the control treatment showed lower combined TBARS values than all other treatments (0.51 vs. 0.77-0.87, respectively) but that this difference was only significant when the control treatment was compared to the vacuum packaging followed 5°C/48 hr incubation treatment. Thus, the authors reached the overall conclusions that a vacuum packaging step followed by incubation steps of 2 hrs at 40.6°C or 48 hrs at 5°C to enhance nitrate-to-nitrite reactions did not result in improved cured characteristics and that even though the no vacuum packaging of jerky slices followed by incubation for 2 hrs at 40.6°C treatment resulted in some cured characteristics, these characteristics were still lacking compared to those of the sodium nitrite-added control. Thus, the natural curing methods evaluated were found to be ineffective for production of a naturally cured whole muscle jerky product with cured characteristics similar to those of a whole muscle jerky product cured with sodium nitrite (148).

It has been suggested that typical quality characteristics of cured meat products such as color, flavor, and flavor stability can be achieved if concentrations of 50 mg/kg ingoing nitrite or higher are used (139). However, the long-term stability of such characteristics could come under question if nitrite levels in the finished product are that low and are depleted during the storage life of the product. Thus, ingoing nitrite levels expected to be attained in the production of naturally cured meat and poultry products could be a cause for concern in terms of cured characteristics and their stability.
As previously established, the roles nitrite plays in the production of processed meat products are many. Color, flavor, aroma, and oxidative stability are all important quality attributes that have been shown to be enhanced by the action of nitrite in meat systems. Due to factors such as the expected variability in ingoing nitrate and nitrite levels as well as the less-than-complete reduction of nitrate to nitrite that is to be expected when a natural source of nitrate and a nitrate-reducing starter culture are utilized, “natural curing” systems that make use of natural sources of nitrate or nitrate are likely to result in cured characteristics that are less predictable and less adequate than those observed in conventional production systems that use the direct addition of nitrite as the curing method of choice.

Safety Aspects

One of the main concerns over the use of natural curing methods that employ vegetable sources of nitrate or nitrite is the potential for nitrosamine formation. Research conducted by Sen and others in 1973 (141) evaluated the formation of nitrosamines in meat curing mixtures and determined that some amine components of spices, especially spices such as black pepper (Piper nigrum) and paprika (Capcicum anuum), can react with nitrite to form nitrosamines in these blends. Thus, the authors concluded that premixing spices with nitrite to create curing mixtures may lead to the formation of dangerous carcinogenic nitrosamines. With no evidence that pre-converted celery powders may or may not react in a similar fashion with the nitrite present in them, the concern over the potential for nitrosamine formation in these novel natural curing systems exists.
A major issue for natural and organic processed meat and poultry products that are manufactured using a natural source of nitrate is that the amount of nitrite derived from the nitrate source is unknown, difficult to measure, variable due to inherent environmental conditions experienced during plant growth, and can also react very quickly with meat components (139). Thus, nitrite concentrations that are higher than those observed in conventional cured products may result. Sindelar and others (146), for example, determined that frankfurter-style cooked sausages manufactured using 0.4% celery powder and an extended incubation time yielded significantly higher residual nitrite levels than a sodium nitrite-cured control throughout 90 days of refrigerated storage. In a product that is going to be subjected to relatively high cooking temperatures (e.g. bacon), higher residual nitrite levels than usual could increase the potential for formation of nitrosamines. Furthermore, the fact that the addition of more than minimally processed cure accelerators such as ascorbate and erythorbate is not allowed in natural and organic meat products takes a known safety measure against the formation of nitrosamines out of the equation.

Nitrite concentration plays an important role in the inhibition of both spoilage and pathogenic microorganisms in cured meats systems. While concentrations of 40-50 mg/kg ingoing nitrite are considered sufficient for the purposes of cured color development (169), a minimum concentration of 120 mg/kg ingoing nitrite is required by USDA for safety of conventionally cured products that fall in the “Keep Refrigerated” category unless the establishment can demonstrate that safety can be assured by some other preservation method, such as pH control, moisture control, or thermal processing, as previously discussed (168). With the limited amount of ingoing nitrite achieved
through the natural curing methods previously described the potential for decreased microbial safety of products manufactured using those methods becomes an issue to consider.

Nitrite is a strong inhibitor of *C. botulinum* growth. Research on the antibotulinal properties of nitrites has shown that other factors such as pH, salt concentration, phosphates concentrations, reductants as well as thermal processing all interact with nitrite in achieving inhibition of *C. botulinum* (161). It has also been suggested that both ingoing and residual nitrite concentrations are important parameters in terms of *C. botulinum* inhibition. However, because ingoing nitrite is depleted over time in cured meat products, the importance of ingoing nitrite likely stems from its direct effect on residual nitrite levels (139). Thus, the reduction of ingoing nitrite levels and/or an increase in the rate of residual nitrite depletion could increase the theoretical risk for *C. botulinum* growth in cured meats (31).

Jackson and others (64) evaluated different brands of commercially available natural and organic frankfurters, hams, and bacon for their efficiency at inhibiting the survival and growth of *C. perfringens* after 10 days of storage at 10°C. In comparison to conventionally cured controls, the natural and organic products showed 1-3 log CFU/g greater *C. perfringens* growth. More specifically, reduced *C. perfringens* inhibition was observed in four of nine bacon brands, seven of ten frankfurter brands, and in six of seven ham brands. Similar trends were observed by Schrader (136) after evaluating the growth and survival of *L. monocytogenes* in commercially available natural and organic meat products. Although there was no control over ingredients added and concentrations as well as a myriad of other extrinsic and intrinsic factors that could have influenced *C.
perfringens and L. monocytogenes growth in commercially available natural and organic meat products, these results indicate that the microbial safety of natural and organic processed meat products is compromised when compared to that of conventional cured products.

The inhibitory effects of nitrite on the growth of L. monocytogenes have been studied but are not as well understood as those nitrite has shown to have on spore formers such as C. botulinum. Buchanan and Phillips (19), after evaluating the effects of sodium nitrite concentrations ranging from 0-1,000 mg/ml of Tryptose Phosphate Broth on the growth kinetics of L. monocytogenes Scott A, concluded that sodium nitrite represents an important parameter that plays a role in the survival and growth of this pathogen. Pelroy and others (122) determined that, among other factors such as packaging atmosphere, storage temperature, and sodium chloride concentration, 190-200 mg/kg sodium nitrite exerted a bacteriostatic effect on L. monocytogenes inoculated onto slices of cold-smoked salmon. Glass and Doyle (45), on the other hand, concluded that combining 103 mg/kg sodium nitrite with 3.5% sodium chloride in beaker sausage and pepperoni stored at 32°C was not enough to inhibit the growth of L. monocytogenes. Thus, the mechanism for L. monocytogenes inhibition by nitrite is still viewed as a complex process that involves several different factors.

Listeria monocytogenes

L. monocytogenes has emerged as an important foodborne pathogen of significant human health concern over the last few years. Listeriosis, although rare, carries a relatively high mortality rate that can be as high as 30% (94). RTE meat and poultry
products have previously been associated with listeriosis outbreaks and, as a result, close attention should be paid to factors that affect the growth and behavior of *L. monocytogenes* in such products. A thorough understanding of the history of this pathogen, some of its defining characteristics as well as the factors that influence its growth will allow for better elucidation of strategies aimed at reducing the risk for contamination of meat products with *L. monocytogenes*.

**History**

The first description of *L. monocytogenes* dates back to 1924 when, after observing six cases of sudden death in young rabbits in the animal breeding establishment at the Department of Pathology at Cambridge, Murray and others (98) referred to the causative bacterium as “*Bacterium monocytogenes*.” In their description of this previously unidentified microorganism, Murray and colleagues concluded that one of the most striking and constant features of *Bacterium monocytogenes* infection was the production of a large mononuclear leucocytosis. Just three years later Pirie (125) discovered a new microorganism responsible for the unusual deaths of gerbils near Johannesburg, South Africa, which she later referred to as “*Listerella hepatolytica*” in honor of Lord Lister, a known bacteriologist of that time that had never been commemorated in bacteriological nomenclature. Pirie also referred to the disease caused by *Listerella hepatolytica* as “the Tiger River disease” (125). Upon sending their respective strains to the National Type Collection at the Lister Institute in London, Murray and Pirie, along with Dr. Leningham, director of the institute, decided that the similarities were so evident that the bacterium was the same and, therefore, settled on the name “*Listerella monocytogenes*” (125). However, due to the fact that the name “*Listerella*” had previously been used to describe
two other microorganisms, the Judicial Commission of the International Committee on Systematic Bacteriology rejected the generic name “Listerella” in 1939. Pirie, thus, proposed the name “Listeria” in 1940 (126).

**Presence in Food Processing Environment**

*L. monocytogenes* has been described as a ubiquitous organism due to the fact that its presence has been detected in a wide variety of environments; soil, dust, air, water, plant material, including silage, among others. In addition, *L. monocytogenes* has been determined to thrive in several different food processing environments such as floors, drains, and standing water, making proper plant sanitation a very important factor to consider when trying to keep this microorganism out of food processing establishments. Without the existence and proper implementation of food safety procedures, *L. monocytogenes* can readily cross-contaminate processing machinery, employee utensils, and many other surfaces with which the food product will come into contact (167). Thus, RTE meat products are of particular concern for contamination with this foodborne pathogen.

**Factors that Affect its Growth and Survival**

*L. monocytogenes* possesses several characteristics that make it a difficult foodborne pathogen to control in food processing environments. For example, *L. monocytogenes* can survive and grow in refrigerated, damp environments where other microorganisms may not and it is also able to survive under frozen conditions. Furthermore, certain degrees of both heat and salt tolerance have been associated with this pathogen. As a result, an understanding of those factors is needed if better control measures are to be developed.
and implemented in an attempt to minimize the chances for the presence of this foodborne pathogen in our food supply.

The temperature to which a food product is exposed from manufacture to consumption may have growth-conducing, preserving, or lethal effects on microorganisms. In the case of *L. monocytogenes*, the temperature range across which it has been found to grow is 0°C to 45°C, but its optimum growth is usually observed at temperatures between 30°C and 37°C. Temperatures below the freezing point of water moderately inactivate or preserve the pathogen, whereas temperatures that rise above 50°C have been shown to be lethal to it (79).

Due to the high degree of difficulty associated with experimentally trying to determine the minimum temperature for growth of *L. monocytogenes* to occur, a mathematical model approach was taken by Tienungoon and others (160). These authors concluded that, under optimum pH and water activity (a_w) conditions, the minimum temperature at which *L. monocytogenes* Scott A can grow is -1.6°C, whereas *L. monocytogenes* L5 can only grow at temperatures of 0.41°C or higher. Furthermore, these same authors determined that medium pH strongly affected the limits for growth at refrigerated temperature.

*L. monocytogenes* is a psychotropic microorganism in that it can survive and grow slowly at refrigeration temperatures (79). This rather unique characteristic presented by this foodborne pathogen has been attributed to the presence of phospholipids in its cellular membrane that remain in a liquid-crystalline state under such temperatures. The fatty acid composition of the phospholipids present in the cellular membrane of *L.*
*L. monocytogenes* has been observed to change as temperatures approach refrigerated conditions. When exposed to a temperature of 37.0°C, for example, the major fatty acids found in the cellular membrane of *L. monocytogenes* are anteiso-C\textsubscript{15:0} (41 to 52%), anteiso-C\textsubscript{17:0} (24 to 51%), and iso-C\textsubscript{15:0} (2 to 18%). On the other hand, when grown at 5°C, the anteiso-C\textsubscript{15:0} form becomes the major fatty acid present in the cellular membrane of *L. monocytogenes*, reaching a concentration of 65-85% of total membrane fatty acids (6, 79, 106). This perceivable reduction in the proportion of long aliphatic chains and the increase in asymmetric branching, thus, allow for the pathogen’s membrane to remain fluid under refrigeration temperatures.

Although storage under refrigerated conditions does not offer any protection against *L. monocytogenes* growth, as previously stated, temperatures above 50°C have been shown to cause irreversible damage to this microorganism (79). Ribosomal damage, protein unfolding, and, consequently, enzyme inactivation are examples of cellular damage observed in *L. monocytogenes* after heating at temperatures above 56°C (79). Dissociation of the 30S and 50S ribosomal subunits as a result of the loss of Mg\textsuperscript{2+} due to heating is known to occur. The denaturation of the 30S ribosomal subunit associated with thermal inactivation has been proposed as the main cause of bacterial death (95). However, the inactivation or the reduction in the activity of enzymes such as superoxide dismutase and catalase in *L. monocytogenes* has also been observed when temperatures reach levels higher than 45 to 50°C and 55 to 60°C, respectively (79). Higher processing temperatures are known to completely inactivate those enzymes and may sensitize the pathogen to aerobic storage conditions (116).
The pH range considered to be optimal for the growth of *L. monocytogenes* to occur is 6 to 8, which should come as no surprise given that most foodborne pathogens thrive at or near neutral conditions. However, growth of this pathogen has been observed at pH values ranging from 4.0 all the way up to 9.6 (79), suggesting that *L. monocytogenes* can survive and/or grow in a wide variety of foods. pH values lower than 6.5 have been suggested to increase the generation time and extend the lag phase of *L. monocytogenes* (19). A decrease in pH can have inhibitory or even fatal effects on the bacterial cell as maintaining a neutral environment within the cell wall is critical to growth and survival. In mildly acidic conditions, for example, cellular enzymatic mechanisms exist to export excess H\(^+\) ions from the cytoplasm. As the pH continues to decrease and more acidic conditions are encountered by the same mechanisms, enzymatic denaturation begins to occur and, as a result, intracellular pH drops to levels that are fatal to the cell (19, 79). However, some acid resistance has been shown to exist in *L. monocytogenes* as its survival in fermented sausages has been documented even though product pH reached levels as low as 4.4 (69). An uncanny ability by this pathogen to survive acidic conditions is further established by the fact that it has been shown to survive in orange juice (pH 3.6) stored at 4°C for 1 to 4 days (113). Cell viability as affected by pH, however, is highly dependent on other environmental factors and on the physiological state of the microorganism (79).

The availability of moisture for microbial growth is more commonly referred to as water activity (a\(_w\)) and it is defined as the ratio of the water vapor pressure of a food substrate to the water vapor pressure of pure water at the same temperature (79). Much like most bacteria, *L. monocytogenes* exhibits optimum growth characteristics at a\(_w\)
values at or above 0.97 (123). Compared to other common foodborne pathogens, however, *L. monocytogenes* is known to possess the unique ability to grow at $a_w$ values as low as 0.90. In fact, even though it may not grow at $a_w$ values lower than 0.90, *L. monocytogenes* can survive under such conditions over long periods of time, particularly under refrigeration (79). The survival of *L. monocytogenes*, for example, has been documented for at least 84 days in fermented hard salami that presented a $a_w$ of 0.79 to 0.86 and that was stored at 4°C (69). Low $a_w$ values (< 0.90) can be considered listeriostatic, but rapid growth may be observed as $a_w$ increases (79). This capacity of *L. monocytogenes* to withstand lower $a_w$ levels than most foodborne pathogens and grow on a processed meat product is thought to stem from the accumulation of high levels of carnitine, betaine, glycine, and proline-containing peptides. These peptides act as osmoprotectants and are essential to *L. monocytogenes* when it comes to maintaining intracellular turgor pressure, an important cell wall growth and cell division factor (5, 79).

Salt (*i.e.* sodium chloride) is an important food ingredient that plays several key roles in the production of processed meat products. Not only does it contribute to the flavor profiles commonly associated with processed meat products, but salt also inhibits microbial growth by decreasing the food’s water activity (79). However, unlike many spoilage and other pathogenic microorganisms, *L. monocytogenes* exhibits halotolerant characteristics in that it has been found to survive in relatively high salt concentrations. One such example is the survival of *L. monocytogenes* for 259 days in a cheese brine that was formulated to contain a total salt concentration of 23.8%, had a pH of 4.9, and was stored at 4°C (81). The presence of 26% salt in brain heart infusion (BHI) broth
decreased *L. monocytogenes* levels by 2 and 3.5 logs after 33 days when the storage temperature was 0 and 4°C, respectively. Such high salt concentrations, however, would not be applicable to the production of processed meat products. Consequently, the use of high levels of salt as a means for inhibiting the growth of *L. monocytogenes* in RTE meat products is not feasible.

As previously established, nitrite is a preservative commonly used in the manufacture of meat and poultry products and, occasionally, also in the manufacture of certain cheeses. That is so due in part to its strong inhibitory properties against anaerobic, spore-forming microorganisms such as *C. botulinum* and other members of the *Clostridia* class. The effects of nitrite on *L. monocytogenes*, however, are not as profound and, at the very least, not well understood at this point (79). Buchanan and Phillips (19) determined that sodium nitrite is a parameter to be considered when it comes to inhibiting the growth of *L. monocytogenes* given that it slightly inhibits its growth. Other authors (48) also concluded that sodium nitrite exerts a significant inhibitory effect on the growth of *L. monocytogenes* after studying its effects on the pathogen in corned beef and ham. These authors determined that products containing 170 mg/kg residual nitrite and stored at 0°C did not support the growth of the pathogen, whereas products that contained only 11 mg/kg sodium nitrite did supports its growth under the same storage conditions (48).

The inhibitory effects nitrite has on the growth of *L. monocytogenes* are enhanced when the addition of nitrite is combined with low pH, temperature, or oxygen level, or when the sodium chloride concentration of the medium or food in question increases (21). For example, vacuum packaged salmon that contained 200 mg/kg nitrite and 5% sodium chloride inhibited the growth of *L. monocytogenes* for 40 days at 5°C (122).
Moreover, hard salami formulated to contain 156 mg/kg sodium nitrite prior to the fermentation of the product and a sodium chloride concentration of 5.0 to 7.8% retarded the growth of *L. monocytogenes* for 12 weeks or more at 4°C (69). Myers and others (99) also concluded that nitrite in combination with salt slows the growth of *L. monocytogenes* after evaluating the effects of sodium nitrite (0 and 200 mg/kg) and sodium chloride (1.8 and 2.4%) concentrations on RTE ham and turkey products stored for 28 days at 4.4°C. Other authors, however, have obtained differing results when it comes to the inhibitory effects of nitrite on *L. monocytogenes* growth. Glass and Doyle (45), for example, determined that sodium chloride concentrations of 3.5% in beaker sausage and pepperoni combined with 103 mg/kg nitrite did not inhibit the growth of this pathogen when the storage temperature was 32°C.

The mechanisms by which nitrite inhibits the growth of not only *L. monocytogenes*, but also that of members of the *Clostridia* class, are still unclear. However, these inhibitory effects nitrite has on microorganisms are thought to stem from reactive species generated as part of curing reactions and not from nitrite itself (79).

**Incidence of Foodborne Listeriosis**

The incidence of listeriosis, the foodborne disease caused by *L. monocytogenes*, in humans is greatest among certain well-defined high-risk populations that include neonates, the elderly, immunocompromised adults, and pregnant women, but cases in individuals with no predisposing or underlying conditions have also been reported (110). Unlike diseases caused by other common foodborne pathogens, listeriosis carries a rather
high mortality rate of approximately 20% (43). Due in part to this very reason foodborne listeriosis has received increased media and regulatory attention around the world.

Starting in 1996, PulseNet, a network run by the Centers for Disease Control and Prevention (CDC) has been conducting molecular subtyping of *L. monocytogenes* isolates by pulsed-field gel electrophoresis (PFGE) on isolates submitted to public health laboratories in an effort to enhance the ability of health officials to detect and investigate outbreaks of listeriosis (28). Since the institution of PulseNet, RTE meats and unpasteurized cheese have been the most commonly identified causative agents of foodborne listeriosis in the United States (110).

The first piece of conclusive evidence linking *L. monocytogenes* to foodborne illness dates back to a 1981 outbreak that took place in Nova Scotia wherein a total of 41 cases (34 pregnant women and 7 adults) of listeriosis occurred over a 6-month period (135). Patients contracted the disease after ingesting contaminated coleslaw. Upon review of the evidence, it was discovered that the cabbage used in the manufacture of the coleslaw was grown on fields that had been fertilized with raw sheep manure from the same farm, which had previously reported cases of ovine listeriosis (110). In the United States alone, a total of 175 separate *L. monocytogenes*-related RTE meat recalls were issued between January 1994 and October 2006 (40). Of these recalls, 74 were associated with deli meats, 42 for sausages, 37 for hot dogs, and 22 for other products.

In 1996, the incidence of listeriosis was 5 per 1 million. By 2004, that number dropped to 2.7 per million. Based on these data, the projected number of deaths per year caused by listeriosis would have declined from 504 in 1997 to 302 in 2004 (110). The
most current estimates indicate that listeriosis in the U.S. results in 1,600 foodborne illnesses, 1,500 hospitalizations, and 260 deaths annually (134). The observed drop in the incidence of foodborne listeriosis is thought to be due to the increased control measures that have been instituted not only by legislators and regulators alike, but also due to food safety initiatives taken by food processors around the world.

**Regulations Regarding *Listeria monocytogenes* in Ready-to-Eat Meat and Poultry Products**

In 2003, the USDA FSIS established a “zero tolerance” policy for the presence of *L. monocytogenes* on RTE meat and poultry products. This policy would later come to be known as the “Listeria Rule.” Under this policy, an RTE meat and poultry product is considered adulterated if it is found to contain *L. monocytogenes* or if it has come into direct contact with a food contact surface which is contaminated with *L. monocytogenes* (34). This policy was established after it became clear that RTE meat and poultry products are of increased concern for contamination with this pathogen due to the fact that they may support its growth, they are at an increased risk for contamination with the pathogen due to exposure to the post-lethality processing environment, and also because they are likely to be consumed without further cooking. Although testing conducted by USDA FSIS shows that the overall incidence of *L. monocytogenes* in RTE meat and poultry products has steadily decreased since the Listeria Rule went into effect, the pathogen continues to be detected at low levels. This has resulted in the maintenance of a zero-tolerance policy by USDA FSIS.
According to the *Listeria* Rule, meat and poultry establishments producing post-lethality exposed RTE products must control *L. monocytogenes*. In order to do so, establishments can address *L. monocytogenes* through their Hazard Analysis and Critical Control Point (HACCP) plans, or prevent the presence of the pathogen in the post-lethality processing environment through a Sanitation Standard Operating Procedure (SOP), or other prerequisite programs (34). Furthermore, the *Listeria* Rule outlined three alternative methods establishments can follow in order to control *L. monocytogenes* contamination of post-lethality exposed RTE products (34):

1. Alternative 1: Application of a post-lethality treatment (PLT) to reduce or eliminate *L. monocytogenes* and an antimicrobial agent or process (AMA or AMP, respectively) to suppress or limit its growth

2. Alternative 2: **Either** application of a PLT **or** an AMA or AMP

3. Alternative 3: The establishment can choose not to apply any PLT, AMA, or AMP as it chooses to rely on its sanitation program to control *L. monocytogenes*

These alternatives increase in the level of stringency of their control from Alternative 3 to Alternative 1. It bears reemphasizing the fact that the *Listeria* Rule only applies to products that are RTE and exposed to the processing environment after the lethality step. Furthermore, the lethality step is often defined as cooking or another process, such as drying or fermentation, that results in a product that is safe for consumption without any further preparation needed (34).

Despite the facts that strict regulations exist for the control of *L. monocytogenes* in RTE meat and poultry products and that meat processors have made considerable efforts
aimed at keeping this dangerous microorganism out of our food supply, recalls associated with *L. monocytogenes* continue to take place in the meat industry. This indicates that further research and regulatory endeavors must be undertaken so as to minimize the prevalence of *L. monocytogenes* in our food supply.

**Control of *Listeria monocytogenes* in Uncured, No-Nitrate-or-Nitrite-Added, Natural or Organic, Ready-to-Eat Meat and Poultry Products**

The control of *L. monocytogenes* in post-lethality exposed RTE meat and poultry products remains a challenge to today’s meat industry. This is supported by the fact that we continue to see recalls associated with this foodborne pathogen in spite of the regulatory controls established by government agencies throughout the world and the extensive efforts that meat processors have gone through in order to address the potential contamination of their products with this microorganism. RTE meat and poultry products that are manufactured under uncured, natural, or organic methods, if contaminated with this microorganism, are at a greater risk for *L. monocytogenes* growth than their conventional counterparts due, mainly, to the required absence of preservatives and antimicrobials traditionally used in the manufacture of conventional products. For example, the use of organic acids such as lactate and diacetate, two antimicrobials commonly found in RTE meat and poultry products and proven to have inhibitory effects on *L. monocytogenes*, is not permitted in the manufacture of natural or organic meat products. As a result, the use of natural antimicrobials and “clean label” technologies or interventions in the manufacture of these types of meat products has received attention from researchers and processors alike (136, 137, 139, 154, 155).
**Sublethal Injury of Bacteria**

The presence of pathogenic microorganisms in food is of obvious concern to government agencies, food manufacturers, and, ultimately, consumers. Several technologies have been developed and are currently being utilized by food manufacturers to address the potential presence of foodborne pathogens in foods. Heating, freezing, drying, freeze-drying, fermentation, addition of antimicrobials, irradiation, and high hydrostatic pressure, among others, are only a few examples of practices currently being implemented by food manufacturers as part of their efforts to combat foodborne pathogens (181). In spite of the proven fact that these technologies do exert deleterious and often deadly effects on a number of foodborne pathogens, a specific population of microorganisms may be able to survive these treatments, escape detection through most conventional detection methods, recover if presented with the appropriate conditions, and, eventually, cause foodborne illness. This population of “sublethally injured” microorganisms is just as important as their intact counterparts and should be taken into consideration when developing product formulations and designing intervention methods.

**Definition**

A sublethally injured, or simply injured, bacterial cell has been defined as a cell that survives a stress such as heating, freezing, acid treatment, or other antimicrobial intervention, but loses some of its defining qualities (24, 181). One of the first descriptions of what an injured microbial cell is was made by Hartsell in 1951 (53). As part of this description, Hartsell defined an injured cell as one capable of forming colonies or growing on nonselective but not on selective media. A sublethally injured cell
has been described further as one that can repair the cellular damage exerted by a stressor (resuscitate) and regain its original ability to grow and form a colony in the presence of selective agents, whereas a dead cell would not be able to grow and form a colony under any circumstances (111).

Relevance

Conservative estimates indicate that foodborne diseases cause between US $10 and $83 billion in illness related costs annually in the United States alone (170). These costs include, but are not limited to, pain and suffering, reduced productivity, and medical costs. The loss of any lives to foodborne illness, needless to say, cannot have a dollar figure associated with it. Thus, our ability to detect any foodborne pathogens that may be present in our foods is of utmost importance, especially when dealing with foods that are RTE and will likely not receive any further cooking from the consumer after purchase. Moreover, the potential presence of sublethally injured pathogens such as *L. monocytogenes* in RTE products, coupled with the inability of current detection methods to allow for the reliable detection of sublethally injured microorganisms, presents a scenario under which false negative results may be obtained. As a result, populations of sublethally injured pathogens and their reliable detection have become an area of focus for researchers in the field of microbiology.

Recovery Methods

Sublethally injured bacterial cells, if presented with favorable conditions, possess the ability to repair themselves (resuscitate) and return to a normal physiological state wherein they can initiate cell division and grow. The term “resuscitation” has been
utilized by researchers because it illustrates the fact that sublethally injured cells come back from apparent death (62). During the process of resuscitation and before normal growth can occur, the restoration of growth capabilities as well as the reversing of any cellular modifications must occur (181). The resuscitation or repair of sublethally injured microorganisms was originally studied using liquid methods, but later came to involve solid methods as well (62, 181). A detailed look into both types of recovery methods is warranted before any one of them is selected for the microorganism and type of injury to be studied.

**Liquid Recovery Methods**

When using liquid repair methods, the food sample will typically be blended and incubated in a nonselective broth to allow for the repair of sublethally injured microorganisms. Time and temperature of this repair period vary with the method of recovery to be used, the microorganism to be studied, and the type of stressor used to inflict injury on the bacterial cells (24, 181). Generally speaking, incubation temperatures will range from 25 to 37°C while the incubation time will typically oscillate between 1 to 5 hours (181). Once the repair or resuscitation period has been completed, enumeration of cells can be carried out using direct plating or most probable number (MPN) techniques. An example of a liquid repair method that has been investigated is the two-fold dilution (2FD) method (73).

It is commonly accepted that, although appealing from a practicality standpoint, the results obtained using liquid repair methods can be misleading. Plating aliquots from food samples after the resuscitation step onto selective media could yield colonies that
could be due to the resuscitation of sublethally injured microorganisms, but these same counts could be due to the growth and/or multiplication of uninjured cells, especially in instances when the time of incubation utilized is prolonged. Furthermore, reculturing and further isolation of the organism to be studied would be required if using the 2FD method, for example (73, 181). Thus, liquid repair methods are expected to receive limited acceptance from regulatory and industry bodies.

**Solid Recovery Methods**

In contrast with liquid recovery methods, solid recovery methods will typically call for either pour-plating or surface-plating of the blended sample with a nonselective medium, followed by incubation for a predetermined amount of time at a suitable temperature for repair to occur. Once the repair step has been completed, some methods call for an overlay with a selective medium that is specific for the microorganism of interest followed by incubation. During this incubation period the selective agents present in the selective medium migrate through the nonselective medium and create a selective environment throughout. Due to the fact that a repair step was instituted at the beginning of the process, those microorganisms that were originally sublethally injured are thought to be able to grow in the presence of the selective agents. Solid recovery methods that employ the previously described procedures are more direct and economical than liquid recovery methods. On the other hand, these methods make picking isolated colonies for further testing difficult and may yield variable results when working with very low (< 10 CFU per g) counts (74, 131, 178, 181). In addition, the temperature of the molten selective agar to be overlaid can further affect sublethally injured microorganisms and
impede their complete recovery (74, 178, 181). Examples of such solid repair methods include the pour-overlay method (130, 131) and the surface-overlay method (52, 152).

A relatively new recovery method is the thin agar layer (TAL) method, developed by Kang and Fung in 1999 (72). This method represents a one-step procedure and involves pouring a thin layer of nonselective agar onto a prepoured and solidified pathogen-specific selective medium. Over the years, the nonselective agar of choice to use as part of the TAL method became tryptic soy agar (TSA) because it provides a favorable environment for sublethally injured cells to resuscitate and regain their original growth capabilities within the first few hours of incubation (71, 179, 180). After resuscitation, the sublethally injured microorganisms can interact with the selective agents in the bottom layer whereas nontarget microorganisms are inhibited by those same agents. Furthermore, the layer of TSA has been shown not to hinder color reactions expected when using selective agents such as those found in modified Oxford (MOX) medium (72, 177, 179, 180, 181).

When using the TAL method, the layer of TSA has been shown not to interfere with typical color reactions or morphology seen in colonies of target microorganisms, which represents an advantage over the two-step overlay method. Additionally, the isolation of individual colonies for further characterization is much easier, given that these colonies would be located on the surface of the TAL plate compared to within or between the two layers of agar as they would be in a layered plate. These advantages, combined with the fact that the TAL method has been demonstrated to allow for the differentiation of target microorganisms even in the presence of background flora, make the TAL method a
promising option in the study sublethally injured foodborne pathogens commonly associated with foodborne illness (29, 177, 179, 180, 181, 182).

**Post-Lethality Interventions**

An intervention can be defined as a process or ingredient that can be applied to a meat and poultry product, or any food product, so as to suppress or limit the potential presence of foodborne pathogens. Thermal processing, freezing, drying, addition of antimicrobial ingredients, and high hydrostatic pressure, among others, are examples of intervention methods commonly used not only in the meat industry, but across the entire food industry, to address the potential presence of pathogenic microorganisms in food products. Intervention methods can be further classified as either lethality interventions or post-lethality interventions based on when during the manufacture of the meat and poultry product they are applied relative to the cooking or thermal treatment of the product. The USDA FSIS (167) has defined a post-lethality treatment as “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.” High hydrostatic pressure processing, for example, is an example of a post-lethality intervention due to the fact that it generally takes place after the product has gone through the lethality or cooking step (167). The use of post-lethality interventions to address the potential presence of *L. monocytogenes* in uncured, no-nitrate-or-nitrite-added, RTE natural or organic meat and poultry products is an area of interest because some of these technologies are allowed for use in these categories of products.
Post-Packaging Thermal Treatment

The application of a thermal treatment to a RTE meat and poultry product that is confined within its final packaging would satisfy alternatives 1, if used in combination with an AMP or AMA, and 2 of the *Listeria* Rule (34). Furthermore, due to the relative heat susceptibility of *L. monocytogenes* and the potentially mild effects heat would have on the quality characteristics of the finished product, efforts to design post-packaging thermal interventions have taken place. One such effort was carried out by Chen and others (30) when they studied the effects of different thermal treatments (71, 81, or 96°C for 30, 60, or 120 sec) and packaging methods (1-, 5-, or 10-link packages), with or without the addition of a pediocin (ALTA 2341), on the survival and growth of *L. monocytogenes* on frankfurters over 12 weeks of storage at 4, 10, or 25°C. These authors concluded that *L. monocytogenes* numbers were reduced by all heat treatments, but that 81°C or more for 60 sec or more in combination with 6,000 AU of pediocin was necessary to achieve a 50% reduction of initial inoculation numbers. Furthermore, the authors were also able to determine that the heat treatments applied were most effective when applied to 1-link packages and least effective when applied to 10-link packages and that they generally did not affect the sensory qualities of frankfurters. Similarly, Selby and others (140) determined that, upon heating vacuum packaged bologna samples to 55, 60, 62.5, and 65°C, the *D*-values at all heating temperatures decreased with increasing temperature. It is likely that thermal inactivation kinetics are affected by product characteristics such as *a*<sub>*w*</sub>, pH, and ingredients.
High Hydrostatic Pressure Processing

High hydrostatic pressure (HHP) treatment, also sometimes referred to as ultra high pressure (UHP) treatment or high pressure processing (HPP), is a relatively novel technology that is utilized to address the potential presence of pathogens in foods, including meats (9). When subjecting a product to HHP treatment, the product in its final packaging is loaded into the pressure vessel and subjected to water pressures ranging from 100 to 900 megapascals (MPa). Given that the pressure is applied uniformly and simultaneously (isostatically) throughout the product, any negative effects of pressure on product size and geometry are minimized. This technology can be considered nonthermal because the adiabatic heating is only 3°C for every 100 MPa (9).

Even though the initial investment may be high for this type of technology, the running cost has been estimated to be around 0.19 US $ per kg of product treated at 600 MPa (9). Commercially available HHP vessels have 300 to 600l chambers and can operate on a semi-continious basis. Companies in Spain (NC Hyperbaric), the USA (Avure Technologies and Multivac), and the UK (Stanstead Fluid Power), among others, currently manufacture and sell HHP units for commercial use (8, 97, 100, 153).

Generally speaking, HHP treatment of foods results in the inactivation of microbial vegetative cells and also in the denaturation of enzymes, all of this while minimally affecting the organoleptic characteristics of the food product, especially cooked meats (9, 42, 60, 96). Damage to the cell membrane seems to be the main mode of action for HHP as the damage this technology causes to bacterial cell membranes can be extensive and often results in cell death (60, 99). Changes in membrane permeability, scarring around
the cell wall, separation of the cell wall from the membrane, protein denaturation, as well as damage to transport systems have been reported in HHP treated microbial populations (114, 132). However, the extent to which HHP will inactivate microorganisms depends on several different factors including, but not limited to, bacterial strain and the growth phase it is in at the time treatment is applied, the characteristics of the food matrix to be treated, temperature of the medium, pressure level, and exposure time (60). When compared to broth systems, for example, nutrient rich meat matrices allow for greater resistance of microorganisms to HHP treatment (59, 144). Thus, it would seem that any HHP treatment parameters would have to be tailored not only to the product to be treated, but also to the specific target microorganism and the expected outcome.

Organic Acids

The antibacterial effectiveness of organic acids and their salts, especially sodium or potassium lactate and diacetate, at controlling *L. monocytogenes* in RTE meats has been studied by different authors (11, 12, 88, 127, 133). Although widely used in the production of RTE meat and poultry products to control the growth of *L. monocytogenes*, lactate and diacetate do not have initial bactericidal effects on this pathogen and, in addition, cannot be used in the manufacture of uncured, no-nitrate-or-nitrite-added, RTE natural or organic meat and poultry products, as previously established. Thus, alternatives to these acids such as octanoic acid and lauric arginate have received attention from researchers and the meat industry.

The USDA FSIS lists lauric arginate (lauramide arginine ethyl ester or LAE) in its list of safe and suitable ingredients for production of meat and poultry products and it allows
for up to 44 mg/kg (plus or minus a 20% tolerance) of lauric arginate by weight of the product to be applied to the inside of a package as a processing aid (166). When used at this level, lauric arginate is considered a processing aid and does not have to be declared on the label of the product and, as a result, can be used in the manufacture of uncured, no-nitrate-or-nitrite-added, RTE natural or organic meat and poultry products.

Previous studies have investigated the effectiveness of lauric arginate to control \textit{L. monocytogenes} in RTE meats. Porto-Fett and others (128), for instance, evaluated the effects of 22 and 44 mg/kg lauric arginate, with or without the addition of potassium lactate and sodium diacetate, on the growth of \textit{L. monocytogenes} on commercially-produced frankfurters. These authors concluded that lauric arginate provides initial lethality towards \textit{L. monocytogenes} when used alone (1.8 log CFU per package) or in combination with lactate and diacetate (2.0 log CFU per package). However, the authors discovered that only when used in combination with said salts will lauric arginate exert a bacteriostatic effect on the pathogen under storage temperatures of 4°C for 120 days. Thus, although it may provide an initial lethality, lauric arginate alone does not inhibit the outgrowth of any \textit{L. monocytogenes} that may survive. Similar results were obtained by Luchansky and others (89) when they researched the effects of lauric arginate on the growth of \textit{L. monocytogenes} on hams.

Short- and medium-chain fatty acids have garnered attention from microbiologist due to their accepted antimicrobial properties. However, the application of fatty acids, especially octanoic acid, to food products with the idea of inhibiting pathogenic microorganisms has not been extensively studied (23). Octanoic acid, sometimes referred to as caprylic acid, is a saturated (C\textsubscript{8:0}) fatty acid (pK\textsubscript{a} 4.89) naturally found in coconut oil
and bovine milk (67). In broth systems, octanoic acid has been shown to effectively inhibit *L. monocytogenes* at pH 5.0 and 5.5 and 20°C, with the lower pH rendering the acid more effective (76). In a study that sought to evaluate the antilisterial effect of octanoic acid delivered to the surface of several different RTE meats within their final packaging, Burnett and others (23) concluded that 1% octanoic acid solutions acidified to pH 2.0 or 4.0 and applied to RTE meat and poultry products at a level of 1.9 ± 0.5 ml of solution per 100 cm² of product surface area resulted in *L. monocytogenes* log reductions ranging from 0.85 to 2.89 log CFU per sample in the different RTE products following 24 ± 4 h of storage at 5 ± 2°C. Furthermore, *L. monocytogenes* populations in all treated samples were significantly lower following treatment with either octanoic solution compared to the controls, the authors also concluded. It should be noted that these authors concluded that the octanoic acid treatments evaluated did not negatively affect the sensory attributed of the RTE meat and poultry products they were applied to (23).

The USDA FSIS also allows for octanoic acid to be used as a processing aid as long as it is applied to the surface of an RTE meat and poultry product at a rate not to exceed 400 mg/kg octanoic acid by weight of the final product (166). Although promising from an initial *L. monocytogenes* lethality standpoint, the bacteriostatic effects of octanoic acid have not been extensively researched and should receive more attention from the scientific community.

**Natural Antimicrobial Ingredients**

The rapid growth seen in the organic foods sector has been attributed to many different factors. The most common ones of these factors seem to be increased consumer
confidence in organic foods and concerns about potential health risks and environmental impacts of conventional food production methods (175). Concerns over the use of pesticides, antibiotics, and other chemicals in conventional production systems have also contributed to the overall sales growth seen in the organic foods sector (39, 143). Antimicrobials that have been proven to effectively inhibit the growth of L. monocytogenes such as lactate and diacetate, among others, are not allowed to be used in the production of natural and organic meat and poultry products. Thus, much emphasis has been placed on the investigation of natural sources of antimicrobials that could potentially replace these ingredients as a means to address L. monocytogenes in these highly restrictive product categories.

Several compounds derived from fruits, spices, oilseeds, and vegetables have been looked at in attempts to elucidate whether they exhibit any bactericidal or bacteriostatic effects on L. monocytogenes and other foodborne pathogens. These compounds often possess Generally Recognized as Safe (GRAS) status. In a study conducted by Ahn and others (4), for example, the authors determined that compounds derived from grape seed extract (ActiVin) and pine bark extract (Pycnogenol) had inhibitory properties against significant foodborne pathogens such as Escherichia coli O157:H7, Salmonella Typhimurium, and L. monocytogenes in an agar dilution test. Furthermore, upon looking at a 1.0% concentration of each ActiVin, Pycnogenol, and rosemary oleoresin in raw ground beef, each of these compounds reduced the levels of those three major pathogens by approximately 1 log CFU/g, and sometimes more, after 9 days of refrigerated storage (4). The presence of bioactive phenolic compounds in these compounds was mentioned as the likely source of antimicrobial properties against the studied foodborne pathogens.
Similar patterns were observed by Lin and others (86) after evaluating the effects of lactic acid, oregano extract, and cranberry extract on the growth of *L. monocytogenes* on fresh beef slices and fresh cod fillets stored at 4°C. Interestingly, although each of the compounds evaluated exhibited inhibitory effects on the growth of *L. monocytogenes* individually, these effects were greater when all three compounds were combined. These authors concluded that the phenolic diversity of the compounds evaluated was the likely source of the increased inhibitory effect and that this diversity could serve as a multipronged approach, similar to the multiple hurdle approach often talked about by regulators and scientists, to food safety (86). Examples of other compounds that have been evaluated for and shown to possess antibacterial and/or bactericidal properties against *L. monocytogenes* in raw meat systems include, but are not limited to, green tea and tamarind rasam (109), and cranberry concentrate (129).

Even though many so-called natural compounds have been shown to possess antilisterial properties, most of these findings have been obtained using liquid broth or foods or raw meat systems. Thus, a bit more interest has been placed on evaluating these compounds in cooked meat systems as of late. Xi and others (183), for instance, evaluated the antilisterial properties of cherry powder at 0.6%, lemon powder at 60 mg/kg, green tea extract at 1000 mg/kg, lime powder at 60 mg/kg, grape seed extract at 0.5%, and concentrations of cranberry powder ranging from 1 to 3% as part of a series of experiments. These authors concluded that, of all compounds evaluated, cranberry powder was most effective at inhibiting the growth of *L. monocytogenes* and that its inhibitory effects was directly proportional to the concentration used. In another study conducted by Hao and others (51), the antilisterial properties of several plant extracts
were evaluated in a cooked beef system. The authors determined that, out of all the natural compounds evaluated, only clove (eugenol) and pimento leaf extract exerted inhibitory effects on *L. monocytogenes* growth after 14 days of storage at 5°C. Furthermore, these authors also concluded that only clove extract limited the growth of *L. monocytogenes* when the storage temperature was 15°C (51). 0.5% finely ground rosemary, 1% rosemary oil, 5% encapsulated rosemary oil, and 0.3 to 0.5% rosemary antioxidant extract have also been shown to possess antilisterial properties after being added as ingredients prior to cooking to RTE pork liver sausage (112).

The differences in antimicrobial potency observed in natural compounds may be due in part to inconsistencies of commercial samples. Another important factor to consider is the food matrix itself, as it has been shown that *L. monocytogenes*, for example, was less sensitive to hop extracts in a food systems compared to a media system and that the fat content of the food caused the antilisterial properties of the hop extracts to vary (82). Thus, the antilisterial properties of natural antimicrobial ingredients used in RTE meat and poultry products are likely to vary based on product characteristics such as fat content, protein content, pH, *a*<sub>w</sub>, and other ingredients added.
Summary of Literature

Uncured, no-nitrate-or-nitrite-added RTE processed meat products have become increasingly popular with consumers over the last few years. The natural and organic processed meat categories continue to experience growth even though prices associated with these products are considerably higher compared to their conventionally produced counterparts. This growth is thought to be mostly due to the perceived safety and health benefits derived from the consumption of foods where the use of chemical preservatives, pesticides, and antimicrobials is restricted or, at least, closely monitored. Nitrite, the ingredient responsible for the characteristics associated with cured meats, is not allowed to be used in processed meat and poultry products seeking to fall under the uncured, natural, or organic categories due to the fact that it is classified as a chemical preservative. Due to nitrite’s proven track record as a potent antimicrobial agent against known foodborne pathogens such as \textit{C. botulinum} and, to a lesser extent, \textit{L. monocytogenes}, its absence from natural and organic processed meat products casts concerns over the safety of said products.

While some products are truly uncured, some may have nitrate or nitrite indirectly added to them in the form of vegetable powders that will result in cured meat characteristics. However, the levels of nitrite found in such meat and poultry products are usually lower than those found in conventional processed meat products. To further complicate matters, the reactions that nitrite undergoes upon its addition to a meat system are not yet fully understood. Thus, the modifications in the processing procedures and formulations of processed meat and poultry products seeking to fall under the natural or organic umbrella are likely to have negative impacts on the safety of these products.
The use of post-lethality interventions such as post-packaging thermal treatment, high hydrostatic pressure processing, octanoic acid, and lauric arginate has garnered interest from the scientific community and the meat industry as a potential means to address *L. monocytogenes* in RTE processed meat and poultry products. Additionally, these post-lethality interventions, if used within certain regulatory limits, could satisfy natural and organic labeling requirements. However, although post-lethality interventions might deliver an initial lethality, some concerns still exist over the potential recovery of sublethally injured microorganisms over the storage life of the product. This scenario creates a clear need for additional hurdles to be implemented along with these post-lethality interventions in order fully address *L. monocytogenes*.

Several compounds derived from fruits, spices, oilseeds, and vegetables that are GRAS substances and could, potentially, be used in the manufacture of natural and organic processed meat and poultry products have been shown to exhibit bactericidal or bacteriostatic effects on *L. monocytogenes* and other foodborne pathogens. Their use in RTE processed meat and poultry products has not been extensively investigated, however. The use of natural antimicrobial interventions alone and in combination with post-lethality interventions as a means to inhibit the recovery and growth of *L. monocytogenes* in naturally cured RTE processed meat products was, therefore, the focus of the work reported in this dissertation.
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CHAPTER 3. EVALUATION OF THE THIN AGAR LAYER (TAL) METHOD FOR THE RECOVERY OF HEAT-INJURED AND PRESSURE-INJURED LISTERIA MONOCytogenES

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Abstract

A sublethally injured bacterial cell has been defined as a cell that survives a stress such as heating, freezing, acid treatment, or other antimicrobial intervention, but loses some of its defining qualities. An injured cell has been described further as one that can repair the cellular damage exerted by a stressor (resuscitate) and regain its original ability to grow and form a colony in the presence of selective agents, whereas a dead cell would not be able to grow and form a colony under any circumstances. Thus, the objective of this study was to evaluate the use of the thin agar layer (TAL) method in the recovery of heat-injured and pressure-injured Listeria monocytogenes in a tryptic soy broth with 0.6% yeast extract (TSBYE) system. The heat-injury experiment consisted of treatment of a L. monocytogenes cocktail at 60 ± 1°C for 3 min, 6 min, or 9 min, while the pressure-injury experiment consisted of treatment of a L. monocytogenes cocktail with high hydrostatic pressure (HHP) at 400 or 600 MPa for 1 s, 2 min, 4 min, or 6 min at 12 ± 2°C. Growth
mediums used were tryptic soy agar with 0.6% yeast extract (TSAYE), modified Oxford medium (MOX), and TAL which consisted of a 7-ml layer of TSA overlaid onto solidified MOX. Results from both experiments indicate that MOX, when compared to TSAYE, may underestimate the number of *L. monocytogenes* that survive heating and HHP treatments similar to those used in our studies. Viable *L. monocytogenes* numbers observed on TAL were greater than those found on MOX in the heat-injury experiment but not in the pressure-injury experiment. The effectiveness of the TAL method may, therefore, be specific to the type of injury applied to the microorganism.

**Introduction**

The presence of pathogenic microorganisms in food is of obvious concern to government agencies, food manufacturers, and, ultimately, consumers. Several technologies have been developed and are currently used by food manufacturers to address the potential presence of foodborne pathogens in foods. Heating, freezing, drying, freeze-drying, fermentation, addition of antimicrobials, irradiation, high hydrostatic pressure, among others, are only a few examples of practices currently being implemented by food manufacturers as part of their efforts to combat foodborne pathogens (19). In spite of the fact that these technologies exert deleterious and often deadly effects on a number of foodborne pathogens, a specific population of microorganisms may be able to survive these treatments, escape detection through most conventional detection methods, recover if presented with the appropriate conditions, and, eventually, cause foodborne illness. This population of “sublethally injured” microorganisms is just as important as their intact counterparts and should be taken into
consideration when developing product formulations and designing microbial intervention methods.

A sublethally injured bacterial cell has been defined as a cell that survives a stress such as heating, freezing, acid treatment, or other antimicrobial intervention, but loses some of its defining qualities (I, 19). One of the first descriptions of an injured microbial cell was made by Hartsell in 1951 (4). As part of this description, Hartsell defined an injured cell as one capable of forming colonies or growing on nonselective media but not on selective media. A sublethally injured cell has been described further as one that can repair the cellular damage exerted by a stressor (resuscitate) and regain its original ability to grow and form a colony in the presence of selective agents, whereas a dead cell would not be able to grow and form a colony under any circumstances (10). Conservative estimates indicate that foodborne diseases cause between $10 and $83 billion in illness-related costs annually in the United States alone (14). These costs include, but are not limited to, pain and suffering, reduced productivity, and medical costs. The loss of any lives to foodborne illness, needless to say, cannot have a dollar figure associated with it. Thus, our ability to detect any foodborne pathogens that may be present in our foods is of utmost importance, especially when dealing with foods that are ready-to-eat (RTE) and will likely not receive any further cooking from the consumer after purchase. Moreover, the potential presence of sublethally injured pathogens such as Listeria monocytogenes in RTE products, coupled with the inability of current detection methods to reliably detect sublethally injured microorganisms, presents a scenario under which false negative results may be obtained. As a result, populations of sublethally injured pathogens and
their reliable detection have become an area of focus for researchers in the field of microbiology.

Sublethally injured bacterial cells, if presented with favorable conditions, possess the ability to repair themselves (resuscitate) and return to a normal physiological state wherein they can initiate cell division and grow. The term “resuscitation” has been utilized by researchers because it illustrates the fact that sublethally injured cells come back from apparent death (5). During the process of resuscitation and before normal growth can occur, the restoration of growth capabilities as well as the reversing of any cellular modifications must occur (19). The resuscitation or repair of sublethally injured microorganisms was originally studied using liquid recovery methods, but later came to involve solid recovery methods as well (5, 19). A detailed look into both types of recovery methods is warranted before any one of them is selected for the microorganism and type of injury to be studied.

When using liquid recovery methods, the food sample is typically blended and incubated in a nonselective broth to allow for the repair of sublethally injured microorganisms. Time and temperature of this recovery period vary with the method of recovery to be used, the microorganism to be studied, and the type of stressor used to inflict injury on the bacterial cells (1, 19). Generally speaking, incubation temperatures will range from 25 to 37°C while the incubation time will typically range between 1 to 5 hours (19). Once the repair or resuscitation period has been completed, enumeration of cells can be carried out using direct plating or most probable number (MPN) techniques. An example of a liquid recovery method that has been investigated is the two-fold dilution (2FD) method (8).
It is commonly accepted that, although appealing from a practicality standpoint, the results obtained using liquid recovery methods can be misleading. Plating aliquots from food samples after the resuscitation step onto selective media could yield colonies that could be due to the resuscitation of sublethally injured microorganisms, but these same counts could be due to the growth and/or multiplication of uninjured cells, especially in instances when the time of incubation utilized is prolonged. Furthermore, reculturing and further isolation of the organism to be studied would be required if using the 2FD method, for example (8, 19). Thus, liquid recovery methods are expected to receive limited acceptance from regulatory and industry bodies.

In contrast with liquid recovery methods, solid recovery methods will typically call for either pour-plating or surface-plating of the blended sample with a nonselective medium, followed by incubation for a predetermined amount of time at a suitable temperature for repair to occur. Once the repair step has been completed, some methods call for an overlay with a selective medium that is specific for the microorganism of interest followed by incubation. During this incubation period the selective agents present in the selective medium migrate through the nonselective medium and create a selective environment throughout. Because a recovery step was instituted at the beginning of the process, those microorganisms that were originally sublethally injured are believed to be able to grow in the presence of the selective agents. Solid recovery methods that employ the previously described procedures are more direct and economical than liquid recovery methods. On the other hand, these methods make picking isolated colonies for further testing difficult and may yield variable results when working with very low (< 10 Colony Forming Units [CFU] per g) counts (9, 12, 16, 19). In addition, the temperature of the
molten selective agar to be overlaid can further affect sublethally injured microorganisms and impede their complete recovery (9, 16, 19). Examples of such solid recovery methods include the pour-overlay method (11, 12) and the surface-overlay method (3, 13).

A relatively new recovery method is the thin agar layer (TAL) method, developed by Kang and Fung in 1999 (7). This method represents a one-step procedure and involves pouring a thin layer of nonselective agar onto a prepoured and solidified pathogen-specific selective medium. Over the years, the nonselective agar of choice to use as part of the TAL method became tryptic soy agar (TSA) because it provides a favorable environment for sublethally injured cells to resuscitate and regain their original growth capabilities within the first few hours of incubation (6, 17, 18). After resuscitation, the sublethally injured microorganisms can interact with the selective agents in the bottom layer whereas nontarget microorganisms are inhibited by those same agents. Furthermore, the layer of TSA does not hinder color reactions expected when using selective agents such as those found in modified Oxford (MOX) medium (7, 15, 17, 18, 19).

When using the TAL method, the layer of TSA has been shown not to interfere with typical color reactions or morphology seen in colonies of target microorganisms, which represents an advantage over the two-step overlay method. Additionally, the isolation of individual colonies for further characterization is much easier, given that these colonies would be located on the surface of the TAL plate compared to within or between the two layers of agar as they would be in a overlayed plate. These advantages, combined with the fact that the TAL method has been demonstrated to allow for the differentiation of target microorganisms even in the presence of background flora, make the TAL method a promising option in the study of sublethally injured foodborne pathogens commonly
associated with foodborne illness (2, 15, 17, 18, 19, 20). Thus, the objective of this study was to evaluate and compare the use of the TAL method for the recovery of heat-injured and pressure-injured \textit{L. monocytogenes} using a tryptic soy broth with 0.6\% yeast extract (TSBYE) system to determine the effectiveness of this recovery system for two different types of sublethal cellular injuries.

**Materials and Methods**

**Preparation of Media**

Tryptic soy agar containing 0.6\% yeast extract (TSAYE) was used as the nonselective medium for comparison with MOX and TAL. Dry ingredients used to manufacture TSAYE medium were 40.0 g of TSA (Difco, Becton Dickinson, Sparks, MD) and 6.0 g of yeast extract (Difco, Becton Dickinson) per liter of de-ionized water. MOX medium was used as the selective medium. Dry ingredients used to manufacture the MOX medium were 42.5 g of Columbia agar base (Difco, Becton Dickinson), 15.0 g of lithium chloride (Difco, Becton Dickinson), 1.0 g of esculin hydrate (Sigma-Aldrich, St. Louis, MO), and 0.5 g of ferric ammonium citrate (Difco, Becton Dickinson) per liter of de-ionized water. MOX was supplemented with modified Oxford antimicrobial supplement (Difco, Becton Dickinson). TAL media that was made according to Kang and Fung (7) with some modifications. MOX was made as previously described. Then, MOX plates to be made into TAL were aseptically overlaid with 7.0 ml of sterile TSA held at 55°C to facilitate even distribution of the molten agar. TAL media was deemed ready for use no fewer than 30 min after 7.0-ml TSA overlay to allow enough time for it to solidify. Plates
used for microbial analyses were sterile and 55 mm in diameter (Fisher Scientific, Waltham, MA).

**Preparation of Inoculum**

*L. monocytogenes* strains Scott A NADC 2045 serotype 4b, H7969 serotype 4b, H7962 serotype 4b, H7596 serotype 4b, and H7762 serotype 4b were obtained from the Iowa State University Food Safety Research Laboratory in the Food Science and Human Nutrition Department. Each strain was cultured separately in TSBYE (Difco, Becton Dickinson) for 24 h at 35°C. A minimum of two consecutive 24-h transfers of each strain to fresh TSBYE (35°C) were performed prior to each experiment. Aliquots (6.0-ml) from each of the five strains were then transferred into a sterile centrifuge tube. The bacterial cells were harvested by centrifugation (10 min at 10,000 rpm and 4°C) in a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT). The supernatant was discarded and the pelleted cells were resuspended in 30.0 ml of sterile 0.1% buffered peptone water (BPW) (Difco, Becton Dickinson). The total concentration of the five-strain inoculum was approximately $10^9$ CFU per ml based on the aerobic plate counts of the washed cell suspension.

**Heat-Injury Experiment**

An aliquot of 1.0 ml of the inoculum, prepared as previously described, was added to each of two flasks containing 99.0-ml of previously heated, sterile TSBYE to result in an initial bacterial concentration of $10^7$ CFU per ml. The temperature of the sterile TSBYE was brought up to and maintained at $60 \pm 1^\circ C$ during the experiment using a reciprocal water bath shaker model R 76 (New Brunswick Scientific, Edison, NJ) set at 50 rpm. At
3, 6, and 9 min time intervals after inoculation, an aliquot of 1.0 ml of inoculated TSBYE from each flask was aseptically transferred into 9.0-ml tubes of sterile 0.1% BPW kept in an ice and water slush. Each of these tubes represented the $10^{-1}$ dilution for each of the time intervals specified. An aliquot of 1.0 ml of the inoculum was added to a non-heated flask containing 99.0 ml of sterile TSBYE to be used as a control. Serial dilutions (10-fold) of each tube were prepared accordingly. An aliquot of 1.0 ml (for $10^0$ dilution, divided into three ~0.33-ml aliquots plated on three separate plates) or 0.1 ml of the appropriate dilution was surfaced plated on MOX, TAL, and TSAYE media. Each sample was plated in duplicate. All inoculated agar plates were incubated in an inverted position at 35°C for 72 h, after which time they were removed from the incubator and colonies typical of *L. monocytogenes* were enumerated. The counts (CFU per ml) were averaged and then converted to $\log_{10}$ CFU per ml. The detection limit of our sampling protocols was ≥ 0.30 log CFU per ml. As part of the heat-injury experiment, TAL was made 48 (TAL 48), 24 (TAL 24), and 0 (TAL 0) h prior to use in order to determine if length of time prior to use had any effects on viable *L. monocytogenes* numbers counted on TAL agar. Two independent replications of this experiment were conducted.

**Pressure-Injury Experiment**

An aliquot of 0.1 ml of the inoculum, prepared as previously described, was aseptically added to 9.9 ml of sterile TSBYE to result in an initial bacterial concentration of $10^7$ CFU per ml. Samples were contained within a 4 fl. oz, 4 by 6 in PET/LLDPE sterile pouch (Kapak Corporation, Minneapolis, MN). The pouches had a water vapor permeability of 4.07 g per m² per 24 h and an O₂ permeability of 118.65 cc per m² per 24 h. Each pouch was sealed immediately after inoculation and placed on ice for transport to
the High Pressure Processing Laboratory at the Iowa State University Food Science and Human Nutrition Department and subjected to the appropriate HHP treatment. HHP treatments evaluated were 400 MPa or 600 MPa for 1 s, 2, 4, or 6 min dwell time at a starting pressurization fluid temperature of 12 ± 2°C and were conducted using a FOOD-LAB 900 Plunger Press system (Standsted Fluid Power Ltd., Standsted, UK). The pressurization fluid was a 50.0% propylene glycol (GWT Koilguard; GWT Global Water Technology, Inc., Indianapolis, IN) and 50.0% water solution (v/v). The average rate of pressurization was 350 MPa per min and depressurization occurred within 7 s. HHP treatment was applied to products within two hours after inoculation. An aliquot of 0.1 ml of the inoculum was added to a pouch containing 9.9 ml of sterile TSBYE to be left untreated and used as a control. Upon completion of HHP treatment, samples were transported on ice to the Iowa State University Food Safety Research Laboratory in the Food Science and Human Nutrition Department for microbial analysis. Samples were aseptically opened and serially diluted (10-fold) accordingly. An aliquot of 1.0 ml (for 10⁰ dilution, divided into three ~0.33-ml aliquots plated on three separate plates) or 0.1 ml of the appropriate dilution was surfaced plated on MOX, TAL, and TSAYE media. TAL media was made less than 48 h prior to use. Each sample was plated in duplicate. All inoculated agar plates were incubated in an inverted position at 35°C for 72 h, after which time they were removed from the incubator and colonies typical of *L. monocytogenes* were enumerated. The counts (CFU per ml) were averaged and then converted to log₁₀ CFU per ml. The detection limit of our sampling protocol was ≥ 0.30 log CFU per ml. Three independent replications of this experiment were conducted.
Statistical Analysis

The overall design of the experiments was a factorial design. The generalized linear mixed models (GLIMMIX) procedure of Statistical Analysis System (version 9.3, SAS Institute Inc., Cary, NC) was used for statistical analysis. Viable \textit{L. monocytogenes} data were analyzed for treatment effects within treatment time and pressure, if applicable. Where significant effects ($P < 0.05$) were found, pair-wise comparisons between the least squares means were computed for each treatment time and pressure, if applicable, using Tukey’s honestly significant difference adjustment.

Results and Discussion

Heat-Injury Experiment

Results obtained after the conduction of the heat-injury experiment can be found on Table 1 and are illustrated on Figure 1. As expected, viable \textit{L. monocytogenes} levels found in the Control treatment ranged from 7.09 to 7.15 log CFU per ml based on the medium used and were near our target inoculation level of $10^7$ CFU per ml. After 3 min at $60 \pm 1^\circ$C, the differences between the numbers of viable \textit{L. monocytogenes} counted on the different media evaluated began to arise. Viable \textit{L. monocytogenes} numbers found on MOX were significantly lower than those counted on all TAL media types as well as those found on TSAYE ($P < 0.05$), indicating that the selective agents found in MOX exerted an inhibitory effect on sublethally injured \textit{L. monocytogenes}. Although there were no significant differences found between the types of TAL tested ($P > 0.05$), viable \textit{L. monocytogenes} counts on TSAYE were higher than those found on all types of TAL.
media ($P < 0.05$). Similar results were obtained after applying a heat-injury treatment of 6 min at 60 ± 1°C.

After 9 min at 60 ± 1°C, similar results were observed with the exception that a significant ($P < 0.05$) difference between the types of TAL was also found. More specifically, TAL 48 exhibited lower ($P < 0.05$) numbers of viable L. monocytogenes than either TAL 0 or TAL 24. These results suggest that the selective components found in the MOX layer of TAL 48 may have migrated through the top TSA layer to a greater extent as a result of time prior to use. Alternatively, a greater degree of heat-injury was likely to have occurred after 9 min at 60 ± 1°C, which may have rendered sublethally injured L. monocytogenes more susceptible to the selective agents from the MOX layer that had migrated through the TSA layer of TAL and, therefore, led to the lower viable L. monocytogenes counts observed.

The overall effect of growth medium on viable L. monocytogenes numbers for the heat-injury experiment is shown in Table 2. Viable L. monocytogenes numbers found on TSAYE were higher than those found in any of the other media studied ($P < 0.05$). This observation allows us to conclude that, of the media evaluated, TSAYE was the most conducive to recovery of those microorganisms that were sublethally injured as a result of the heating treatments employed. Viable L. monocytogenes levels found on MOX were lower than those found in any of the other growth media evaluated ($P < 0.05$), which suggests that selective agents found in MOX inhibited the recovery and subsequent formation of colonies by sublethally injured L. monocytogenes. Kang and Fung (7) observed that while viable L. monocytogenes numbers found on MOX were lower than those found on TSA and TAL after heating treatment at 55.0°C for 15 min in both 0.1%
peptone water and sterile skim milk, numbers found on the latter two did not significantly differ. Our results, as previously mentioned, showed that TSAYE yielded higher viable \textit{L. monocytogenes} counts than TAL. This was likely due to the supplementation of TSA with 0.6\% YE, which is high in B-complex vitamins, and to the shorter heating times employed in our experiment.

Furthermore, of the types of TAL media investigated, TAL 24 yielded the highest viable \textit{L. monocytogenes} counts and TAL 48 the lowest ($P < 0.05$). These results suggest that selective agents found in MOX may migrate through the top (TSA) layer of TAL over time and exert an inhibitory effect on the recovery and growth of sublethally injured microorganisms. Viable \textit{L. monocytogenes} counts obtained using all types of TAL were still higher than those obtained using MOX ($P < 0.05$). Given that viable \textit{L. monocytogenes} levels found on the types of TAL studied ranged from 4.78 to 5.03 log CFU per ml, the practical benefits of preparing TAL media up to 48 h prior to use should be considered when developing microbial analysis protocols. To our knowledge, no other work has been conducted on this subject and further research is warranted.

Overall, under the conditions of this study, our results allow us to conclude that MOX may underestimate the number of \textit{L. monocytogenes} cells that survive heating treatments similar to those applied in this study compared to TSAYE and also to TAL that is made up to 48 h prior to use. Furthermore, the use of TAL media that is made up to 48 h prior to use may result in viable bacterial numbers that are closer to the actual number of \textit{L. monocytogenes} that survive heating treatments similar to those investigated in this experiment. Thus, the use of TAL media in the study of heat-injury of \textit{L. monocytogenes} represents a useful method for researchers to employ.
Pressure-Injury Experiment

As expected, viable *L. monocytogenes* levels found in the Control treatment of the pressure-injury experiment ranged from 7.16 to 7.23 log CFU per ml based on the medium used and were near our target inoculation level of $10^7$ CFU per ml (Tables 3 and 5). The effects of growth medium and HHP treatment time at 400 MPa on viable *L. monocytogenes* counts are described in Table 3 and illustrated in Figure 2. No significant differences in viable *L. monocytogenes* numbers were found between MOX, TAL, and TSAYE after 1 s, 2 min, or 4 min of HHP treatment at 400 MPa ($P > 0.05$). After 6 min of HHP treatment at 400 MPa, on the other hand, viable *L. monocytogenes* levels observed on TSAYE were higher than those observed on MOX ($P < 0.05$), indicating that selective agents found in MOX may inhibit the growth of pressure-injured *L. monocytogenes* compared to TSAYE under these conditions. No significant differences were found between viable *L. monocytogenes* numbers obtained using TAL compared to either MOX or TSAYE ($P > 0.05$). Viable *L. monocytogenes* numbers obtained on TAL, although not significantly different ($P > 0.05$), were numerically higher than those obtained using MOX by 0.64 log CFU per ml.

Analysis of the pooled least squares means for all 400 MPa HHP treatments investigated (Table 4) showed that viable *L. monocytogenes* counts obtained using TSAYE were higher than those obtained using either MOX or TAL ($P < 0.05$). MOX and TAL, in turn, yielded viable counts that were not different from one another ($P > 0.05$).

The effects of growth medium and HHP treatment time at 600 MPa on viable *L. monocytogenes* counts are described in Table 5 and illustrated in Figure 3. No significant
differences in viable *L. monocytogenes* numbers were found between MOX, TAL, and TSAYE after 1 s of HHP treatment at 600 MPa (*P* > 0.05). After 2 min of HHP treatment at 600 MPa, on the other hand, viable *L. monocytogenes* levels observed on TSAYE were higher than those observed on both MOX and TAL (*P* < 0.05). Viable *L. monocytogenes* levels observed on TAL were significantly higher than those found on MOX (*P* < 0.05), however. Furthermore, after 4 and 6 min of HHP treatment at 600 MPa, viable *L. monocytogenes* counts observed on TSAYE were higher (*P* < 0.05) than those found using both MOX and TAL, whereas no significant differences were found between the latter two (*P* > 0.05). These observations suggest that selective agents found in MOX may inhibit the growth of pressure-injured *L. monocytogenes* compared to TSAYE. Additionally, TAL allowed for the recovery of pressure-injured *L. monocytogenes* to a greater degree than MOX did after 2 min of HHP treatment at 600 MPa but not after 4 or 6 min of HHP treatment at the same pressure.

The comparison of pooled least squares means for all 600 MPa HHP treatments is shown on Table 6 and indicates that TSAYE yielded higher (*P* < 0.05) viable *L. monocytogenes* numbers than both MOX and TAL. Additionally, viable *L. monocytogenes* levels obtained using TAL, although numerically higher, were not significantly different (*P* > 0.05) from those obtained using MOX. These results indicate that, under the conditions of this study, selective agents found in MOX and TAL media inhibit the recovery and growth of pressure-injured *L. monocytogenes* when compared to TSAYE and that TAL allows only for a limited recovery and growth of such microorganisms.
Overall, under the conditions of this experiment, results obtained in our laboratory allow us to conclude that MOX may underestimate the number of L. monocytogenes cells that survive HHP treatments similar to those applied in this study compared to TSAYE. Furthermore, the use of TAL medium when evaluating the pressure-injury of L. monocytogenes offers limited advantages. TSAYE provided best recovery of L. monocytogenes following HHP at 600 MPa.

The two types of injury (heat vs. pressure) exerted on L. monocytogenes as part of our experiments yielded differing results in terms of the ability of the TAL method to allow for the recovery of sublethally injured microorganisms. It is likely that the metabolic pathways and/or cellular components such as ribosomes, enzymes, and nucleic acids affected by the types of injury herein investigated are responsible for the differences observed. Although the value of the TAL method in the study in heat-injured L. monocytogenes is well established, its use in the study of other types of sublethal injury of L. monocytogenes requires more attention from the scientific community and more research should be conducted on the recovery of sublethally injured bacterial cells.

Acknowledgements

This project was supported by the American Meat Institute Foundation. Special thanks to Devin Maurer and Daniel Fortin for the invaluable contributions made throughout the conduction of these experiments.
References


**TABLE 1. Effects of growth medium on viable Listeria monocytogenes numbers after treatment at 60.0 ± 1.0°C in TSBYE broth for 3, 6, or 9 min**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Control</th>
<th>3 min</th>
<th>6 min</th>
<th>9 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOX</td>
<td>7.15 ± 0.03</td>
<td>4.74 ± 0.35&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.19 ± 0.25&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.90 ± 0.35&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>TAL 0</td>
<td>7.09 ± 0.03</td>
<td>5.79 ± 0.11&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.02 ± 0.26&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.90 ± 0.49&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>TAL 24</td>
<td>7.11 ± 0.05</td>
<td>5.92 ± 0.22&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.19 ± 0.21&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.98 ± 0.71&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>TAL 48</td>
<td>7.14 ± 0.06</td>
<td>5.86 ± 0.11&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.97 ± 0.19&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.52 ± 0.48&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>TSAYE</td>
<td>7.11 ± 0.03</td>
<td>6.49 ± 0.22&lt;sup&gt;C&lt;/sup&gt;</td>
<td>5.94 ± 0.10&lt;sup&gt;C&lt;/sup&gt;</td>
<td>4.87 ± 0.56&lt;sup&gt;D&lt;/sup&gt;</td>
</tr>
<tr>
<td>SE&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.10</td>
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</tbody>
</table>

<sup>a</sup> Values are least squares means ± standard deviation. Within a column, means with different superscripts (A through D) are significantly different (P < 0.05).

<sup>b</sup> Medium: MOX, modified Oxford medium base supplemented with modified Oxford antimicrobial supplement; TAL 0, 0-h-old thin agar layer medium; TAL 24, 24-h-old thin agar layer medium; TAL 48, 48-h-old thin agar layer medium; TSAYE, tryptic soy agar supplemented with 0.6% yeast extract.

<sup>c</sup> Standard error of the differences of least squares means.
FIGURE 1. Effects of growth medium on viable Listeria monocytogenes numbers after treatment at 60.0 ± 1.0°C in TSBYE broth for 3, 6, or 9 min.

Medium: MOX, modified Oxford medium base supplemented with modified Oxford antimicrobial supplement; TAL 0, 0-h-old thin agar layer medium; TAL 24, 24-h-old thin agar layer medium; TAL 48, 48-h-old thin agar layer medium; TSAYE, tryptic soy agar supplemented with 0.6% yeast extract.

a–d Within a treatment time, columns with different superscripts are significantly different (P < 0.05).
TABLE 2. *Pooled least squares means of viable* Listeria monocytogenes *counts by growth medium after inoculation with a 5-strain cocktail at* $10^7$ *CFU per ml followed by 3, 6, and 9 min at 60.0 ± 1.0°C treatments*<sup>a</sup>

<table>
<thead>
<tr>
<th>Medium&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Log CFU per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOX</td>
<td>3.28&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>TAL 0</td>
<td>4.90&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>TAL 24</td>
<td>5.03&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>TAL 48</td>
<td>4.78&lt;sup&gt;D&lt;/sup&gt;</td>
</tr>
<tr>
<td>TSAYE</td>
<td>5.77&lt;sup&gt;E&lt;/sup&gt;</td>
</tr>
<tr>
<td>SE&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.06</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are pooled least squares means. Within a column, means with different superscripts (A through E) are significantly different ($P < 0.05$).

<sup>b</sup> Medium: MOX, modified Oxford medium base supplemented with modified Oxford antimicrobial supplement; TAL 0, 0-h-old thin agar layer medium; TAL 24, 24-h-old thin agar layer medium; TAL 48, 48-h-old thin agar layer medium; TSAYE, tryptic soy agar supplemented with 0.6% yeast extract.

<sup>c</sup> Standard error of the differences of least squares means.
TABLE 3. Effects of growth medium on viable Listeria monocytogenes numbers after HHP treatment consisting of 400 MPa for 1 s, 2, 4, or 6 min at 12 ± 2°C in TSBYE broth

<table>
<thead>
<tr>
<th>Medium</th>
<th>Control</th>
<th>1 s</th>
<th>2 min</th>
<th>4 min</th>
<th>6 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOX</td>
<td>7.22 ± 0.05</td>
<td>7.38 ± 0.09</td>
<td>7.23 ± 0.07</td>
<td>6.09 ± 0.51</td>
<td>5.21 ± 0.61\textsuperscript{A}</td>
</tr>
<tr>
<td>TAL</td>
<td>7.23 ± 0.04</td>
<td>7.47 ± 0.06</td>
<td>7.27 ± 0.04</td>
<td>6.03 ± 0.65</td>
<td>5.85 ± 0.67\textsuperscript{AB}</td>
</tr>
<tr>
<td>TSAYE</td>
<td>7.16 ± 0.05</td>
<td>7.41 ± 0.14</td>
<td>7.07 ± 0.04</td>
<td>6.63 ± 0.13</td>
<td>6.29 ± 0.45\textsuperscript{B}</td>
</tr>
<tr>
<td>SE\textsuperscript{c}</td>
<td>0.30</td>
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<td></td>
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</tr>
</tbody>
</table>

\textsuperscript{a} Values are least squares means ± standard deviation. Within a column, means with different superscripts (A through B) are significantly different ($P < 0.05$).

\textsuperscript{b} Medium: MOX, modified Oxford medium base supplemented with modified Oxford antimicrobial supplement; TAL, thin agar layer medium; TSAYE, tryptic soy agar supplemented with 0.6% yeast extract.

\textsuperscript{c} Standard error of the differences of least squares means.
FIGURE 2. Effects of growth medium on viable Listeria monocytogenes numbers after HHP treatment consisting of 400 MPa for 1 s, 2, 4, or 6 min at 12 ± 2°C in TSBYE broth.

Medium: MOX, modified Oxford medium base supplemented with modified Oxford antimicrobial supplement; TAL, thin agar layer medium; TSAYE, tryptic soy agar supplemented with 0.6% yeast extract.

\( \text{a} - \text{b} \) Within a treatment time, columns with different superscripts are significantly different \( (P < 0.05) \).
TABLE 4. **Pooled least squares means of viable Listeria monocytogenes counts by growth medium after inoculation with a 5-strain cocktail at 10⁷ CFU per ml followed by HHP treatment consisting of 400 MPa for 1 s, 2, 4, and 6 min at 12 ± 2°C**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Log CFU per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOX</td>
<td>6.48&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>TAL</td>
<td>6.66&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>TSAYE</td>
<td>6.85&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>SE&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.09</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are pooled least squares means. Within a column, means with different superscripts (A through B) are significantly different ($P < 0.05$).

<sup>b</sup> Medium: MOX, modified Oxford medium base supplemented with modified Oxford antimicrobial supplement; TAL, thin agar layer medium; TSAYE, tryptic soy agar supplemented with 0.6% yeast extract.

<sup>c</sup> Standard error of the differences of least squares means.
TABLE 5. Effects of growth medium on viable Listeria monocytogenes numbers after HHP treatment consisting of 600 MPa for 1 s, 2, 4, or 6 min at 12 ± 2°C in TSBYE broth

<table>
<thead>
<tr>
<th>Medium</th>
<th>Control</th>
<th>1 s</th>
<th>2 min</th>
<th>4 min</th>
<th>6 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOX</td>
<td>7.22 ± 0.05</td>
<td>6.21 ± 0.46</td>
<td>0.93 ± 0.33&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.64 ± 0.73&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.85 ± 0.54&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>TAL</td>
<td>7.23 ± 0.04</td>
<td>6.41 ± 0.36</td>
<td>1.85 ± 0.41&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.73 ± 0.50&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.69 ± 0.53&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>TSAYE</td>
<td>7.16 ± 0.05</td>
<td>6.71 ± 0.19</td>
<td>3.10 ± 0.25&lt;sup&gt;C&lt;/sup&gt;</td>
<td>1.48 ± 0.36&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.72 ± 0.75&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>SE&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are least squares means ± standard deviation. Within a column, means with different superscripts (A through C) are significantly different (P < 0.05).

<sup>b</sup> Medium: MOX, modified Oxford medium base supplemented with modified Oxford antimicrobial supplement; TAL, thin agar layer medium; TSAYE, tryptic soy agar supplemented with 0.6% yeast extract.

<sup>c</sup> Standard error of the differences of least squares means.
FIGURE 3. Effects of growth medium on viable Listeria monocytogenes numbers after HHP treatment consisting of 600 MPa for 1 s, 2, 4, or 6 min at 12 ± 2°C in TSBYE broth.

Medium: MOX, modified Oxford medium base supplemented with modified Oxford antimicrobial supplement; TAL, thin agar layer medium; TSAYE, tryptic soy agar supplemented with 0.6% yeast extract.

a–c Within a treatment time, columns with different superscripts are significantly different ($P < 0.05$).
TABLE 6. Pooled least squares means of viable *Listeria monocytogenes* counts by growth medium after inoculation with a 5-strain cocktail at $10^7$ CFU per ml followed by HHP treatment consisting of 600 MPa for 1 s, 2, 4, and 6 min at $12 \pm 2^\circ C$\textsuperscript{a}

<table>
<thead>
<tr>
<th>Medium\textsuperscript{b}</th>
<th>Log CFU per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOX</td>
<td>2.15\textsuperscript{A}</td>
</tr>
<tr>
<td>TAL</td>
<td>2.42\textsuperscript{A}</td>
</tr>
<tr>
<td>TSAYE</td>
<td>3.25\textsuperscript{B}</td>
</tr>
<tr>
<td>SE\textsuperscript{c}</td>
<td>0.19</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Values are pooled least squares means. Within a column, means with different superscripts (A through B) are significantly different ($P < 0.05$).

\textsuperscript{b} Medium: MOX, modified Oxford medium base supplemented with modified Oxford antimicrobial supplement; TAL, thin agar layer medium; TSAYE, tryptic soy agar supplemented with 0.6% yeast extract.

\textsuperscript{c} Standard error of the differences of least squares means.
CHAPTER 4. INVESTIGATING THE CONTROL OF LISTERIA MONOCYTOGENES ON NATURALLY CURED FRANKFURTERS USING NATURAL ANTIMICROBIAL INGREDIENTS AND POST-LETHALITY INTERVENTIONS

A paper to be submitted to the Journal of Food Protection

Nicolas A. Lavieri, Joseph G. Sebranek, Joseph C. Cordray, James S. Dickson, Ashley M. Horsch, Stephanie Jung, Elaine M. Larson, David K. Manu, and Aubrey F. Mendonça

Abstract

Ready-to-Eat (RTE) meat and poultry products manufactured with natural or organic methods are at greater risk for Listeria monocytogenes growth, if contaminated, than their conventional counterparts due to the required absence of preservatives and antimicrobials. Thus, the objective of this study was to investigate the use of commercially available natural antimicrobials and post-lethality interventions in the control of L. monocytogenes growth and recovery on naturally cured frankfurters. Antimicrobials evaluated were cranberry powder (90MX), vinegar (DV), and vinegar and lemon juice concentrate (LV1X). Post-lethality interventions studied were high hydrostatic pressure at 400 (HHP400) or 600 (HHP600) MPa, lauric arginate (LAE), octanoic acid (OA), and post-packaging thermal treatment (PPTT). Parameters evaluated through 98 days of storage at 4 ± 1°C were residual nitrite concentrations, CIE L*, a*, and b* values, and viable L. monocytogenes on modified Oxford (MOX) and thin agar
layer (TAL) media. On day 1, OA, 90MX, and LV1X yielded lower residual nitrite concentrations than the Control, whereas HHP400, HHP600, LAE, and DV did not. Counts on MOX and TAL did not differ. LAE, OA, and HHP400 reduced *L. monocytogenes* levels compared to the Control after 1 day of storage by 2.28, 2.03, and 1.88 log CFU per g, respectively. PPTT did not achieve a significant reduction in bacterial numbers. *L. monocytogenes* recovered and grew in all post-lethality intervention treatments, except HHP600. 90MX did not inhibit the growth of *L. monocytogenes*, while DV and LV1X did. Results of this study demonstrate the bactericidal properties of HHP, OA, and LAE and the bacteriostatic potential of natural antimicrobial ingredients such as DV and LV1X against *L. monocytogenes*. Further research aimed at addressing the survival and recovery of *L. monocytogenes* by combining ingredients and post-lethality interventions suitable for use in natural and organic meat and poultry products is needed.

**Introduction**

In many parts of the world, natural and organic foods have been experiencing noticeable market growth over the last few decades (31, 41). Processed meat products within both of those categories have accounted for a significant part of that growth. In fact, in 2011 in the United States, meat, fish, and poultry represented the fastest growing of the eight organic food categories after experiencing a 13% increase in sales over 2010 (24). Sales growth predictions for the entire organic foods sector indicated that the ≥ 9% yearly growth observed over the last few years is expected to be maintained through 2013, at least (24). This growth is expected even though price premiums associated with organic products have been estimated to range between 10-40% (41) and those of organic meat and poultry products to sometimes reach 200% or higher (5).
Although many natural and organic products resemble their conventionally produced counterparts, the stringent regulations that apply to natural and organic foods may render the use of certain ingredients illegal. The use of nitrate and nitrite in the production of cured processed meat products such as ham, frankfurters, among others, is one such example as the use of either is not permitted when manufacturing natural and organic processed meat products.

Because the quality and safety benefits derived from meat curing are unquestionable, the indirect addition of nitrate or nitrite to natural and organic processed meat products, sometimes referred to as “natural curing,” represents a new technology that has garnered interest from processors, consumers and scientists alike (31, 33). Some fruits and vegetables are known to contain relatively high levels of nitrate. Potatoes, lettuce, melons, cabbage, celery, spinach, beets, carrots, cauliflower, and broccoli are only a few examples of such vegetables and fruits (40). However, due to concerns over the flavor and/or color compatibility or clash that may stem from using some of these as sources of nitrate or nitrite in the production of natural and organic processed meat products, more emphasis has been placed on celery (Apium graveolens var. dulce) than on any other vegetable or fruit.

The use of natural sources of nitrate and nitrate-reducing starter cultures, and the ensuing need for an incubation step for the reduction of nitrate to nitrite, in the production of natural or organic processed meat products result in increased production times. This scenario is not very compatible with today’s high throughput production systems and consumers’ increased demand for these categories of products. Thus, manufacturers of celery powders have begun to add nitrate-reducing starter cultures such as
Staphylococcus carnosus directly to the celery purees before the drying step and, as a result, have started to market “pre-converted” nitrite versions of celery powders. Once dried or slightly condensed, pre-converted celery powders or juices will contain 10,000-15,000 mg/Kg, or 1.0-1.5%, nitrite. Recommended usage levels are different depending on not only the product but also the manufacturer of the celery powder or juice and range from 0.2-1.0% based on green (raw) meat weight. Using a pre-converted celery powder in which the active ingredient is nitrite instead of nitrate will effectively eliminate the need for a nitrate reduction step and, therefore, result in decreased production times.

The meat industry has derived unquantifiable benefits from the use of nitrite. Increased food safety, improved flavor and lipid stability, and an overall increased shelf-life of cured meat products are a few of the advantages we have come to expect from cured meat products (30, 33). Thus, it is safe to say that the use of nitrite in cured meat and poultry production has led to the existence of products whose specific flavors, colors, and textures cannot be reproduced by using any other ingredient (25, 30, 33). Although the color and flavor stability benefits derived from using nitrite are clear, of greater significance are its antimicrobial properties. L. monocytogenes has emerged as an important foodborne pathogen of significant human health concern over the last few years. Listeriosis, although rare, carries a relatively high mortality rate that can be as high as 30% (21). Ready-to-Eat (RTE) meat and poultry products have previously been associated with listeriosis outbreaks and, as a result, close attention should be paid to factors that affect the growth and behavior of L. monocytogenes in such products. Buchanan and Phillips (6), after evaluating the effects of sodium nitrite concentrations ranging from 0-1,000 mg/ml of Tryptose Phosphate Broth on the growth kinetics of L.
Scott A, concluded that sodium nitrite represents an important parameter that plays a role in the survival and growth of this pathogen. Pelroy and others (26) determined that, among other factors such as packaging atmosphere, storage temperature, and sodium chloride concentration, 190-200 mg/kg sodium nitrite exerted a bacteriostatic effect on \textit{L. monocytogenes} inoculated onto slices of cold-smoked salmon. In 2003, the United States Department of Agriculture Food Safety and Inspection Services (USDA FSIS) established a “zero tolerance” policy for the presence of \textit{L. monocytogenes} on RTE meat and poultry products (10). This policy would later come to be known as the “\textit{Listeria Rule}.” Under this policy, an RTE meat and poultry product is considered adulterated if it is found to contain \textit{L. monocytogenes} or if it has come into direct contact with a food contact surface which is contaminated with \textit{L. monocytogenes} (10).

RTE meat and poultry products that are manufactured under uncured, natural, or organic methods are at a greater risk for \textit{L. monocytogenes} growth if contaminated than their conventional counterparts due, mainly, to the required absence of preservatives and antimicrobials traditionally used in the manufacture of conventional products (28, 35). For example, the use of lactate and diacetate, two antimicrobials commonly found in RTE meat and poultry products and proven to have inhibitory effects on \textit{L. monocytogenes}, is not permitted in the manufacture of natural or organic meat products. As a result, the use of natural antimicrobials and “clean label” technologies or interventions in the manufacture of these types of meat products has received attention from researchers and processors alike (28, 29, 31, 34, 36).

The USDA FSIS (38) has defined a post-lethality treatment as “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.” High hydrostatic pressure processing (HHP), for example, is an example of a post-lethality intervention due to the fact that it generally takes place after the product has gone through the lethality or cooking step (38). Post-packaging thermal processing, freezing, drying, among others, are additional examples of intervention methods commonly used not only in the meat industry, but across the entire food industry, to address the potential presence of pathogenic microorganisms in food products. The use of post-lethality interventions to address the potential presence of *L. monocytogenes* in uncured, no-nitrate-or-nitrite-added, RTE natural or organic meat and poultry products is an area of interest because some of these technologies are allowed for use in these categories of products.

The USDA FSIS lists lauric arginate (lauramide arginine ethyl ester or LAE) as a safe and suitable ingredient for the production of meat and poultry products and allows up to 44 mg/kg (plus or minus a 20% tolerance) of lauric arginate by weight of the product to be applied to the inside of a package as a processing aid (37). When used at this level, lauric arginate would not have to be declared on the label of the product and, as a result, can be used in the manufacture of uncured, no-nitrate-or-nitrite-added, RTE natural or organic meat and poultry products. The USDA FSIS also allows for octanoic acid to be used as a processing aid as long as it is applied to the surface of an RTE meat and poultry product at a rate not to exceed 400 mg/kg octanoic acid by weight of the final product (37). Octanoic acid, sometimes referred to as caprylic acid, is a saturated (C\(_{8:0}\)) fatty acid (pK\(_a\) 4.89) naturally found in coconut oil and bovine milk (14). Although promising from an initial *L. monocytogenes* lethality standpoint, the bacteriostatic effects of octanoic acid
have not been extensively researched and should receive more attention from the scientific community.

Much emphasis has been placed on the investigation of natural sources of antimicrobials that could potentially replace chemical preservatives and synthetic antimicrobial ingredients as a means to address *L. monocytogenes* in the highly restrictive natural and organic categories. Several compounds derived from fruits, spices, oilseeds, and vegetables have been looked at in attempts to elucidate whether they exhibit any bactericidal or bacteriostatic effects on *L. monocytogenes* and other foodborne pathogens. These compounds often possess Generally Recognized as Safe (GRAS) status. The differences in antimicrobial potency observed in natural compounds may be due in part to inconsistencies of commercial samples. Another important factor to consider is the food matrix itself, as it has been shown that *L. monocytogenes*, for example, was less sensitive to hop extracts in a food systems compared to a media system and that the fat content of the food caused the antilisterial properties of the hop extracts to vary (16). Thus, the antilisterial properties of natural antimicrobial ingredients used in RTE meat and poultry products are likely to vary based on product characteristics such as fat content, protein content, pH, *a*<sub>w</sub>, and other ingredients added.

Although post-lethality interventions might deliver an initial lethality and natural antimicrobials may have a bacteriostatic effect, some concerns still exist over the potential recovery and growth of sublethally injured *L. monocytogenes* over the storage life of the product. These concerns create a clear need for additional hurdles to be investigated and, eventually, implemented in order to fully address *L. monocytogenes* in RTE meat and poultry products. Investigating the use of natural antimicrobial ingredients
and post-lethality interventions that are currently allowed for use under the highly restrictive natural and organic meat and poultry products manufacturing practices as a means to inhibit the recovery and growth of *L. monocytogenes* on RTE frankfurters was, therefore, the focus of our work.

**Materials and Methods**

**Manufacture of Frankfurters**

Nine frankfurter treatments (eight experimental and one control treatment) were manufactured to evaluate the inhibition of *L. monocytogenes* recovery and growth by natural antimicrobial ingredients and post-lethality interventions. Frankfurters were produced at the Iowa State University Meat Laboratory targeting a final fat content of 30% by blending 90% lean beef trimmings and 50% lean pork trimmings, using formulations found in Table 1. Pre-converted celery powder (VegStable 504, Florida Food Products, Inc., Eustis, FL) was used as the natural source of nitrite. Based on analysis, VegStable 504 is 1.5% (wt/wt) nitrite. The beef and pork trimmings were obtained from a local processor and frozen prior to use to ensure uniformity of raw materials. The beef and pork trimmings were tempered to -2°C and then were coarse ground through a plate with 9.53-mm-diameter holes (Biro MFG Co., Marblehead, OH). The ground beef and pork trimmings were then ground through a plate with 3.18-mm-diameter holes (Biro MFG Co.). The ground beef trimmings were then chopped (VSM65, Krämer & Grebe GmbH & Co. KG., Biendenkopf-Wallau, Germany) with the salt, 50 mg/kg natural nitrite, and half of the ice/water under vacuum until a temperature of 3°C was achieved. Then, ground pork trimmings, dextrose, spices, the rest of the ice/water,
and natural antimicrobial (if applicable) were added and chopping continued until a
temperature of 14°C was attained. The emulsion was then stuffed into 21-mm-diameter
cellulose casings (RP 21/95, Viscofan, Danville, IL) using a rotary vane vacuum-filling
machine (RS 1040 C, Risco USA Corp., South Eaton, MA) and linked into
approximately 7.4 cm units to accommodate later high hydrostatic pressure (HHP)
treatments. Thorough rinsing with cold water of all of the equipment utilized was
conducted after each frankfurter formulation was manufactured so as to avoid cross-
contamination between product formulations. All treatments were then placed in a single-
truck smokehouse (MT EVD RSE 4, Alkar Engineering Corp., Lodi, WI) and heated to
an internal temperature of 71.1°C. The frankfurters were then placed in a 0°C cooler
overnight to stabilize. The next day, which marked day 0 of the experiment, the
frankfurters were stripped of the casing, placed into barrier bags (B2470, Cryovac Sealed
Air Corporation, Duncan, SC) with an oxygen transmission rate of 3-6 cc at 4°C (m², 24
hrs atm @ 4°C, 0% RH) and a water vapor transmission rate of 0.5-0.6 g at 38°C (100%
RH, 0.6 m², 24 hrs), and vacuum sealed (UV 2100, Multivac, Inc., Kansas City, MO).
The frankfurters destined for analyses other than microbiological were placed in boxes,
transferred to a holding cooler in the Iowa State University Meat Laboratory, and stored
at 4 ± 1°C for the duration of the experiment. The frankfurters for microbial analyses
were placed in boxes with vacuum packaged ice, transferred to the Iowa State University
Food Safety Research Laboratory in the Food Science and Human Nutrition Department
for subsequent inoculation, and stored at 4 ± 1°C for the duration of the experiment. Two
independent replications were produced.
Mean Weight and Surface Area Calculations

On day 0, a total of five randomly selected frankfurter links from the Control, 90MX, DV, and LV1X formulations (Table 1) were weighed and measured \((n = 20\) per replication) so as to obtain representative average weights and surface area measurements. The surface area \((\text{cm}^2)\) of the frankfurter links was modeled by the equation of the surface area of a cylinder \((\text{area} = 2\pi r^2 \text{ [side only]})\) plus two half spheres \((\text{area} = 4 \pi r^2)\), where \(\pi = 3.142, r = \text{radius}, \text{ and } h = \text{height}\). Ends were removed and then the length/height was measured. Microstructures created by folds at each end of the frankfurter links were not considered in surface area calculations. Average weight and surface area measurements would then be used to calculate log CFU per g and octanoic acid (OA) and lauric arginate (LAE) volumes per link to be used in the study, respectively.

Proximate Analysis

Proximate analysis was conducted for moisture, fat, and protein of homogenized Control, 90MX, DV, and LV1X formulations (Table 1) on day 0 using AOAC methods 950.46, 960.63, and 992.15, respectively \((1, 2, 3)\). Samples were prepared in duplicate for each frankfurter formulation.

pH

Product pH was measured by placing a pH probe (FC20, Hanna Instruments, Woonsocket, RI) into homogenized (KFP715 food processor, Kitchenaid, St. Joseph, MI) samples from Control, 90MX, DV, and LV1X formulations (Table 1) that were prepared by first blending the ground frankfurters with distilled, de-ionized water in a 1:9 ratio,
and then measuring the pH with a pH/ion meter (Accumet 925 pH/ion meter, Fisher Scientific). Calibration was conducted using phosphate buffers of pH 4.0, 7.0, and 10.0. Duplicate readings were taken for each product formulation on day 0.

**Water Activity**

Available moisture was determined using a water activity meter (AquaLab 4TE, Decagon Devices Inc., Pullman, WA). Samples were cut into small pieces, placed in disposable sample cups, covered, and allowed to equilibrate to room temperature (5-10 min). Measurements were obtained on day 0 and were performed in duplicate for Control, 90MX, DV, and LV1X formulations (Table 1). Calibration was performed using 1.00 and 0.76 sodium chloride water activity standards.

**Color Measurements**

External and internal color measurements were performed using a Hunterlab LabScan XE spectrocolorimeter (HunterLab, Reston, VA) at two randomly selected locations on the frankfurter links in duplicate, and the resulting average was used in data analysis. Color measurements were obtained at days 1, 14, 28, 42, 56, 70, 84, and 98. The colorimeter was calibrated using the same packaging material as used on the samples and placed over a white standard tile. Values for the white standard tile were X = 81.72, Y = 86.80, and Z = 91.46. External color of the frankfurter links was measured while they were still inside the packaging material under vacuum. Internal color of the frankfurter links was evaluated by slicing individual links longitudinally followed by immediate measurement. Illuminant A, 10° standard observer with a 1.27 cm viewing area and a 1.78 cm port size was used to evaluate frankfurter samples. Commission International
d’Eclairage (CIE) L* (lightness), a* (redness), and b* (yellowness) values were determined by reflectance ratio of wavelengths 650/670 nm.

**Residual Nitrite Analysis**

Residual nitrite was determined utilizing AOAC method 973.31 (4). Samples from each treatment were evaluated in duplicate and measurements were obtained at days 1, 14, 28, 42, 56, 70, 84, and 98.

**Natural Antimicrobial Ingredients**

Three commercially available natural antimicrobial ingredients were evaluated in this study; 1.0% cranberry powder (90MX; Ocean Spray International, Middleboro, MA), 1.0% vinegar (DV; WTI Ingredients, Inc., Jefferson, GA), and 2.5% vinegar and lemon juice concentrate (LV1X; WTI Ingredients, Inc., Jefferson, GA) (wt/wt). Each ingredient was added at levels recommended by the respective supplier (Table 1). The pH of 10% solutions (w/v) of the 90MX, DV, and LV1X ingredients were 3.89, 5.87, and 5.57, respectively.

**Preparation of Inoculum**

*L. monocytogenes* strains Scott A NADC 2045 serotype 4b, H7969 serotype 4b, H7962 serotype 4b, H7596 serotype 4b, and H7762 serotype 4b were obtained from the Iowa State University Food Safety Research Laboratory in the Food Science and Human Nutrition Department. Each strain was cultured separately in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) (Difco, Becton Dickinson, Sparks, MD) for 24 h at 35°C. A minimum of two consecutive 24-h transfers of each strain to fresh
TSBYE (35°C) were performed prior to each experiment. Aliquots (6.0-ml) from each of the five strains were then transferred into a sterile centrifuge tube. The bacterial cells were harvested by centrifugation (10 min at 10,000 rpm and 4°C) in a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT). The supernatant was discarded and the pelleted cells were resuspended in 30.0 ml of sterile buffered peptone water (BPW) (Difco, Becton Dickinson). The total concentration of the five-strain cocktail was approximately $10^9$ colony forming units (CFU) per ml based on the aerobic plate counts of the washed cell suspension. Two serial dilutions (100-fold each) of the cell suspension were prepared in BPW to give a final inoculum concentration of $10^5$ CFU per ml. This diluted five-strain cocktail was used to inoculate frankfurter links.

**Sample Inoculation**

While in the Food Safety Research Laboratory, each packaged sample was reopened and the surface of the product was aseptically inoculated with a 0.2-ml aliquot of the diluted five-strain cocktail. The cell concentration at inoculation was approximately $10^3$ CFU per gram. The bags were then vacuum sealed using a model A300/52 vacuum packaging machine (Multivac, Inc.) and stored at 4 ± 1°C for the duration of the experiment.

**Post-Lethality Interventions**

Four clean-label post-lethality interventions were evaluated in this study; HHP, octanoic acid (OA), lauric arginate (LAE), and post-packaging thermal treatment (PPTT). Frankfurter links from the Control formulation were randomly assigned to these post-lethality interventions. For frankfurters that were to be subjected to microbial analyses,
all post-lethality interventions were applied to the product within two hours after inoculation.

HHP was evaluated under two different sets of parameters; 400 MPa, 4 min dwell time at 12 ± 2°C initial fluid temperature or 600 MPa, 4 min dwell time at 12 ± 2°C initial fluid temperature. Frankfurters were transported on ice to the High Pressure Processing Laboratory at the Iowa State University Food Science and Human Nutrition Department and subjected to the appropriate HHP treatment using a FOOD-LAB 900 Plunger Press system (Standsted Fluid Power Ltd., Standsted, UK). The pressurization fluid was a 50.0% propylene glycol (GWT Koilguard; GWT Global Water Technology, Inc., Indianapolis, IN) and 50.0% water solution (v/v). The average rate of pressurization was 350 MPa per min and depressurization occurred within 7 s.

OA (Octa-Gone; EcoLab, Inc., Eagan, MN) was applied according to the supplier’s recommendations. According to the manufacturer, Octa-Gone contains approximately 3.6% octanoic acid (v/v). A 23.4% Octa-Gone and 76.6% water solution (v/v) was prepared by mixing Octa-Gone with sterile de-ionized water at 4 ± 1°C. Based on average surface area measurements obtained per replication as previously described, the OA solution was aseptically dispensed into the bag containing the frankfurter link (0.0186 ml per cm²) and vacuum sealed. Vacuum sealing evenly distributed the solution throughout the bag. The pH of the working solution of Octa-Gone was 3.01.

LAE (Protect-M; Purac America, Lincolnshire, IL) was applied according to the supplier’s recommendations. According to the manufacturer, Protect-M contains approximately 10.0% laurie arginate (v/v). A 2.5% Protect-M and 97.5% water solution
(v/v) was prepared by mixing Protect-M with sterile de-ionized water at 4 ± 1°C. Based on average surface area measurements obtained per replication as previously described, the LAE solution was aseptically dispensed into the bag containing the frankfurter link (0.007192 ml per cm²) and vacuum sealed. Vacuum sealing evenly distributed the solution throughout the bag. The pH of the working solution of Protect-M was 3.92.

PPTT was conducted by immersing packages of frankfurters in water at 71.0 ± 1.0°C for 30 s using a water bath (Isotemp-228, Fisher Scientific). Seven packages were immersed as a group so as not to affect water temperature by more than 1.0°C. Water temperature was monitored throughout the process. Packages were held in heated water for the prescribed length of time and then placed on ice immediately after to chill before placement in refrigerated storage.

**Microbial Analysis**

Microbial analysis of frankfurter samples for viable *L. monocytogenes* was conducted on days 1, 14, 28, 42, 56, 70, 84, and 98. On the appropriate day, two packages for each treatment were removed from the holding cooler, opened aseptically, and their contents placed inside a sterile Whirl-Pak stomacher bag (Nasco, Ft. Atkinson, WI). Sample preparation was performed by adding 50.0 ml of sterile BPW to each bag, closing the bag so as to form a “pillow,” and then shaking the sample for approximately 30 s. The wash solution from each ham sample was then serially diluted (10-fold) in BPW to obtain predetermined dilutions of the samples according to the sampling day. An aliquot of 1.0 ml (for 10⁰ dilution, divided into three ~0.33-ml aliquots plated on three separate plates) or 0.1 ml of the appropriate dilution was surfaced plated on modified Oxford medium base
(MOX) supplemented with modified Oxford antimicrobial supplement (Difco, Becton Dickinson). The dry ingredients used to manufacture the MOX were 42.5 g of Columbia agar base (Difco, Becton Dickinson), 15.0 g of lithium chloride (Difco, Becton Dickinson), 1.0 g of esculin hydrate (Sigma-Aldrich, St. Louis, MO), and 0.5 g of ferric ammonium citrate (Difco, Becton Dickinson) per liter of de-ionized water. Additionally, 0.1 ml of the appropriate dilution was surfaced plated on thin agar layer medium base (TAL) that was made according to Kang and Fung (15) with some modifications. MOX was made as previously described. Then, within 48 hr before sampling was to be conducted, MOX plates to be made into TAL were aseptically overlaid with 7.0 ml of sterile tryptic soy agar (Difco, Becton Dickinson) held at 55°C to facilitate the even distribution of the molten agar. Each sample was plated in duplicate. Plates used for microbial analyses were sterile and 55 mm in diameter (Fisher Scientific, Waltham, MA). All inoculated agar plates were incubated in an inverted position at 35°C for 48 hr, after which time they were removed from the incubator and colonies typical of \textit{L. monocytogenes} were enumerated. The counts (CFU per ml) were averaged and then converted to log CFU per g using the average weight of the frankfurter links from the two replications of the experiment ($n = 40$). The detection limit of our sampling protocols was $\geq 0.30$ log CFU per g based on a sample weight of 25.0 g.

**Statistical Analysis**

The overall design of the experiment was a factorial design. The generalized linear mixed models (GLIMMIX) procedure of Statistical Analysis System (version 9.3, SAS Institute Inc., Cary, NC) was used for statistical analysis. \textit{L. monocytogenes} growth and analytical data were analyzed for treatment effects within day. Day and treatment x day
interactions were also analyzed. Where significant effects ($P < 0.05$) were found, pairwise comparisons between the least squares means were computed for each day using Tukey’s honestly significant difference adjustment.

**Results and Discussion**

**Mean Surface Area and Weight Results**

The mean weight of the frankfurters was $23.76 \pm 0.92$ g, while the mean diameter, length, and surface area were $1.95 \pm 0.03$ cm, $7.36 \pm 0.23$ cm, and $57.03 \pm 1.64$ cm$^2$, respectively (data not shown and $n = 40$ for all measurements). These mean frankfurter dimensions resulted in LAE and OA treatment volumes of 0.41 and 1.06 ml per package, respectively, after dosages of each compound were calculated according to the respective manufacturer’s recommendations as previously described. These dosages resulted in LAE and OA treatment concentrations of 43.16 and 376.11 mg/kg, respectively.

**Physicochemical Traits**

Physicochemical characteristics of the frankfurters can be found in Table 2. The only trait for which significant ($P < 0.05$) differences were detected was product pH. The 90MX treatment resulted in the lowest pH, followed by the LV1X, the DV, and the Control treatments. Cranberry has been reported to contain phenolic acids and exhibit a high titratable acidity ($17$). Xi and others obtained similar results when using different ingoing levels of cranberry powder in a cooked meat model system ($43$) and in frankfurters ($42$). Similarly, the vinegar and vinegar and lemon juice concentrate used in this study are also likely reservoirs of phenolic and other acidic compounds, such as acetic and citric acid, expected to cause the observed lower pH in frankfurters made with
those ingredients. The proximate composition of the different frankfurter formulations manufactured was not significantly different ($P > 0.05$).

**Residual Nitrite Concentration Results**

The effects of treatment on residual nitrite concentration are found in Table 3 and Figure 1. Although all frankfurter formulations were manufactured with 50 mg/kg natural nitrite on an ingoing basis, the highest residual nitrite concentration observed on day 1 of the study was 25.84 mg/kg (HHP600 treatment). This indicates that part of the ingoing nitrite was depleted in curing and other reactions that took place during product manufacture. Honikel (11) reported that as much as 65% of the ingoing nitrite can be depleted during product manufacture. Similarly, Xi and others (42) reported that as much as 75% of the ingoing nitrite can be depleted during the manufacture of frankfurters. Factors such as product pH, cooking temperature, and reducing agents used have been shown to affect residual nitrite concentrations in meat systems (8). Sebranek (30) indicated that decreases in pH as small as 0.2 units during product manufacture can result in a doubling of the rate at which curing reactions occur. Thus, the significant ($P < 0.05$) decrease in pH brought about by the natural antimicrobial ingredients used in this study, especially cranberry powder, was expected to influence residual nitrite concentrations.

On day 1, the OA, 90MX, and LV1X treatments exhibited lower ($P < 0.05$) residual nitrite concentrations than the Control treatment, whereas the HHP400, HHP600, LAE, and DV treatments did not ($P > 0.05$). The acidity of compounds founds in the cranberry powder, the vinegar and lemon juice concentrate, and in OA and their effect on product pH are likely responsible for the observed lower concentrations of nitrite observed in
these treatments on day 1 of the study. These results are in agreement with Xi and others (43), authors who found that varying cranberry powder levels from 1.0 to 3.0% resulted in lower residual nitrite levels in a cooked meat model system. The same authors obtained similar results when evaluating a series of antimicrobial ingredients, which included cranberry powder, in the manufacture of frankfurters (42).

Throughout storage of the products at 4 ± 1°C, all treatments showed significant \( P < 0.05 \) decreases in residual nitrite concentrations. The residual nitrite concentration observed in the Control treatment significantly \( (P < 0.05) \) decreased after 28 days of storage and continued to decrease over time until reaching its lowest level, 5.78 mg/Kg, by day 98. Similar results were observed in the HHP400, HHP600, LAE, PPTT, DV, and LV1X treatments. On the other hand, the OA and 90MX treatments experienced significant \( (P < 0.05) \) decreases in residual nitrite concentrations as early as day 14 of the study. Furthermore, these same two treatments reached their lowest residual nitrite concentrations by day 70, indicating that cranberry powder and octanoic acid may speed up the rate at which residual nitrite is degraded compared to the other treatments and under the conditions of this study. Similar patterns were reported by Xi and others (42) after evaluating different concentrations of cranberry powder in combination with other ingredients and their effects on residual nitrite levels of frankfurters stored under refrigeration over 49 days.

**External L*, a*, and b* Values Results**

External L* values can be found on Table 4 and Figure 2. The 90MX treatment resulted in lower \( (P < 0.05) \) external L* values than the Control treatment on days 42, 56,
and 84 and, although not significant \((P > 0.05)\) in most cases, exhibited numerically lower external L* values when compared to all other treatments evaluated throughout the entire duration of the study. The post-lethality interventions studied did not significantly affect external L* values of frankfurters under the conditions of this study \((P > 0.05)\). These results indicate that a darker exterior is likely to result from the use of cranberry powder as an ingredient in the manufacture of frankfurters. Similar patterns were observed by Xi and others \((42)\) when using cranberry powder in the manufacture of naturally cured frankfurters.

Table 5 and Figure 3 describe the effects of treatment on external a* values. Although no discernible treatment effects were observed in the data, all treatments evaluated experienced a significant \((P < 0.05)\) increase in external a* values by day 28 of the study. No further changes were observed within any of the treatments thereafter, however. Neither natural antimicrobial ingredient nor post-lethality intervention implemented significantly affected external a* values of frankfurters under the conditions of this study \((P > 0.05)\). These results slightly contrast those obtained by Xi and others \((42)\) in that cranberry powder, under the conditions of this study, did not cause a significant decrease in external a* values compared to the naturally-cured Control treatment. Although bright red in color in its powdered form, cranberry powder has been shown to turn dark purple upon addition to meat systems due to an increase in cranberry powder pH \((44)\). This color shift is thought to stem from a partial red-to-blue shift in the color of the anthocyanin pigments of cranberry as a result of increased pH \((44)\).

External b* values as affected by treatment can be found in Table 6 and Figure 4. The 90MX treatment yielded lower external b* values than the other natural antimicrobial
ingredients evaluated in this study on day 1 ($P < 0.05$). This pattern was not observed on any other day throughout storage of the products. No differences between treatments were detected on days 28, 56, 84, and 98 of the study. The 90MX treatment, although not significant ($P > 0.05$) in most instances, exhibited numerically lower external $b^*$ values when compared to all other treatments evaluated throughout the entire duration of the study, except for day 84.

**Internal $L^*$, $a^*$, and $b^*$ Values Results**

The effects of treatment on internal $L^*$ values are described in Table 7 and Figure 5. In similar fashion to external $L^*$ value results obtained in this study, the 90MX treatment consistently yielded the lowest internal $L^*$ values of all treatments across the duration of the study. Internal $L^*$ values shown by the 90MX treatment were significantly lower ($P < 0.05$) compared to those shown by frankfurters manufactured using the other natural antimicrobials on days 1, 70, and 98. Additionally, the 90MX treatment resulted in significantly lower internal $L^*$ values than the Control treatment on days 1, 28, 84, and 98 of the study ($P < 0.05$). These results indicate that a decrease in both external and internal lightness of frankfurters is likely to occur as a consequence of using cranberry powder as an ingredient.

No significant differences ($P > 0.05$) were detected between treatments on days 1, 14, 28, 56, 70, and 98 of the study when it came to internal $a^*$ values (Table 8 and Figure 6). All treatments evaluated exhibited a significant ($P < 0.05$) increase in internal $a^*$ values by day 14 of the study. Similar patterns were observed on external $a^*$ value results, as
previously discussed. Furthermore, compared to day 14, another significant ($P < 0.05$) increase in internal $a^*$ values was observed in all treatments by day 56.

Similar to external $b^*$ value results obtained in this study, the 90MX treatment consistently resulted in the lowest internal $b^*$ values throughout the duration of the study (Table 9 and Figure 7). In fact, the 90MX treatment resulted in lower $b^*$ values than all other treatments on days 1 and 84 and, when compared to the Control treatment, the same was also observed on days 14, 28, and 70 ($P < 0.05$). Similar results were obtained by Xi and others (42) upon evaluating the effects of varying concentrations of cranberry powder on the internal $b^*$ values of naturally-cured frankfurters. None of the post-lethality intervention evaluated exerted a significant effect on the internal $b^*$ values when compared to the Control treatment ($P > 0.05$).

**Viable *Listeria monocytogenes***

Viable *L. monocytogenes* numbers on MOX (Table 10) and TAL (Table 11) media were monitored throughout the duration of the study and are illustrated in Figure 8 and Figure 9, respectively. The growth mediums used did not significantly differ ($P > 0.05$) within treatment on any given day, indicating that, under the conditions of this study, the use of the TAL technique offers limited advantages compared to using a traditional medium such as MOX. Thus, the discussion about viable *L. monocytogenes* numbers is based on results obtained using MOX.

Of the post-lethality interventions evaluated, all except PPTT significantly decreased *L. monocytogenes* numbers after 1 day of storage at $4 \pm 1^\circ C$ ($P < 0.05$) when compared to the Control treatment. The greatest reduction was seen in the LAE treatment, where a
decrease of 2.28 log CFU per g compared to the untreated Control treatment was observed. Porto-Fett and others (27) evaluated the effects of 22 and 44 mg/kg lauric arginate, with or without the addition of potassium lactate and sodium diacetate, on the growth of *L. monocytogenes* on commercially-produced frankfurters. These authors concluded that lauric arginate provides initial lethality towards *L. monocytogenes* when used alone (1.8 log CFU per package) or in combination with lactate and diacetate (2.0 log CFU per package). Similar results were obtained by Luchansky and others (20) when they researched the effects of lauric arginate on the growth of *L. monocytogenes* on hams.

Similarly, the OA treatment resulted in a 2.03 log CFU per g reduction in *L. monocytogenes* numbers compared to the Control treatment. In a study that sought to evaluate the antilisterial effect of octanoic acid delivered to the surface of several different RTE meats within their final packaging, Burnett and others (7) concluded that 1% octanoic acid solutions acidified to pH 2.0 or 4.0 and applied to RTE meat and poultry products at 1.9 ± 0.5 ml per 100 cm² of product surface area resulted in *L. monocytogenes* log reductions ranging from 0.85 to 2.89 log CFU per sample in the different RTE products following 24 ± 4 h of storage at 5 ± 2°C. Furthermore, *L. monocytogenes* populations in all treated samples were significantly lower following treatment with either octanoic solution compared to the controls.

The HHP400 treatment resulted in a 1.88 log CFU per g reduction in *L. monocytogenes* numbers compared to the Control treatment while the HHP600 treatment resulted in reduction of *L. monocytogenes* numbers to levels below the detection limit of our sampling protocols (≥ 0.30 log CFU per g) throughout the entire duration of the study. These results agree with those obtained by Myers and others (23), as these authors
found that an HHP treatment of 600 MPa for 3 min and 17°C resulted in a 3.85-4.35 log CFU per g reduction in *L. monocytogenes* numbers on RTE meat products. Similarly, Myers and others (22) also concluded that HHP treatment with 600 MPa for 3 min and 17°C resulted in a 3.9-4.3 log CFU per g reduction in *L. monocytogenes* numbers on RTE sliced ham. The same authors, however, concluded that 400 MPa HHP treatment of RTE sliced ham for 3 min at 17°C resulted in less than a 1 log CFU per g reduction in *L. monocytogenes* numbers. Slight variations in product physicochemical characteristics such as aw, pH, salt concentration, among others, between studies may account for these differences. Furthermore, the extent to which HHP will inactivate microorganisms depends on several different factors including, but not limited to, bacterial strain and the growth phase it is in at the time treatment is applied, the characteristics of the food matrix to be treated, temperature of the medium, pressure level, and exposure time (13). When compared to broth systems, for example, nutrient-rich meat matrices allow for greater resistance of microorganisms to HHP treatment (12, 32). Thus, it would seem that any HHP treatment parameters would have to be tailored not only to the product to be treated, but also to the specific target microorganism and the expected outcome.

Additionally, PPTT did not significantly decrease initial *L. monocytogenes* numbers (*P* > 0.05) and resulted in similar *L. monocytogenes* growth patterns compared to the Control treatment throughout the duration of the study. These results contrast those obtained by Chen and others (9), as these authors concluded that a post-packaging thermal treatment of 71 ± 1°C for 30 sec would result in a 1.4 log CFU per g reduction in *L. monocytogenes* numbers on 1-link packages of frankfurters when using a 3.4 log CFU per g initial inoculation level.
Although an initial bactericidal effect of the HHP400, OA, and LAE treatments was clearly observed, the bacteriostatic properties of these treatments come under question as evidenced by our results. The HHP400 treatment experienced a significant ($P < 0.05$) increase in *L. monocytogenes* numbers after 56 days of storage. In fact, by day 98 of the study there was no significant difference between the Control and the HHP400 treatments ($P > 0.05$). Similar results were reported by Myers and others (22). These authors reported that after 400 MPa HHP treatment of RTE sliced ham for 3 min at 17°C, which resulted in less than a 1 log CFU per g reduction in *L. monocytogenes* numbers, the pathogen was able to grow to numbers above inoculation levels upon storage under refrigeration.

Similarly, the OA and LAE treatments showed significant ($P < 0.05$) increases in *L. monocytogenes* numbers by days 42 and 28 of the study, respectively, with the latter also showing no significant difference in *L. monocytogenes* levels compared to the Control treatment on day 98 ($P > 0.05$). These findings are in agreement with those of Porto-Fett and others (27) as these authors discovered that only when used in combination with lactate or diacetate will lauric arginate exert a bacteriostatic effect on the pathogen under storage temperatures of 4°C for 120 days. Similar results were obtained by Luchansky and others (20) when they researched the effects of lauric arginate on the growth of *L. monocytogenes* on hams. Burnett and others (7) also reported bacteriocidal effects of octanoic acid solutions on *L. monocytogenes*, but no information was provided as to its bacteriostatic effects on the same microorganism. Thus, although they may provide an initial lethality, lauric arginate and octanoic acid alone do not inhibit the outgrowth of any *L. monocytogenes* that may survive and their bacteriostatic effects should receive
more attention from the scientific community. The results of our study indicate that although beneficial from the standpoint of initial lethality, HHP400, OA, and LAE post-lethality interventions do not offer protection against the growth of surviving *L. monocytogenes* upon storage of the product and under the conditions of this study.

The natural antimicrobials evaluated in this study did not significantly affect *L. monocytogenes* levels after 1 day of storage ($P > 0.05$) when compared to the Control treatment. The bacteriostatic properties of these ingredients, however, varied greatly. The 90MX treatment, for example, experienced significant ($P < 0.05$) increases in *L. monocytogenes* levels as soon as day 28 of the study. These levels continued to increase ($P < 0.05$) through day 56 and reached maximum levels on day 84. These results indicate that, at the level used and under the conditions of this study, cranberry powder does not exert bacteriostatic effects on *L. monocytogenes*. Lin and others (19) concluded that cranberry extract alone was not inhibitory of *L. monocytogenes* growth upon refrigerated storage of both inoculated fish and beef slices. Similarly, results by Xi and others (43) indicate that cranberry powder, also when used at a level of 1.0% (wt/wt), will not inhibit the growth of *L. monocytogenes* completely. In contrast, the DV and LV1X treatments did not experience significant changes in *L. monocytogenes* levels throughout the duration of the study ($P > 0.05$). Research on similar buffered vinegar and buffered vinegar and lemon juice concentrate products and their inhibitory effects on *C. perfringens* in ground turkey roast (39) and roast beef (18) concluded that these ingredients exhibit inhibitory properties on that microorganism. However, additional research on the subject is needed as these effects are likely to vary based on product characteristics and microorganism of concern.
In conclusion, at the levels used and under the conditions of this study, DV and LV1X exhibit strong bacteriostatic properties against *L. monocytogenes* and represent viable options that could be instituted by manufacturers of organic and natural RTE processed meat and poultry products in their *L. monocytogenes* control plans. These natural antimicrobial ingredients, however, did not exhibit bactericidal properties under the conditions or this study. Additionally, although beneficial from the standpoint of initial lethality, the HHP400, OA, and LAE post-lethality interventions do not offer protection against the growth of surviving *L. monocytogenes* upon storage of the product and under the conditions of this study. Thus, additional research aimed at combining natural antimicrobial ingredients and post-lethality interventions that are suitable for use in the manufacture of organic and natural processed meat and poultry products is warranted.

**Acknowledgements**

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References


# TABLE 1. Naturally cured frankfurter formulations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>90/10 Beef (kg)</th>
<th>50/50 Pork (kg)</th>
<th>Ice/Water (kg)</th>
<th>Salt (kg)</th>
<th>Dextrose (kg)</th>
<th>Pre-converted Celery Powder&lt;sup&gt;a&lt;/sup&gt; (g)</th>
<th>Spices&lt;sup&gt;b&lt;/sup&gt; (g)</th>
<th>Antimicrobial A&lt;sup&gt;c&lt;/sup&gt; (g)</th>
<th>Antimicrobial B&lt;sup&gt;d&lt;/sup&gt; (g)</th>
<th>Antimicrobial C&lt;sup&gt;e&lt;/sup&gt; (g)</th>
<th>Post-Lethality Intervention</th>
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<td>Control</td>
<td>8.95</td>
<td>8.95</td>
<td>3.61</td>
<td>0.40</td>
<td>0.36</td>
<td>74.84</td>
<td>320.57</td>
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<td>HHP400</td>
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<td>High Hydrostatic Pressure (400 MPa, 4 min dwell time at 12 ± 2°C).</td>
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<td>HHP600</td>
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<td></td>
<td>High Hydrostatic Pressure (600 MPa, 4 min dwell time at 12 ± 2°C).</td>
</tr>
<tr>
<td>LAE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Octa-Gone (Octanoic Acid; EcoLab, Inc., Eagan, MN).</td>
</tr>
<tr>
<td>PPTT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Post-Packaging Thermal Treatment (71.0 ± 1°C for 30 s).</td>
</tr>
<tr>
<td>90MX</td>
<td>8.95</td>
<td>8.95</td>
<td>3.61</td>
<td>0.40</td>
<td>0.36</td>
<td>74.84</td>
<td>320.57</td>
<td>226.80</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DV</td>
<td>8.95</td>
<td>8.95</td>
<td>3.61</td>
<td>0.40</td>
<td>0.36</td>
<td>74.84</td>
<td>320.57</td>
<td>226.80</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LV1X</td>
<td>8.95</td>
<td>8.95</td>
<td>3.61</td>
<td>0.40</td>
<td>0.36</td>
<td>74.84</td>
<td>320.57</td>
<td>-</td>
<td>-</td>
<td>567.02</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Vegstable 504 (Natural Nitrite; Florida Food Products, Inc., Eustis, FL).

<sup>b</sup> Blend TG-05-405-000 (Mustard, spices, garlic powder; A.C. Legg Packing Co., Calera, AL).

<sup>c</sup> 90MX (Cranberry Powder; Ocean Spray International, Middleboro, MA).

<sup>d</sup> LV1X (Vinegar and Lemon Juice Concentrate; WTI Ingredients, Inc., Jefferson, GA).

<sup>e</sup> Post-Packaging Thermal Treatment (71.0 ± 1°C for 30 s).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>(a_w)</th>
<th>pH</th>
<th>Fat %</th>
<th>Moisture %</th>
<th>Protein %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.9703</td>
<td>6.11&lt;sup&gt;A&lt;/sup&gt;</td>
<td>25.39</td>
<td>55.30</td>
<td>13.29</td>
</tr>
<tr>
<td>90MX</td>
<td>0.9724</td>
<td>5.70&lt;sup&gt;B&lt;/sup&gt;</td>
<td>25.38</td>
<td>54.85</td>
<td>13.42</td>
</tr>
<tr>
<td>DV</td>
<td>0.9672</td>
<td>6.06&lt;sup&gt;C&lt;/sup&gt;</td>
<td>24.47</td>
<td>55.83</td>
<td>13.38</td>
</tr>
<tr>
<td>LV1X</td>
<td>0.9704</td>
<td>5.95&lt;sup&gt;D&lt;/sup&gt;</td>
<td>23.89</td>
<td>56.16</td>
<td>13.39</td>
</tr>
<tr>
<td>SE&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.003</td>
<td>0.01</td>
<td>0.64</td>
<td>0.47</td>
<td>0.40</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through D) are significantly different \((P < 0.05)\).

<sup>b</sup> Control, naturally cured control; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

<sup>c</sup> Standard error of the differences of least squares means.
TABLE 3. *Effect of treatment on residual nitrite concentrations of naturally cured frankfurters stored at 4 ± 1°C* \(^{a}\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.01(^{AD,Z})</td>
<td>26.68(^{A,Z})</td>
<td>21.02(^{AD,Y})</td>
<td>17.00(^{A,X})</td>
<td>11.45(^{AD,W})</td>
<td>9.33(^{A,W})</td>
<td>9.36(^{AD,W})</td>
<td>5.78(^{ABD,V})</td>
</tr>
<tr>
<td>HHP400</td>
<td>24.76(^{AD,Z})</td>
<td>25.82(^{AB,Z})</td>
<td>19.89(^{AD,Y})</td>
<td>15.81(^{A,X})</td>
<td>14.38(^{B,X})</td>
<td>9.39(^{A,W})</td>
<td>8.34(^{AB,W})</td>
<td>7.25(^{AD,W})</td>
</tr>
<tr>
<td>HHP600</td>
<td>25.84(^{AZ})</td>
<td>24.77(^{AZ})</td>
<td>18.86(^{D,Y})</td>
<td>15.01(^{A,X})</td>
<td>12.88(^{AB,X})</td>
<td>7.77(^{AD,W})</td>
<td>7.02(^{BE,W})</td>
<td>6.34(^{ACD,W})</td>
</tr>
<tr>
<td>OA</td>
<td>22.39(^{BE,Z})</td>
<td>18.45(^{C,Y})</td>
<td>13.18(^{BC,X})</td>
<td>9.14(^{BC,W})</td>
<td>8.05(^{CE,W})</td>
<td>4.55(^{BC,V})</td>
<td>4.30(^{C,V})</td>
<td>4.97(^{DE,V})</td>
</tr>
<tr>
<td>LAE</td>
<td>24.56(^{AB,Z})</td>
<td>25.72(^{AB,Z})</td>
<td>20.68(^{AD,Y})</td>
<td>16.38(^{A,X})</td>
<td>14.36(^{B,X})</td>
<td>8.95(^{A,W})</td>
<td>10.68(^{D,W})</td>
<td>7.34(^{A,V})</td>
</tr>
<tr>
<td>PPTT</td>
<td>23.49(^{DBE,YZ})</td>
<td>24.60(^{AZ})</td>
<td>21.28(^{A,Y})</td>
<td>16.23(^{A,X})</td>
<td>14.08(^{B,X})</td>
<td>9.81(^{A,W})</td>
<td>5.82(^{CE,V})</td>
<td>3.72(^{BE,V})</td>
</tr>
<tr>
<td>90MX</td>
<td>17.31(^{C,Z})</td>
<td>13.99(^{D,Y})</td>
<td>9.91(^{C,X})</td>
<td>7.39(^{B,W})</td>
<td>6.39(^{E,W})</td>
<td>3.65(^{C,V})</td>
<td>4.13(^{C,V})</td>
<td>4.01(^{BE,V})</td>
</tr>
<tr>
<td>DV</td>
<td>24.34(^{AB,Z})</td>
<td>24.07(^{B,Z})</td>
<td>18.96(^{D,Y})</td>
<td>15.22(^{A,X})</td>
<td>13.65(^{AB,X})</td>
<td>8.88(^{A,W})</td>
<td>7.56(^{AE,W})</td>
<td>7.46(^{A,W})</td>
</tr>
<tr>
<td>LV1X</td>
<td>21.58(^{EZ})</td>
<td>20.08(^{C,Z})</td>
<td>14.55(^{B,Y})</td>
<td>11.22(^{C,X})</td>
<td>9.86(^{DC,X})</td>
<td>5.97(^{BD,W})</td>
<td>5.38(^{CE,W})</td>
<td>4.86(^{BCE,W})</td>
</tr>
<tr>
<td>SE(^{c})</td>
<td>0.73</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Values are least squares means. Within a column, means with different superscripts (A through E) are significantly different \((P < 0.05)\). Within a row, means with different superscripts (V through Z) are significantly different \((P < 0.05)\).

\(^{b}\) Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

\(^{c}\) Standard error of the differences of least squares means.
FIGURE 1. Effect of treatment on residual nitrite concentrations of naturally cured frankfurters stored at 4 ± 1°C

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
TABLE 4. Effect of treatment on external L* values of naturally cured frankfurters stored at 4 ± 1°C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>66.09&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.94&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>66.24&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>66.52&lt;sup&gt;A&lt;/sup&gt;</td>
<td>67.35&lt;sup&gt;A&lt;/sup&gt;</td>
<td>65.97</td>
<td>68.15&lt;sup&gt;A&lt;/sup&gt;</td>
<td>65.65</td>
</tr>
<tr>
<td>HHP400</td>
<td>14</td>
<td>66.22&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.29&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.92&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.60&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.12&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.25</td>
<td>65.00&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>64.33</td>
</tr>
<tr>
<td>HHP600</td>
<td>28</td>
<td>64.76&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.45&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>66.43&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>64.70&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>64.58&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.26</td>
<td>65.35&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.54</td>
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<tr>
<td>OA</td>
<td>42</td>
<td>65.96&lt;sup&gt;AB,YZ&lt;/sup&gt;</td>
<td>68.60&lt;sup&gt;A,Z&lt;/sup&gt;</td>
<td>66.80&lt;sup&gt;AB,YZ&lt;/sup&gt;</td>
<td>65.89&lt;sup&gt;AB,YZ&lt;/sup&gt;</td>
<td>65.76&lt;sup&gt;AB,YZ&lt;/sup&gt;</td>
<td>64.79&lt;sup&gt;Y&lt;/sup&gt;</td>
<td>64.55&lt;sup&gt;BC,Y&lt;/sup&gt;</td>
<td>64.80&lt;sup&gt;Y&lt;/sup&gt;</td>
</tr>
<tr>
<td>LAE</td>
<td>56</td>
<td>66.68&lt;sup&gt;A&lt;/sup&gt;</td>
<td>67.58&lt;sup&gt;A&lt;/sup&gt;</td>
<td>67.17&lt;sup&gt;A&lt;/sup&gt;</td>
<td>64.93&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.12&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>66.95</td>
<td>65.78&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>66.29</td>
</tr>
<tr>
<td>PPTT</td>
<td>70</td>
<td>65.64&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.65&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.87&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>66.34&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.54&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.44</td>
<td>66.38&lt;sup&gt;AC&lt;/sup&gt;</td>
<td>65.68</td>
</tr>
<tr>
<td>90MX</td>
<td>84</td>
<td>62.93&lt;sup&gt;B&lt;/sup&gt;</td>
<td>63.56&lt;sup&gt;B&lt;/sup&gt;</td>
<td>63.56&lt;sup&gt;B&lt;/sup&gt;</td>
<td>62.90&lt;sup&gt;B&lt;/sup&gt;</td>
<td>62.86&lt;sup&gt;B&lt;/sup&gt;</td>
<td>63.49</td>
<td>62.75&lt;sup&gt;B&lt;/sup&gt;</td>
<td>63.34</td>
</tr>
<tr>
<td>DV</td>
<td>98</td>
<td>66.97&lt;sup&gt;A&lt;/sup&gt;</td>
<td>66.08&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>67.90&lt;sup&gt;A&lt;/sup&gt;</td>
<td>65.63&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>66.14&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>66.98</td>
<td>65.76&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>66.50</td>
</tr>
<tr>
<td>LV1X</td>
<td></td>
<td>65.37&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>67.21&lt;sup&gt;A&lt;/sup&gt;</td>
<td>66.87&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.24&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.36&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>64.90</td>
<td>65.34&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>65.60</td>
</tr>
</tbody>
</table>

SE<sup>c</sup> 1.12

<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different (<i>P</i> < 0.05). Within a row, means with different superscripts (Y through Z) are significantly different (<i>P</i> < 0.05).

<sup>b</sup> Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentration.

<sup>c</sup> Standard error of the differences of least squares means.
FIGURE 2. Effect of treatment on external $L^*$ values of naturally cured frankfurters stored at $4 \pm 1 ^\circ C$

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
TABLE 5. Effect of treatment on external \( a^* \) values of naturally cured frankfurters stored at 4 ± 1°C\(^a\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.64(^{Z})</td>
<td>13.90(^{AB,Y})</td>
<td>14.35(^{Y})</td>
<td>13.95(^{Y})</td>
<td>13.56(^{Y})</td>
<td>14.59(^{Y})</td>
<td>13.31(^{A,Y})</td>
<td>14.69(^{Y})</td>
</tr>
<tr>
<td>HHP400</td>
<td>11.02(^{Z})</td>
<td>13.22(^{AB,Y})</td>
<td>14.07(^{Y})</td>
<td>14.26(^{Y})</td>
<td>14.16(^{Y})</td>
<td>14.40(^{Y})</td>
<td>14.49(^{AB,Y})</td>
<td>14.07(^{Y})</td>
</tr>
<tr>
<td>HHP600</td>
<td>11.55(^{Z})</td>
<td>13.67(^{AB,Y})</td>
<td>13.42(^{Y})</td>
<td>14.26(^{Y})</td>
<td>13.97(^{Y})</td>
<td>14.63(^{Y})</td>
<td>13.66(^{AB,Y})</td>
<td>13.72(^{Y})</td>
</tr>
<tr>
<td>OA</td>
<td>11.79(^{Z})</td>
<td>12.67(^{AB,Z})</td>
<td>14.22(^{Y})</td>
<td>14.49(^{Y})</td>
<td>14.57(^{Y})</td>
<td>15.32(^{Y})</td>
<td>14.88(^{AB,Y})</td>
<td>14.29(^{Y})</td>
</tr>
<tr>
<td>LAE</td>
<td>11.08(^{Z})</td>
<td>12.62(^{A,XY})</td>
<td>13.84(^{XY})</td>
<td>14.76(^{Y})</td>
<td>14.82(^{Y})</td>
<td>14.39(^{Y})</td>
<td>14.46(^{AB,Y})</td>
<td>14.30(^{Y})</td>
</tr>
<tr>
<td>PPTT</td>
<td>11.53(^{Z})</td>
<td>13.22(^{AB,Y})</td>
<td>14.45(^{XY})</td>
<td>14.03(^{XY})</td>
<td>14.10(^{XY})</td>
<td>14.80(^{X})</td>
<td>14.29(^{AB,XY})</td>
<td>14.34(^{XY})</td>
</tr>
<tr>
<td>90MX</td>
<td>12.43(^{Z})</td>
<td>14.21(^{B,Y})</td>
<td>14.76(^{Y})</td>
<td>14.76(^{Y})</td>
<td>14.37(^{Y})</td>
<td>14.11(^{Y})</td>
<td>14.64(^{AB,Y})</td>
<td>13.76(^{ZY})</td>
</tr>
<tr>
<td>DV</td>
<td>11.40(^{Z})</td>
<td>13.81(^{AB,Y})</td>
<td>14.20(^{Y})</td>
<td>14.72(^{Y})</td>
<td>14.63(^{Y})</td>
<td>14.65(^{Y})</td>
<td>15.06(^{B,Y})</td>
<td>13.93(^{Y})</td>
</tr>
<tr>
<td>LV1X</td>
<td>11.74(^{Z})</td>
<td>13.52(^{AB,Y})</td>
<td>14.35(^{XY})</td>
<td>14.81(^{XY})</td>
<td>14.56(^{XY})</td>
<td>15.07(^{X})</td>
<td>14.46(^{AB,XY})</td>
<td>14.25(^{XY})</td>
</tr>
<tr>
<td>SE(^c)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Values are least squares means. Within a column, means with different superscripts (A through B) are significantly different (\( P < 0.05 \)). Within a row, means with different superscripts (X through Z) are significantly different (\( P < 0.05 \)).

\(^b\) Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

\(^c\) Standard error of the differences of least squares means.
FIGURE 3. Effect of treatment on external $a^*$ values of naturally cured frankfurters stored at 4 ± 1°C

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.


### TABLE 6. Effect of treatment on external b* values of naturally cured frankfurters stored at 4 ± 1°C<sup>a</sup>

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.54&lt;sup&gt;ABD,Z&lt;/sup&gt;</td>
<td>20.55&lt;sup&gt;AZ&lt;/sup&gt;</td>
<td>19.39&lt;sup&gt;XZ&lt;/sup&gt;</td>
<td>18.25&lt;sup&gt;AB,XY&lt;/sup&gt;</td>
<td>17.60&lt;sup&gt;Y&lt;/sup&gt;</td>
<td>18.43&lt;sup&gt;AB,XY&lt;/sup&gt;</td>
<td>17.50&lt;sup&gt;Y&lt;/sup&gt;</td>
<td>17.89&lt;sup&gt;XY&lt;/sup&gt;</td>
</tr>
<tr>
<td>HHP400</td>
<td>19.97&lt;sup&gt;BD,Z&lt;/sup&gt;</td>
<td>19.63&lt;sup&gt;AC,XZ&lt;/sup&gt;</td>
<td>18.84&lt;sup&gt;YZ&lt;/sup&gt;</td>
<td>18.26&lt;sup&gt;AB,XY&lt;/sup&gt;</td>
<td>18.16&lt;sup&gt;XY&lt;/sup&gt;</td>
<td>18.31&lt;sup&gt;AB,XY&lt;/sup&gt;</td>
<td>18.54&lt;sup&gt;WXZ&lt;/sup&gt;</td>
<td>17.92&lt;sup&gt;WY&lt;/sup&gt;</td>
</tr>
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<td>HHP600</td>
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<td>18.91&lt;sup&gt;XY&lt;/sup&gt;</td>
<td>18.77&lt;sup&gt;AB,XY&lt;/sup&gt;</td>
<td>18.18&lt;sup&gt;Y&lt;/sup&gt;</td>
<td>18.89&lt;sup&gt;A,XY&lt;/sup&gt;</td>
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<td>18.59&lt;sup&gt;BCF,XY&lt;/sup&gt;</td>
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<td>18.58&lt;sup&gt;AB,XY&lt;/sup&gt;</td>
<td>18.32&lt;sup&gt;XY&lt;/sup&gt;</td>
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<td>18.56&lt;sup&gt;YZ&lt;/sup&gt;</td>
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<td>18.33&lt;sup&gt;YZ&lt;/sup&gt;</td>
<td>18.01&lt;sup&gt;AB,Y&lt;/sup&gt;</td>
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<td>17.93&lt;sup&gt;YZ&lt;/sup&gt;</td>
<td>17.55&lt;sup&gt;BYZ&lt;/sup&gt;</td>
<td>17.39&lt;sup&gt;XY&lt;/sup&gt;</td>
<td>17.14&lt;sup&gt;B,XY&lt;/sup&gt;</td>
<td>17.82&lt;sup&gt;XZ&lt;/sup&gt;</td>
<td>17.09&lt;sup&gt;XY&lt;/sup&gt;</td>
</tr>
<tr>
<td>DV</td>
<td>21.14&lt;sup&gt;CD,Z&lt;/sup&gt;</td>
<td>20.43&lt;sup&gt;AD,XZ&lt;/sup&gt;</td>
<td>18.96&lt;sup&gt;XY&lt;/sup&gt;</td>
<td>19.24&lt;sup&gt;AXY&lt;/sup&gt;</td>
<td>18.63&lt;sup&gt;Y&lt;/sup&gt;</td>
<td>18.58&lt;sup&gt;AB,Y&lt;/sup&gt;</td>
<td>19.00&lt;sup&gt;XY&lt;/sup&gt;</td>
<td>18.18&lt;sup&gt;Y&lt;/sup&gt;</td>
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<tr>
<td>LV1X</td>
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<td>19.11&lt;sup&gt;ACE,Y&lt;/sup&gt;</td>
<td>19.26&lt;sup&gt;Y&lt;/sup&gt;</td>
<td>19.44&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>18.93&lt;sup&gt;Y&lt;/sup&gt;</td>
<td>19.44&lt;sup&gt;A,Y&lt;/sup&gt;</td>
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</tbody>
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<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through F) are significantly different ($P < 0.05$). Within a row, means with different superscripts (W through Z) are significantly different ($P < 0.05$).

<sup>b</sup> Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

<sup>c</sup> Standard error of the differences of least squares means.
FIGURE 4. Effect of treatment on external $b^*$ values of naturally cured frankfurters stored at 4 ± 1°C

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
TABLE 7. Effect of treatment on internal $L^*$ values of naturally cured frankfurters stored at 4 ± 1°C$^a$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
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<th>98</th>
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<td>68.80$^{ABE,YZ}$</td>
<td>67.55$^{ABC,YZ}$</td>
<td>67.26$^{AB,YZ}$</td>
<td>66.19$^{ABC,Y}$</td>
<td>67.61$^{AC,YZ}$</td>
<td>67.50$^{AC,YZ}$</td>
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<tr>
<td>HHP400</td>
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<td>67.87$^{AB,YZ}$</td>
<td>67.90$^{ABCE,YZ}$</td>
<td>66.59$^{BC,YZ}$</td>
<td>65.41$^{AB,Y}$</td>
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<td>65.40$^{AB,Y}$</td>
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<td>66.11$^{BCD}$</td>
<td>66.50$^{BC}$</td>
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<td>66.36$^{ABC}$</td>
<td>66.19$^{AB}$</td>
<td>66.37$^{ABC}$</td>
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<td>68.61$^{AB}$</td>
<td>68.56$^{ABE}$</td>
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<td>68.87$^{AC}$</td>
<td>67.36$^{ABC}$</td>
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<td>66.80$^{AB,Y}$</td>
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<td>67.27$^{ABC,YZ}$</td>
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<td>65.73$^{B}$</td>
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<td>65.08$^{C}$</td>
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<td>63.41$^{B}$</td>
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<td>67.78$^{A,Y}$</td>
<td>69.16$^{C,YZ}$</td>
<td>69.99$^{C,YZ}$</td>
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<td>66.39$^{BC,YZ}$</td>
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</table>

$^a$ Values are least squares means. Within a column, means with different superscripts (A through E) are significantly different ($P < 0.05$). Within a row, means with different superscripts (Y through Z) are significantly different ($P < 0.05$).

$^b$ Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

$^c$ Standard error of the differences of least squares means.
FIGURE 5. Effect of treatment on internal $L^*$ values of naturally cured frankfurters stored at 4 ± 1°C

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
TABLE 8. Effect of treatment on internal $a^*$ values of naturally cured frankfurters stored at 4 ± 1°C$^a$

<table>
<thead>
<tr>
<th>Treatment$^b$</th>
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<th>42</th>
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<th>70</th>
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<th>98</th>
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<td>13.19$^{A,VWX}$</td>
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<td>14.09$^V$</td>
<td>13.04$^{AB,WX}$</td>
<td>12.87$^{WX}$</td>
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<td>13.04$^{VWX}$</td>
<td>13.67$^V$</td>
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<td>12.00$^Y$</td>
<td>12.83$^{XY}$</td>
<td>12.78$^{AB,YX}$</td>
<td>13.42$^{WX}$</td>
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</tr>
</tbody>
</table>

$^a$ Values are least squares means. Within a column, means with different superscripts (A through B) are significantly different ($P < 0.05$). Within a row, means with different superscripts (U through Z) are significantly different ($P < 0.05$).

$^b$ Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

$^c$ Standard error of the differences of least squares means.
FIGURE 6. Effect of treatment on internal $a^*$ values of naturally cured frankfurters stored at $4 \pm 1^\circ C$

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
### Table 9. Effect of treatment on internal $b^*$ values of naturally cured frankfurters stored at 4 ± 1°C$^a$

<table>
<thead>
<tr>
<th>Treatment$^b$</th>
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<th>28</th>
<th>42</th>
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<th>70</th>
<th>84</th>
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<td>15.46$^{A,YZ}$</td>
<td>15.34$^{A,YZ}$</td>
<td>14.64$^{A,XY}$</td>
<td>15.24$^{AB,XZ}$</td>
<td>15.14$^{A,XY}$</td>
<td>14.88$^{A,XY}$</td>
<td>14.42$^{AB,XY}$</td>
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<td>15.73$^{A,YZ}$</td>
<td>14.76$^{AB,XY}$</td>
<td>15.40$^{A,XZ}$</td>
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<td>14.94$^{A,XY}$</td>
<td>14.57$^{AB,XY}$</td>
<td>14.55$^{AB,XY}$</td>
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<td>14.59$^{A,XY}$</td>
<td>15.17$^{AB,XZ}$</td>
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<td>15.32$^{A,XZ}$</td>
<td>15.18$^{A,XZ}$</td>
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<td>16.06$^{A,XZ}$</td>
<td>15.12$^{AB,XY}$</td>
<td>15.15$^{A,XY}$</td>
<td>15.44$^{A,WXZ}$</td>
<td>15.26$^{A,XZ}$</td>
<td>14.99$^{A,XY}$</td>
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<td>15.92$^{A,XZ}$</td>
<td>15.21$^{AB,YZ}$</td>
<td>14.59$^{A,XY}$</td>
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<td>15.00$^{AB,YZ}$</td>
<td>14.93$^{AB,Y}$</td>
<td>14.17$^{AB,Y}$</td>
<td>14.90$^{AB,Y}$</td>
<td>14.27$^{AB,Y}$</td>
<td>14.69$^{A,Y}$</td>
<td>14.26$^{AB,Y}$</td>
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<td>13.93$^{B,YZ}$</td>
<td>13.92$^{B,YZ}$</td>
<td>12.94$^{B,Y}$</td>
<td>14.01$^{B,YZ}$</td>
<td>13.60$^{B,YZ}$</td>
<td>13.37$^{B,YZ}$</td>
<td>13.67$^{B,YZ}$</td>
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<td>DV</td>
<td>16.34$^{A,Z}$</td>
<td>15.51$^{A,YZ}$</td>
<td>15.09$^{AB,YZ}$</td>
<td>14.62$^{A,Y}$</td>
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<td>15.88$^{A,YZ}$</td>
<td>15.61$^{A,YZ}$</td>
<td>15.32$^{A,Y}$</td>
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$^a$ Values are least squares means. Within a column, means with different superscripts (A through B) are significantly different ($P < 0.05$). Within a row, means with different superscripts (W through Z) are significantly different ($P < 0.05$).

$^b$ Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

$^c$ Standard error of the differences of least squares means.
FIGURE 7. Effect of treatment on internal $b^*$ values of naturally cured frankfurters stored at 4 ± 1°C

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
### TABLE 10. Effect of treatment on viable \textit{Listeria monocytogenes} (log CFU per gram) on modified Oxford medium on naturally cured frankfurters stored at 4 ± 1°C$^a$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
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<th>98</th>
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<td>6.90$^{A,X}$</td>
<td>7.97$^{A,X}$</td>
<td>8.06$^{A,X}$</td>
<td>8.24$^{A,X}$</td>
<td>8.32$^{A,X}$</td>
<td>8.20$^{A,X}$</td>
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<td>0.47$^{B,Z}$</td>
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<td>1.81$^{B,Z}$</td>
<td>2.85$^{B,Z}$</td>
<td>4.16$^{B,W}$</td>
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<td>0.08$^{B,W}$</td>
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<td>3.77$^{B,Y}$</td>
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<td>7.80$^{AD,W}$</td>
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<td>3.40$^{AD,Z}$</td>
<td>5.64$^{AC,Y}$</td>
<td>7.66$^{AD,X}$</td>
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<td>4.97$^{C,Y}$</td>
<td>6.40$^{CD,WY}$</td>
<td>7.56$^{A,WX}$</td>
<td>7.84$^{A,WX}$</td>
<td>8.00$^{A,X}$</td>
<td>7.90$^{AB,X}$</td>
</tr>
<tr>
<td>DV</td>
<td>2.60$^A$</td>
<td>2.58$^{CD}$</td>
<td>2.47$^B$</td>
<td>2.48$^B$</td>
<td>2.40$^B$</td>
<td>2.47$^D$</td>
<td>3.49$^C$</td>
<td>2.54$^C$</td>
</tr>
<tr>
<td>LV1X</td>
<td>2.57$^A$</td>
<td>2.51$^{CD}$</td>
<td>2.46$^B$</td>
<td>2.59$^B$</td>
<td>2.50$^B$</td>
<td>2.78$^{BD}$</td>
<td>3.39$^C$</td>
<td>3.01$^C$</td>
</tr>
<tr>
<td>SE$^d$</td>
<td>0.47</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Values are least squares means. Within a column, means with different superscripts (A through D) are significantly different ($P < 0.05$). Within a row, means with different superscripts (W through Z) are significantly different ($P < 0.05$).

$^b$ Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

$^c$ Not detected.

$^d$ Standard error of the differences of least squares means.
FIGURE 8. Effect of treatment on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured frankfurters stored at 4 ± 1°C

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
TABLE 11. Effect of treatment on viable *Listeria monocytogenes* (log CFU per gram) on thin agar layer medium on naturally cured frankfurters stored at 4 ± 1°C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.42\textsuperscript{A,Z}</td>
<td>4.17\textsuperscript{A,Y}</td>
<td>6.89\textsuperscript{A,X}</td>
<td>7.98\textsuperscript{A,X}</td>
<td>8.03\textsuperscript{A,X}</td>
<td>8.19\textsuperscript{A,X}</td>
<td>8.30\textsuperscript{A,X}</td>
<td>8.16\textsuperscript{A,X}</td>
</tr>
<tr>
<td>HHP400</td>
<td>0.91\textsuperscript{B,C,Z}</td>
<td>0.91\textsuperscript{B,Z}</td>
<td>1.38\textsuperscript{B,XZ}</td>
<td>1.77\textsuperscript{B,XZ}</td>
<td>2.66\textsuperscript{B,XY}</td>
<td>4.14\textsuperscript{B,Y}</td>
<td>6.21\textsuperscript{B,W}</td>
<td>7.26\textsuperscript{A,W}</td>
</tr>
<tr>
<td>HHP600</td>
<td>ND\textsuperscript{e}</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>OA</td>
<td>0.47\textsuperscript{B,Z}</td>
<td>0.45\textsuperscript{B,Z}</td>
<td>1.35\textsuperscript{B,Z}</td>
<td>1.67\textsuperscript{B,Z}</td>
<td>3.73\textsuperscript{B,Y}</td>
<td>6.14\textsuperscript{C,X}</td>
<td>6.44\textsuperscript{B,X}</td>
<td>6.71\textsuperscript{A,X}</td>
</tr>
<tr>
<td>LAE</td>
<td>ND</td>
<td>1.39\textsuperscript{BD,Z}</td>
<td>4.22\textsuperscript{C,Y}</td>
<td>6.13\textsuperscript{C,X}</td>
<td>7.36\textsuperscript{A,WX}</td>
<td>7.57\textsuperscript{A,C,WX}</td>
<td>8.00\textsuperscript{A,W}</td>
<td>7.79\textsuperscript{A,W}</td>
</tr>
<tr>
<td>PPTT</td>
<td>2.10\textsuperscript{AC,Z}</td>
<td>3.34\textsuperscript{AC,Z}</td>
<td>5.58\textsuperscript{AC,Y}</td>
<td>7.66\textsuperscript{AD,X}</td>
<td>8.26\textsuperscript{A,X}</td>
<td>8.19\textsuperscript{A,X}</td>
<td>8.22\textsuperscript{A,X}</td>
<td>8.07\textsuperscript{A,X}</td>
</tr>
<tr>
<td>90MX</td>
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<td>3.05\textsuperscript{AC,Z}</td>
<td>4.91\textsuperscript{C,Y}</td>
<td>6.42\textsuperscript{CD,W}</td>
<td>7.52\textsuperscript{A,WX}</td>
<td>7.77\textsuperscript{A,A,WX}</td>
<td>7.96\textsuperscript{A,X}</td>
<td>7.81\textsuperscript{A,WX}</td>
</tr>
<tr>
<td>DV</td>
<td>2.34\textsuperscript{AC}</td>
<td>2.54\textsuperscript{CD}</td>
<td>2.51\textsuperscript{B}</td>
<td>2.46\textsuperscript{B}</td>
<td>2.31\textsuperscript{B}</td>
<td>2.33\textsuperscript{D}</td>
<td>3.47\textsuperscript{C}</td>
<td>2.48\textsuperscript{B}</td>
</tr>
<tr>
<td>LV1X</td>
<td>2.44\textsuperscript{A}</td>
<td>2.54\textsuperscript{CD}</td>
<td>2.39\textsuperscript{B}</td>
<td>2.60\textsuperscript{B}</td>
<td>2.45\textsuperscript{B}</td>
<td>2.69\textsuperscript{BD}</td>
<td>3.29\textsuperscript{C}</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Values are least squares means. Within a column, means with different superscripts (A through D) are significantly different \((P < 0.05)\). Within a row, means with different superscripts (W through Z) are significantly different \((P < 0.05)\).

\textsuperscript{b} Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

\textsuperscript{c} Not detected.

\textsuperscript{d} Standard error of the differences of least squares means.
FIGURE 9. Effect of treatment on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured frankfurters stored at 4 ± 1°C

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
CHAPTER 5. INVESTIGATING THE CONTROL OF LISTERIA MONOCYTGENES ON NATURALLY CURED READY-TO-EAT HAM USING NATURAL ANTIMICROBIAL INGREDIENTS AND POST-LETHALITY INTERVENTIONS

A paper to be submitted to the Journal of Food Protection

Nicolas A. Lavieri, Joseph G. Sebranek, Joseph C. Cordray, James S. Dickson, Ashley M. Horsch, Stephanie Jung, David K. Manu, and Aubrey F. Mendonça

Abstract

Ready-to-Eat (RTE) meat and poultry products manufactured with natural or organic methods are at greater risk for Listeria monocytogenes growth, if contaminated, than their conventional counterparts due to the required absence of preservatives and antimicrobials. Thus, the objective of this study was to investigate the use of commercially available natural antimicrobials and post-lethality interventions in the control of L. monocytogenes growth and recovery on naturally cured RTE ham. Antimicrobials evaluated were cranberry powder (90MX), vinegar (DV), and vinegar and lemon juice concentrate (LV1X). Post-lethality interventions studied were high hydrostatic pressure at 400 (HHP400) or 600 (HHP600) MPa, lauric arginate (LAE), octanoic acid (OA), and post-packaging thermal treatment (PPTT). Parameters evaluated through 98 days of storage at 4 ± 1°C were residual nitrite concentrations, CIE L*, a*, and b* values, and viable L. monocytogenes on modified Oxford (MOX) and thin agar
layer (TAL) media. On day 1, OA, 90MX, DV, and LV1X yielded lower residual nitrite concentrations than the Control, whereas HHP400, HHP600, and LAE did not. Counts on MOX and TAL did not differ. LAE, HHP400, and OA reduced *L. monocytogenes* levels compared to the Control after 1 day of storage by 2.38, 2.21, and 1.73 log CFU per g, respectively. PPTT did not achieve a significant reduction in bacterial numbers. *L. monocytogenes* recovered and grew in all post-lethality intervention treatments, except HHP600. 90MX did not inhibit the growth of *L. monocytogenes*, while DV and LV1X did. Results of this study demonstrate the bactericidal properties of HHP, OA, and LAE and the bacteriostatic potential of natural antimicrobial ingredients such as DV and LV1X against *L. monocytogenes*. Further research aimed at addressing the survival and recovery of *L. monocytogenes* by combining ingredients and post-lethality interventions suitable for use in natural and organic meat and poultry products is needed.

**Introduction**

The popularity of natural and organic foods has increased and led to noticeable market growth of these food categories over the last few decades (31, 42). Natural and organic meat products, in particular, have accounted for a significant part of that growth as in 2011 in the United States, meat, fish, and poultry represented the fastest growing of the eight organic food categories after experiencing a 13% increase in sales compared to the previous year (25). This increase in sales is expected to continue in spite of the fact that price premiums associated with organic products have been estimated to range between 10-40% (42) and those of organic meat and poultry products sometimes reach ≥200% (5).
Stringent regulations that govern the production of natural and organic foods have rendered the use of certain ingredients illegal. In the manufacture of natural and organic processed meat products such as boneless ham and frankfurters, the direct addition of nitrite or nitrate, curing ingredients used in the manufacture of such products, is not permitted. Because the quality and safety benefits derived from meat curing are unquestionable, the indirect addition of nitrate or nitrite to natural and organic processed meat products, sometimes referred to as “natural curing,” represents a relatively new technology that has garnered interest from processors and scientists (31, 33). Concerns over the flavor and color compatibility or clash that may stem from using certain vegetables and fruits as sources of nitrate or nitrite in the production of natural and organic processed meat products have led to more emphasis being placed on the use of celery (*Apium graveolens* var. dulce) (41). The use of natural sources of nitrate and nitrate-reducing starter cultures, and the subsequent need for an incubation step for the reduction of nitrate to nitrite to occur, in the production of natural or organic processed meat products results in increased production times. This scenario is not compatible with today’s production systems and consumers’ increased demand for these categories of products. As a result, celery powder or concentrate manufacturers have started to market “pre-converted” nitrite versions of celery powders that are made by adding nitrate-reducing starter cultures such as *Staphylococcus carnosus* directly to the celery purees before the drying step. Pre-converted celery powders or juices will contain 10,000-15,000 mg/kg (1.0-1.5%) nitrite once dried or slightly condensed. Depending on not only the meat product but also the manufacturer, recommended usage levels of the celery powder or juice range from 0.2-1.0% based on green (raw) meat weight. Using a pre-converted
celery powder or concentrate in which the active ingredient is nitrite will effectively eliminate the need for a nitrate reduction step and, inevitably, result in decreased production times.

The use of nitrite in the manufacture of cured meat and poultry products has led to the existence of products whose specific flavors, colors, and textures cannot be reproduced by using any other ingredient (26, 30, 33). Although the color and flavor stability benefits derived from using nitrite are clear, of greater significance are its antimicrobial properties (14). *L. monocytogenes* has risen as a foodborne pathogen of significant human health concern over the last few years. As a result, in 2003, the United States Department of Agriculture Food Safety and Inspection Services (USDA FSIS) established a “zero tolerance” policy for the presence of *L. monocytogenes* on ready-to-eat (RTE) meat and poultry products (10). Under this policy, which later came to be known as the “Listeria Rule,” an RTE meat and poultry product is considered adulterated if it is found to contain *L. monocytogenes* or if it has come into direct contact with a food contact surface which is contaminated with *L. monocytogenes* (10). Listeriosis, although rare, carries a relatively high mortality rate that can be as high as 30% (22). RTE meat and poultry products have previously been associated with listeriosis outbreaks and, as a result, close attention should be paid to factors that affect the growth and behavior of *L. monocytogenes* in such products.

RTE meat and poultry products manufactured under uncured, natural, or organic methods and requirements are at a greater risk for *L. monocytogenes* contamination and subsequent growth than their conventional counterparts mainly due to the required absence of preservatives and antimicrobials traditionally used in the manufacture of
conventional products (28, 35). Lactate and diacetate, antimicrobials commonly found in RTE meat and poultry products and proven to have inhibitory effects on *L. monocytogenes*, are not permitted in the manufacture of natural or organic meat products (39). As a result, the use of natural antimicrobials and “clean label” technologies or post-lethality interventions in the manufacture of these types of meat products has received attention from researchers and processors alike (28, 29, 31, 34, 36).

The USDA FSIS (38) defines a post-lethality treatment as “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.” The use of post-lethality interventions to address the potential presence of *L. monocytogenes* in uncured, no-nitrate-or-nitrite-added, RTE natural or organic meat and poultry products is an area of interest because some of these technologies are allowed for use in these categories of products. High hydrostatic pressure processing (HHP), for example, is an example of a post-lethality intervention as it generally takes place after the product has gone through the lethality or cooking step (38). Other examples of post-lethality interventions include sprays or solutions that can be applied to the finished product such as lauric arginate (lauramide arginine ethyl ester or LAE) and octanoic acid (sometimes referred to as caprylic acid or OA) as well as post-packaging thermal treatment or pasteurization. The USDA FSIS lists lauric arginate as a safe and suitable ingredient for the production of meat and poultry products and allows for up to 44 mg/kg (± a 20% tolerance) by weight of the product to be applied to the inside of a package as a processing aid (37). When used at this level, lauric arginate would not have to be declared on the label of the product and could be
used in the manufacture of uncured, no-nitrate-or-nitrite-added, RTE natural or organic meat and poultry products. Similarly, the USDA FSIS also allows for octanoic acid to be used as a processing aid if applied to the surface of an RTE meat and poultry product at a rate not to exceed 400 mg/kg octanoic acid by weight of the final product (37). Octanoic acid is a saturated (C\text{8:0}) fatty acid (pK\text{a} 4.89) naturally found in coconut oil and bovine milk (15). Although promising from an initial \textit{L. monocytogenes} lethality standpoint, the bacteriostatic effects of lauric arginate and octanoic acid have not been extensively researched and should receive more attention from the scientific community.

Some emphasis has also been placed on the investigation of natural sources of antimicrobials that could potentially replace chemical preservatives and synthetic antimicrobial ingredients as a means to address \textit{L. monocytogenes} in the highly restrictive natural and organic categories (36, 43, 44). These compounds often possess Generally Recognized as Safe (GRAS) status. However, differences in antimicrobial potency observed in natural compounds may result in part from inconsistencies of commercial samples. Another important factor to consider is the food matrix itself, as it has been shown that the antilisterial properties of natural antimicrobials can vary as a result of the fat content of the food (17). Thus, the antilisterial properties of natural antimicrobial ingredients used in RTE meat and poultry products are likely to vary based on product characteristics such as fat content, protein content, pH, a\text{w}, and other ingredients added.

Although post-lethality interventions might deliver an initial lethality and natural antimicrobials may have a bacteriostatic effect, concerns still exist over the potential recovery and growth of sublethally injured and uninjured \textit{L. monocytogenes} over the storage life of the product. Such concerns create a clear need for additional hurdles or a
combination of hurdles to be investigated and, eventually, implemented in order to fully address *L. monocytogenes* in RTE meat and poultry products. Investigating the use of commercially available natural antimicrobial ingredients and post-lethality interventions that are currently allowed for use under the highly restrictive natural and organic meat and poultry products manufacturing practices as a means to inhibit the recovery and growth of *L. monocytogenes* in naturally cured RTE ham was, therefore, the focus of our work.

**Materials and Methods**

**Manufacture of Hams**

Nine ham treatments (eight experimental and one control treatment) were manufactured to evaluate the inhibition of *L. monocytogenes* recovery and growth by clean-label natural antimicrobial ingredients and post-lethality interventions. Hams were produced at the Iowa State University Meat Laboratory with inside ham muscles, using formulations found in Table 1. The ham muscles were obtained from a local processor and frozen prior to use to ensure uniformity of raw materials. The ham muscles were tempered to -2°C and then were coarse ground through a plate with 9.53-mm-diameter holes (Biro MFG Co., Marblehead, OH). Nonmeat ingredients (Table 1) were added and mixed with ground ham muscles at 26 rpm for 2 min using a double action mixer (Leland Southwest, Fort Worth, TX). Pre-converted celery powder (VegStable 504, Florida Food Products, Inc., Eustis, FL) was used as the natural source of nitrite. Based on analysis, VegStable 504 is 1.5% (wt/wt) nitrite. All products were formulated to contain 50 mg/kg ingoing natural nitrite. Mixed samples were then reground using a plate with 6.35-mm-
diameter holes and stuffed into a 50-mm-diameter impermeable plastic casing (Nalobar APM 45, Kalle USA, Gurnee, IL) using a rotary vane vacuum-filling machine (RS 1040 C, Risco USA Corp., South Eaton, MA). Thorough rinsing with cold water of all of the equipment utilized was conducted after each ham formulation was manufactured so as to avoid cross-contamination between product formulations. All treatments were then placed in a single-truck smokehouse (Maurer, AG, Reichenau, Germany) and heated to an internal temperature of 71.1°C. The hams were then placed in a 0°C cooler overnight to stabilize. The next day, which marked day 0 of the experiment, the hams were sliced into approximately 12.0-mm-thick slices using a hand slicer (SE 12 D, Bizerba, Piscataway, NJ), placed into barrier bags (B2470, Cryovac Sealed Air Corporation, Duncan, SC) with an oxygen transmission rate of 3-6 cc at 4°C (m², 24 hrs atm @ 4°C, 0% RH) and a water vapor transmission rate of 0.5-0.6 g at 38°C (100% RH, 0.6 m², 24 hrs), and vacuum sealed (UV 2100, Multivac, Inc., Kansas City, MO). Hams for analytical analyses were placed in boxes and transferred to a holding cooler in the Iowa State University Meat Laboratory and stored at 4 ± 1°C for the duration of the experiment. Hams for microbial analyses were placed in boxes with vacuum packaged ice, transferred to the Iowa State University Food Safety Research Laboratory in the Food Science and Human Nutrition Department for subsequent inoculation, and stored at 4 ± 1°C for the duration of the experiment. Two independent replications were produced.

**Mean Weight and Surface Area Calculations**

On day 0, a total of five randomly selected slices of ham from the Control, 90MX, DV, and LV1X ham formulations (Table 1) were weighed and measured ($n = 20$ per replication) so as to obtain representative average weights and surface area
measurements. The surface area (cm$^2$) of the ham slices was modeled by the equation of the surface area of a cylinder: area = $2\pi r^2 + 2\pi rh$, where $\pi = 3.142$, $r =$ radius, and $h =$ height. Average weight and surface area measurements would then be used to calculate log CFU per g and octanoic acid (OA) and lauric arginate (LAE) volumes per slice to be used in the study, respectively.

**Proximate Analysis**

Proximate analysis was conducted for moisture, fat, and protein of homogenized Control, 90MX, DV, and LV1X formulations (Table 1) on day 0 using AOAC methods 950.46, 960.63, and 992.15, respectively (1, 2, 3). Samples were prepared in duplicate for each ham formulation.

**pH**

Product pH was measured by placing a pH probe (FC20, Hanna Instruments, Woonsocket, RI) into homogenized (KFP715 food processor, Kitchenaid, St. Joseph, MI) samples from Control, 90MX, DV, and LV1X formulations (Table 1) that were prepared by first blending the ground ham with distilled, de-ionized water in a 1:9 ratio, and then measuring the pH with a pH/ion meter (Accumet 925 pH/ion meter, Fisher Scientific). Calibration was conducted using phosphate buffers of pH 4.0, 7.0, and 10.0. Duplicate readings were taken for each product formulation on day 0.

**Water Activity**

Available moisture was determined using a water activity meter (AquaLab 4TE, Decagon Devices Inc., Pullman, WA). Samples were cut into small pieces, placed in
disposable sample cups, covered, and allowed to equilibrate to room temperature (5-10 min). Measurements were obtained on day 0 and were performed in duplicate for Control, 90MX, DV, and LV1X formulations (Table 1). Calibration was performed using 1.00 and 0.76 sodium chloride water activity standards.

**Color Measurements**

Color measurements were performed using a Hunterlab LabScan XE spectrocolorimeter (HunterLab, Reston, VA) at two randomly selected locations on the ham slices in duplicate, and the resulting average was used in data analysis. Color measurements were obtained at days 1, 14, 28, 42, 56, 70, 84, and 98. The colorimeter was calibrated using the same packaging material as used on the samples and placed over a white standard tile. Values for the white standard tile were X = 81.72, Y = 86.80, and Z = 91.46. Internal color of the hams was measured while they were still inside the packaging material under vacuum. Illuminant A, 10° standard observer with a 1.27 cm viewing area and a 1.78 cm port size was used to evaluate ham samples. Commission International d’Eclairage (CIE) L* (lightness), a* (redness), and b* (yellowness) values were determined by reflectance ratio of wavelengths 650/670 nm.

**Residual Nitrite Analysis**

Residual nitrite was determined utilizing AOAC method 973.31 (4). Samples from each treatment were evaluated in duplicate and measurements were obtained at days 1, 14, 28, 42, 56, 70, 84, and 98.
Natural Antimicrobial Ingredients

Three commercially available natural antimicrobial ingredients were evaluated in this study; 1.0% cranberry powder (90MX; Ocean Spray International, Middleboro, MA), 1.0% vinegar (DV; WTI Ingredients, Inc., Jefferson, GA), and 2.5% vinegar and lemon juice concentrate (LV1X; WTI Ingredients, Inc., Jefferson, GA) (wt/wt). Each ingredient was added at levels recommended by the respective supplier (Table 1). The pH of 10% solutions (w/v) of the 90MX, DV, and LV1X ingredients were 3.89, 5.87, and 5.57, respectively.

Preparation of Inoculum

*L. monocytogenes* strains Scott A NADC 2045 serotype 4b, H7969 serotype 4b, H7962 serotype 4b, H7596 serotype 4b, and H7762 serotype 4b were obtained from the Iowa State University Food Safety Research Laboratory in the Food Science and Human Nutrition Department. Each strain was cultured separately in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) (Difco, Becton Dickinson, Sparks, MD) for 24 h at 35°C. A minimum of two consecutive 24-h transfers of each strain to fresh TSBYE (35°C) were performed prior to each experiment. Aliquots (6.0-ml) from each of the five strains were then transferred into a sterile centrifuge tube. The bacterial cells were harvested by centrifugation (10 min at 10,000 rpm and 4°C) in a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT). The supernatant was discarded and the pelleted cells were resuspended in 30.0 ml of sterile buffered peptone water (BPW) (Difco, Becton Dickinson). The total concentration of the five-strain cocktail was approximately 10⁹ colony forming units (CFU) per ml based on the aerobic
plate counts of the washed cell suspension. Two serial dilutions (100-fold each) of the cell suspension were prepared in BPW to give a final inoculum concentration of $10^5$ CFU per ml. This diluted five-strain cocktail was used to inoculate samples of ham.

**Sample Inoculation**

While in the Food Safety Research Laboratory, each packaged sample was reopened and the surface of the product was aseptically inoculated with a 0.2-ml aliquot of the diluted five-strain cocktail. The cell concentration at inoculation was approximately $10^3$ CFU per gram. The bags were then vacuum sealed using a model A300/52 vacuum packaging machine (Multivac, Inc.) and stored at $4 \pm 1^\circ C$ for the duration of the experiment.

**Post-Lethality Interventions**

Four clean-label post-lethality interventions were evaluated in this study; high hydrostatic pressure (HHP), octanoic acid (OA), lauric arginate (LAE), and post-packaging thermal treatment (PPTT). Ham slices from the Control formulation were randomly assigned to these post-lethality interventions. For hams that were to be subjected to microbial analyses, all post-lethality interventions were applied to the product within two hours after inoculation.

HHP was evaluated under two different sets of parameters; 400 MPa, 4 min dwell time at $12 \pm 2^\circ C$ initial fluid temperature or 600 MPa, 4 min dwell time at $12 \pm 2^\circ C$ initial fluid temperature. Hams were transported on ice to the Food Safety Research Laboratory for inoculation and then to the High Pressure Processing Laboratory at the Iowa State University Food Science and Human Nutrition Department and subjected to
the appropriate HHP treatment using a FOOD-LAB 900 Plunger Press system (Standsted Fluid Power Ltd., Standsted, UK). The pressurization fluid was a 50.0% propylene glycol (GWT Koilguard; GWT Global Water Technology, Inc., Indianapolis, IN) and 50.0% water solution (v/v). The average rate of pressurization was 350 MPa per min and depressurization occurred within 7 s.

OA (Octa-Gone; EcoLab, Inc., Eagan, MN) was applied according to the supplier’s recommendations. According to the manufacturer, Octa-Gone contains approximately 3.6% octanoic acid (v/v). A 23.4% Octa-Gone and 76.6% water solution (v/v) was prepared by mixing Octa-Gone with sterile de-ionized water at 4 ± 1°C. Based on average surface area measurements obtained per replication as previously described, the OA solution was aseptically dispensed into the bag containing the ham slice (0.0186 ml per cm²) and vacuum sealed. Vacuum sealing evenly distributed the solution throughout the bag. The pH of the working solution of Octa-Gone was 3.01.

LAE (Protect-M; Purac America, Lincolnshire, IL) was applied according to the supplier’s recommendations. According to the manufacturer, Protect-M contains approximately 10.0% lauric arginate (v/v). A 2.5% Protect-M and 97.5% water solution (v/v) was prepared by mixing Protect-M with sterile de-ionized water at 4 ± 1°C. Based on average surface area measurements obtained per replication as previously described, the LAE solution was aseptically dispensed into the bag containing the ham slice (0.007192 ml per cm²) and vacuum sealed. Vacuum sealing evenly distributed the solution throughout the bag. The pH of the working solution of Protect-M was 3.92.
PPTT was conducted by immersing packages of ham in water at 71.0 ± 1.0°C for 30 s using a water bath (Isotemp-228, Fisher Scientific). Seven packages were immersed as a group so as not to affect water temperature by more than 1.0°C. Water temperature was monitored throughout the process. Packages were held in heated water for the prescribed length of time and then placed on ice immediately after to chill before placement in refrigerated storage.

**Microbial Analysis**

Microbial analysis of ham samples for viable *L. monocytogenes* was conducted on days 1, 14, 28, 42, 56, 70, 84, and 98. On the appropriate day, two packages for each treatment were removed from the holding cooler, opened aseptically, and their contents placed inside a sterile Whirl-Pak stomacher bag (Nasco, Ft. Atkinson, WI). Sample preparation was performed by adding 50.0 ml of sterile BPW to each bag, closing the bag so as to form a “pillow,” and then shaking the sample for approximately 30 s. The wash solution from each ham sample was then serially diluted (10-fold) in BPW to obtain predetermined dilutions of the samples according to the sampling day. An aliquot of 1.0 ml (for 10^0 dilution, divided into three ~0.33-ml aliquots plated on three separate plates) or 0.1 ml of the appropriate dilution was surfaced plated on modified Oxford medium base (MOX) supplemented with modified Oxford antimicrobial supplement (Difco, Becton Dickinson). The dry ingredients used to manufacture the MOX were 42.5 g of Columbia agar base (Difco, Becton Dickinson), 15.0 g of lithium chloride (Difco, Becton Dickinson), 1.0 g of esculin hydrate (Sigma-Aldrich, St. Louis, MO), and 0.5 g of ferric ammonium citrate (Difco, Becton Dickinson) per liter of de-ionized water. Additionally, 0.1 ml of the appropriate dilution was surfaced plated on thin agar layer medium base
(TAL) that was made according to Kang and Fung (16) with some modifications. MOX was made as previously described. Then, never more than 48 hr before sampling was to be conducted, MOX plates to be made into TAL were aseptically overlaid with 7.0 ml of sterile tryptic soy agar (Difco, Becton Dickinson) held at 55°C to facilitate the even distribution of the molten agar. Each sample was plated in duplicate. Plates used for microbial analyses were sterile and 55 mm in diameter (Fisher Scientific, Waltham, MA). All inoculated agar plates were incubated in an inverted position at 35°C for 48 hr, after which time they were removed from the incubator and colonies typical of *L. monocytogenes* were enumerated. The counts (CFU per ml) were averaged and then converted to log CFU per g using the average weight of the sliced ham from the two replications of the experiment (*n* = 40). The detection limit of our sampling protocols was ≥ 0.30 log CFU per g based on a sample weight of 25.0 g.

**Statistical Analysis**

The overall design of the experiment was a factorial design. The generalized linear mixed models (GLIMMIX) procedure of Statistical Analysis System (version 9.3, SAS Institute Inc., Cary, NC) was used for statistical analysis. *L. monocytogenes* growth and analytical data were analyzed for treatment effects within day. Day and treatment x day interactions were also analyzed. Where significant effects (*P* < 0.05) were found, pairwise comparisons between the least squares means were computed for each day using Tukey’s honestly significant difference adjustment.
Results and Discussion

Mean Surface Area and Weight Results

The mean weight of the ham slices was 25.24 ± 0.58 g, while the mean diameter, height, and surface area were 4.66 ± 0.01 cm, 1.33 ± 0.08 cm, and 53.68 ± 1.44 cm$^2$, respectively (data not shown and $n = 40$ for all measurements). These mean ham slice dimensions resulted in LAE and OA treatment volumes of 0.39 and 1.00 ml per package, respectively, after dosages of each compound were calculated according to the respective manufacturer’s recommendations as previously described. These dosages resulted in LAE and OA treatment concentrations of 38.24 and 333.27 mg/kg, respectively.

Physicochemical Traits

Physicochemical characteristics of the hams can be found in Table 2. The LV1X and DV treatments resulted in significantly lower $a_w$ values than the 90MX and Control treatments ($P < 0.05$). The 90MX treatment, in turn, resulted in significantly lower $a_w$ values when compared to the Control treatment ($P < 0.05$). Final product pH was also affected by natural antimicrobial compound added. The pH of the Control treatment was not significantly different from that of the DV treatment ($P > 0.05$), but did significantly differ from both the LV1X and the 90MX treatments ($P < 0.05$). These differences may stem from the addition of the natural antimicrobial compounds to the products and the subsequent decline in product pH observed. Cranberry has been reported to contain phenolic acids and exhibit a high titratable acidity (18). Xi and others obtained similar results when using different ingoing levels of cranberry powder in a cooked meat model system (44) and in frankfurters (43). Similarly, the vinegar and vinegar and lemon juice
concentrate used in this study are also likely reservoirs of phenolic and other acidic compounds, such as acetic and citric acid, expected to cause the observed lower pH in products made with those ingredients. No significant differences in fat % and protein % were found between the treatments ($P > 0.05$). Moisture %, however, was significantly lower in the DV treatment compared to the Control ($P < 0.05$). These slight differences in the proximate composition of the ham formulations were not expected to influence the results of this study.

**L*, a*, and b* Values Results**

$L*$ value results can be found in Table 3 and Figure 1. Treatment and day exerted a significant effect on $L*$ values ($P < 0.05$). There were no differences in $L*$ values between treatments on days 1 and 84 of the study ($P > 0.05$). Although not significant most of the times ($P > 0.05$), the 90MX treatment resulted in the highest $L*$ values of any treatment on all days except for day 28. This is an indication that, under the conditions of this study, cranberry powder may induce an increase in objective lightness scores of RTE ham. Different patterns were observed by Xi and others (43) when using cranberry powder in the manufacture of naturally-cured frankfurters, as their results indicated that external $L*$ values of the frankfurters containing cranberry powder decreased as cranberry powder concentration increased. Furthermore, these authors observed that in treatments where cranberry powder was used as an ingredient, external $L*$ values ranged from 53.65 to 54.75, whereas $L*$ values observed in the 90MX treatment throughout the duration of our study ranged from 60.73 to 66.56. Xi and others (43), nonetheless, used cranberry powder levels greater than that which was used in this study and, additionally, used cranberry powder in combination with other compounds such as cherry powder and
lime powder, which may have affected the color attributes of the frankfurters. Differences in meat and nonmeat ingredients used may also account for these differences.

Results of the objective measurement of $a^*$ values (Table 4 and Figure 2) indicate that, under the conditions of this study, the use of cranberry powder may induced a reddening effect in RTE ham as $a^*$ values for the 90MX treatment were significantly higher than at least two of the other treatments evaluated on days 1, 28, 42, 56, 70, and 84 of our study. Furthermore, although not always significant ($P > 0.05$), the 90MX treatment resulted in higher $a^*$ values than all other treatments on every sampling day except for day 1. Although bright red in color in its powdered form, cranberry powder has been shown to turn dark purple upon addition to meat systems due to an increase in cranberry powder pH (45). This color shift is thought to stem from a partial red-to-blue shift in the color of the anthocyanin pigments of cranberry as a result of increased pH (45) and may have resulted in the pattern in $a^*$ values observed in this study.

Table 5 and Figure 3 describe $b^*$ values obtained in our study. Treatment and day exerted a significant effect on $b^*$ values of hams ($P < 0.05$), but there was not a significant treatment by day interaction ($P > 0.05$). Although not always significant ($P > 0.05$), the 90MX and LV1X treatments consistently resulted in the highest $b^*$ values throughout the 98 days of refrigerated storage. In fact, the LV1X treatment exhibited significantly higher ($P < 0.05$) $b^*$ values than all other treatments, except for the 90MX treatment, on days 1, 14, 28, 42, 70, and 84 of the study, suggesting that vinegar and lemon juice concentrate may have a yellowing effect on RTE ham, under the conditions of our study. Our results contrast those obtained by Xi and others (43), who reported a
decrease in $b^*$ values upon evaluating the effects of varying concentrations of cranberry powder on the external $b^*$ values of naturally-cured frankfurters.

CIE $L^*$, $a^*$, and $b^*$ results obtained in this study indicate that cranberry powder and, to a lesser extent, vinegar and lemon juice concentrate may influence the objective color characteristics of RTE ham. These effects, however, may be product-specific and the effects of these antimicrobial ingredients on other types of processed meat products should be studied further. On the other hand, under the conditions of this study, the post-lethality interventions evaluated seemed to exert no deleterious effects on $L^*$, $a^*$, or $b^*$ values, suggesting their use in RTE ham or similar products could be of value.

**Residual Nitrite Concentration Results**

The effects of treatment on residual nitrite concentration are found in Table 6 and Figure 4. Treatment and day exerted a significant effect on residual nitrite concentration ($P < 0.05$). Additionally, a significant treatment by day interaction was found to occur ($P < 0.05$).

Although all ham formulations were manufactured with 50 mg/kg natural nitrite on an ingoing basis, the highest residual nitrite concentration observed in all of the treatments on day 1 of the study was 41.67 mg/kg (Control treatment). This indicates that part of the ingoing nitrite was depleted in curing and other reactions that took place during product manufacture. Honikel (11) reported that as much as 65% of the ingoing nitrite can be depleted during product manufacture. Similarly, Xi and others (43) reported that as much as 75% of the ingoing nitrite can be depleted during the manufacture of frankfurters. Factors such as product pH, cooking temperature, and reducing agents used have been
shown to affect residual nitrite concentrations in meat systems (8). Sebranek (30) indicated that decreases in pH as small as 0.2 units during product manufacture can result in a doubling of the rate at which curing reactions occur. Thus, the significant ($P < 0.05$) decrease in pH brought about by the natural antimicrobial ingredients used in this study, especially cranberry powder, was expected to influence residual nitrite concentrations.

On day 1 of the study, the OA, 90MX, DV, and LV1X treatments exhibited lower residual nitrite concentrations than the Control treatment ($P < 0.05$). This pattern prevailed through day 42 of the study ($P < 0.05$). These results indicate that the acidic nature of octanoic acid and the natural antimicrobials investigated in this study inversely affect residual nitrite concentrations in RTE ham under the conditions of our study. As previously mentioned, the significant ($P < 0.05$) decrease in pH brought about by the natural antimicrobial ingredients used in this study, especially cranberry powder, was expected to influence residual nitrite concentrations. OA, under the conditions of this study, may have a similar effect on product pH and, as a result, on residual nitrite concentrations.

The 90MX treatment displayed the lowest residual nitrite concentrations throughout the duration of the study. In fact, when compared to those in the Control treatment, residual nitrite concentrations in the 90MX treatment were significantly lower ($P < 0.05$) on all days. These results are in agreement with Xi and others (44), authors who found that varying cranberry powder levels from 1.0 to 3.0% resulted in lower residual nitrite levels in a cooked meat model system. The same authors obtained similar results when evaluating a series of antimicrobial ingredients, which included cranberry powder, in the manufacture of naturally cured frankfurters (43).
Neither one of the HHP treatments seemed to affect residual nitrite concentrations throughout the storage period when compared to the Control treatment, as no significant differences were found to exist between these treatments on any of the sampling days ($P > 0.05$). Bruun-Jensen and Skibsted (6) concluded that the rate of oxidation of nitrosylmyoglobin by oxygen decreases with increasing hydrostatic pressure and that, as a result, cured meat products may derive the greatest benefit from high pressure technology. Residual nitrite concentration and CIE L*, a*, and b* results obtained in this study indicate that nitrosylmyochromogen, the pigment responsible for the pink color associated with cured meats, is not significantly affected by either of the HHP treatments evaluated and under the conditions of this study.

Throughout storage of the products at $4 \pm 1^\circ\text{C}$, all treatments showed significant ($P < 0.05$) decreases in residual nitrite concentrations. The residual nitrite concentration observed in the Control treatment significantly ($P < 0.05$) decreased after 28 days of storage. The same pattern was observed in the HHP400, OA, and PPTT treatments ($P < 0.05$), which suggests that these post-lethality interventions did not affect the rate at which residual nitrite concentration declines over the storage life of the product. Similarly, the LAE treatment did not experience a significant decrease in residual nitrite concentration until day 42 ($P < 0.05$).

**Viable Listeria monocytogenes**

Viable *L. monocytogenes* numbers on MOX (Table 7 and Figure 5) and TAL (Table 8 and Figure 6) media were monitored throughout the duration of the study. The growth mediums used did not significantly differ ($P > 0.05$) within treatment on any given day,
indicating that, under the conditions of this study, the use of the TAL technique offers limited advantages compared to using a traditional medium such as MOX. Thus, the discussion about viable *L. monocytogenes* numbers as affected by treatment is based on results obtained using MOX.

Treatment and day had a significant effect on viable *L. monocytogenes* counts (*P* < 0.05). Additionally, the treatment by day interaction was also found to be significant (*P* < 0.05). The natural antimicrobials evaluated in this study did not significantly affect *L. monocytogenes* levels after 1 day of storage (*P* > 0.05) when compared to the Control treatment. The bacteriostatic properties of these ingredients, however, varied greatly. The 90MX treatment, for example, experienced significant (*P* < 0.05) increases in *L. monocytogenes* levels as soon as day 28 of the study. These levels continued to increase (*P* < 0.05) through day 42 and reached maximum levels on day 70. These results indicate that, at the level used and under the conditions of this study, cranberry powder does not exert bacteriostatic effects on *L. monocytogenes*. Lin and others (20) concluded that cranberry extract alone was not inhibitory of *L. monocytogenes* growth upon refrigerated storage of both inoculated fish and beef slices. Similarly, results by Xi and others (44) indicated that cranberry powder, also when used at a level of 1% (wt/wt), did not inhibit the growth of *L. monocytogenes* completely.

The DV treatment did not experience significant changes in *L. monocytogenes* levels throughout the duration of the study (*P* > 0.05), while the LV1X treatment did not exhibit a significant increase in *L. monocytogenes* counts until day 84 of the study (*P* < 0.05). *L. monocytogenes* levels found in the LV1X treatment on days 84 and 98, however, were still lower than those found in the Control treatment (*P* < 0.05). These results allow us to
conclude that, at the levels used and under the conditions of this study, DV and LV1X exhibit strong bacteriostatic properties against *L. monocytogenes* under the conditions of our study. To our knowledge, no other peer-reviewed research articles have previously evaluated these compounds and their effects on the growth of *L. monocytogenes* on similar meat and poultry products. Research on similar buffered vinegar and buffered vinegar and lemon juice concentrate products and their inhibitory effects on *C. perfringens* in ground turkey roast (40) and roast beef (19) concluded that these ingredients exhibit inhibitory properties on that microorganism. However, additional research on the subject is needed as these effects are likely to vary based on product characteristics and microorganism of concern.

The HHP600 treatment resulted in *L. monocytogenes* numbers below the detection limit of our sampling protocols (≥ 0.30 log CFU per g) throughout the entire duration of the study. These results agree with those obtained by Myers and others (24), as these authors found that an HHP treatment of 600 MPa for 3 min and 17°C resulted in a 3.85-4.35 log CFU per g reduction in *L. monocytogenes* numbers on RTE meat products. Similarly, Myers and others (23) also concluded that HHP treatment with 600 MPa for 3 min and 17°C resulted in a 3.9-4.3 log CFU per g reduction in *L. monocytogenes* numbers on RTE sliced ham.

The LAE, HHP400, and OA treatments resulted in 2.38, 2.21, and 1.73 log CFU per g reductions in viable *L. monocytogenes* numbers, respectively, compared to the Control treatment, on day 1 of the study. All of these reductions were found to be significant (*P* < 0.05), demonstrating the bactericidal potential of these post-lethality interventions under the conditions of this study. Myers and other (23), in contrast, concluded that 400 MPa
HHP treatment of RTE sliced ham for 3 min at 17°C resulted in less than a 1 log CFU per g reduction in *L. monocytogenes* numbers. Slight variations in product physicochemical characteristics such as *a*<sub>w</sub>, pH, salt concentration, among others, between studies may account for these differences. Furthermore, the extent to which HHP will inactivate microorganisms depends on several different factors including, but not limited to, bacterial strain and the growth phase it is in at the time treatment is applied, the characteristics of the food matrix to be treated, temperature of the medium, pressure level, and exposure time (13). When compared to broth systems, for example, nutrient rich meat matrices allow for greater resistance of microorganisms to HHP treatment (12, 32). Thus, it would seem that any HHP treatment parameters would have to be tailored not only to the product to be treated, but also to the specific target microorganism and the expected outcome.

Additionally, PPTT did not significantly decrease initial viable *L. monocytogenes* numbers (*P* > 0.05) and resulted a similar *L. monocytogenes* behavior patterns compared to the Control treatment throughout the duration of the study. These results contrast those obtained by Chen and others (9), as these authors concluded that a post-packaging thermal treatment of 71 ± 1°C for 30 sec would result in a 1.4 log CFU per g reduction in *L. monocytogenes* numbers on 1-link packages of frankfurters when using a 3.4 log CFU per g initial inoculation level.

Although an initial bactericidal effect of the HHP400, OA, and LAE treatments was observed, the bacteriostatic properties of these treatments come under question as evidenced by our results. The HHP400 treatment experienced a significant (*P* < 0.05) increase in viable *L. monocytogenes* numbers after 56 days of storage. In fact, by day 98
of the study there was no significant difference between the Control and the HHP400 treatments \((P > 0.05)\). Similar results were reported by Myers and others \((23)\). These authors reported that after 400 MPa HHP treatment of RTE sliced ham for 3 min at 17°C, which resulted in less than a 1 log CFU per g reduction in \(L.\) monocytogenes numbers, the pathogen was able to grow to numbers above inoculation levels upon storage under refrigeration.

Similarly, the OA and LAE treatments showed significant \((P < 0.05)\) increases in \(L.\) monocytogenes numbers by days 28 and 14 of the study, respectively, with both of these treatments showing no significant difference in viable \(L.\) monocytogenes levels compared to the Control treatment by day 70 \((P > 0.05)\). The LAE treatment, in fact, resulted in viable \(L.\) monocytogenes levels that were not significantly different from those found in the Control treatment as early as day 28 of the study \((P > 0.05)\). These findings are in agreement with those of Porto-Fett and others \((27)\), as these authors discovered that only when used in combination with lactate or diacetate will lauric arginate exert a bacteriostatic effect on the pathogen under storage temperatures of 4°C for 120 days. Similar results were obtained by Luchansky and others \((21)\) when they researched the effects of lauric arginate on the growth of \(L.\) monocytogenes on hams. Burnett and others \((7)\) also reported bacteriocidal effects of octanoic acid solutions on \(L.\) monocytogenes, but no information was provided as to its bacteriostatic effects on the same microorganism. Thus, although they may provide an initial lethality, lauric arginate and octanoic acid alone do not inhibit the outgrowth of any \(L.\) monocytogenes that may survive and their bacteriostatic effects should receive more attention from the scientific community. The results of our study indicate that although beneficial from the standpoint of initial
lethality, HHP400, OA, and LAE post-lethality interventions do not offer protection against the growth of surviving *L. monocytogenes* upon storage of the product under the conditions of this study.

In conclusion, at the levels used and under the conditions of this study, DV and LV1X exhibit strong bacteriostatic properties against *L. monocytogenes* and represent viable options that could be instituted by manufacturers of organic and natural processed meat and poultry products in their *L. monocytogenes* control plans. These natural antimicrobial ingredients, however, did not exhibit bactericidal properties under the conditions or this study. Additionally, although beneficial from the standpoint of initial lethality, the HHP400, OA, and LAE post-lethality interventions do not offer protection against the growth of surviving *L. monocytogenes* upon storage of the product and under the conditions of this study. Thus, additional research aimed at combining natural antimicrobial ingredients and post-lethality interventions that are suitable for use in the manufacture of organic and natural processed meat and poultry products is warranted.

**Acknowledgements**

This project was supported by the American Meat Institute Foundation. The authors would like to recognize the companies that donated research materials: Florida Food Products, WTI Ingredients, Ocean Spray International, EcoLab, Purac America, and Kalle USA. Special thanks to Devin Maurer and Daniel Fortin for the invaluable contributions made throughout the conduction of these experiments.
References


natural antimicrobials in no-nitrate-or-nitrite-added ham. J. Food Prot. 75:1071–1076.


### TABLE 1. Naturally cured RTE ham formulations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ham (kg)</th>
<th>Water (kg)</th>
<th>Salt (kg)</th>
<th>Sugar (kg)</th>
<th>Pre-converted Celery Powder&lt;sup&gt;a&lt;/sup&gt; (g)</th>
<th>Antimicrobial A&lt;sup&gt;b&lt;/sup&gt; (g)</th>
<th>Antimicrobial B&lt;sup&gt;c&lt;/sup&gt; (g)</th>
<th>Antimicrobial C&lt;sup&gt;d&lt;/sup&gt; (g)</th>
<th>Post-Lethality Intervention</th>
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<tr>
<td>Control</td>
<td>18.14</td>
<td>3.66</td>
<td>0.50</td>
<td>0.30</td>
<td>74.84</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HHP400</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>HHP&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>HHP600</td>
<td></td>
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<td>HHP&lt;sup&gt;f&lt;/sup&gt;</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>-</td>
<td>OA&lt;sup&gt;g&lt;/sup&gt;</td>
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<tr>
<td>LAE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>LAE&lt;sup&gt;h&lt;/sup&gt;</td>
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<td>18.14</td>
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<td>74.84</td>
<td>226.80</td>
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<td>-</td>
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<tr>
<td>DV</td>
<td>18.14</td>
<td>3.66</td>
<td>0.50</td>
<td>0.30</td>
<td>74.84</td>
<td>-</td>
<td>226.80</td>
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<tr>
<td>LV1X</td>
<td>18.14</td>
<td>3.66</td>
<td>0.50</td>
<td>0.30</td>
<td>74.84</td>
<td>-</td>
<td>-</td>
<td>567.02</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Vegetable 504 (Natural Nitrite; Florida Food Products, Inc., Eustis, FL).

<sup>b</sup> 90MX (Cranberry Powder; Ocean Spray International, Middleboro, MA).

<sup>c</sup> DV (Vinegar; WTI Ingredients, Inc., Jefferson, GA).

<sup>d</sup> LV1X (Vinegar and Lemon Juice Concentrate; WTI Ingredients, Inc., Jefferson, GA).

<sup>e</sup> High Hydrostatic Pressure (400 MPa, 4 min dwell time at 12 ± 2°C).

<sup>f</sup> High Hydrostatic Pressure (600 MPa, 4 min dwell time at 12 ± 2°C).

<sup>g</sup> Octa-Gone (Octanoic Acid; EcoLab, Inc., Eagan, MN).

<sup>h</sup> Protect-M (Lauric Arginate; Purac America, Lincolnshire, IL).

<sup>i</sup> Post-Packaging Thermal Treatment (71.0 ± 1°C for 30 s).
TABLE 2. Effect of natural antimicrobial ingredients on physicochemical properties of naturally cured RTE ham

<table>
<thead>
<tr>
<th>Treatment</th>
<th>a&lt;sub&gt;w&lt;/sub&gt;</th>
<th>pH</th>
<th>Fat %</th>
<th>Moisture %</th>
<th>Protein %</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.9745&lt;sup&gt;A&lt;/sup&gt;</td>
<td>6.14&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>1.95</td>
<td>76.29&lt;sup&gt;B&lt;/sup&gt;</td>
<td>17.85</td>
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<td>5.85&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.85</td>
<td>75.60&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>18.04</td>
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<tr>
<td>DV</td>
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<td>6.04&lt;sup&gt;CD&lt;/sup&gt;</td>
<td>2.04</td>
<td>75.49&lt;sup&gt;A&lt;/sup&gt;</td>
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<tr>
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<td>6.03&lt;sup&gt;D&lt;/sup&gt;</td>
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<td>0.04</td>
<td>0.12</td>
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<td>0.22</td>
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<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through D) are significantly different (P < 0.05).

<sup>b</sup> Control, naturally cured control; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

<sup>c</sup> Standard error of the differences of least squares means.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
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<td>64.07AB,X</td>
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<td>60.17ABC,YZ</td>
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<td>58.07AC,Z</td>
<td>57.46B,Z</td>
<td>57.96Z</td>
<td>57.86AZ</td>
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<tr>
<td>HHP600</td>
<td>61.13XZ</td>
<td>62.98AB,X</td>
<td>58.21AYZ</td>
<td>59.05AYZ</td>
<td>57.35C,YW</td>
<td>58.41BC,WZ</td>
<td>57.31WY</td>
<td>57.90AB,WZ</td>
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<td>60.55AB,Z</td>
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<td>LAE</td>
<td>62.20Z</td>
<td>63.02AB,Z</td>
<td>61.32AB,Z</td>
<td>61.47AB,Z</td>
<td>60.49ABC,Z</td>
<td>60.14AB,Z</td>
<td>59.94Z</td>
<td>59.83AB,Z</td>
</tr>
<tr>
<td>PPTT</td>
<td>62.80YZ</td>
<td>63.77AB,Y</td>
<td>59.96AB,Z</td>
<td>60.65AB,YZ</td>
<td>61.38AB,YZ</td>
<td>60.15AB,YZ</td>
<td>60.28YZ</td>
<td>59.92AB,Z</td>
</tr>
<tr>
<td>90MX</td>
<td>63.79YZ</td>
<td>66.56B,Y</td>
<td>61.73AB,Z</td>
<td>63.38B,YZ</td>
<td>61.96B,Z</td>
<td>62.79AB,Z</td>
<td>60.73Z</td>
<td>62.56B,Z</td>
</tr>
<tr>
<td>DV</td>
<td>63.76WZ</td>
<td>65.29AB,Z</td>
<td>60.06AB,XY</td>
<td>62.34AB,WXZ</td>
<td>59.76ABC,X</td>
<td>60.73AB,WXY</td>
<td>59.52X</td>
<td>59.74AB,X</td>
</tr>
<tr>
<td>LV1X</td>
<td>61.29YZ</td>
<td>64.79AB,Y</td>
<td>62.13B,YZ</td>
<td>62.16AB,YZ</td>
<td>60.62AB,CZ</td>
<td>61.62AC,YZ</td>
<td>59.88Z</td>
<td>60.80AB,Z</td>
</tr>
<tr>
<td>SE⁵</td>
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<td>1.20</td>
</tr>
</tbody>
</table>

⁵ Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different ($P < 0.05$). Within a row, means with different superscripts (W through Z) are significantly different ($P < 0.05$).

⁶ Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

⁷ Standard error of the differences of least squares means.
FIGURE 1. Effect of treatment on $L^*$ values of naturally cured RTE ham stored at 4 ± 1°C

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
TABLE 4. Effect of treatment on $a^*$ values of naturally cured RTE ham stored at 4 ± 1°C$^a$

<table>
<thead>
<tr>
<th>Treatment $^b$</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.35$^{AB}$</td>
<td>5.62$^{A}$</td>
<td>5.11$^{A}$</td>
<td>5.10$^{A}$</td>
<td>5.84$^{AB}$</td>
<td>5.55$^{AB}$</td>
<td>5.22$^{A}$</td>
<td>6.03</td>
</tr>
<tr>
<td>HHP400</td>
<td>4.87$^A$</td>
<td>6.12$^{AB}$</td>
<td>5.97$^{AB}$</td>
<td>4.80$^{A}$</td>
<td>4.78$^{AB}$</td>
<td>5.19$^{A}$</td>
<td>5.81$^{AB}$</td>
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</tr>
<tr>
<td>HHP600</td>
<td>4.89$^A$</td>
<td>5.90$^{AB}$</td>
<td>4.90$^{A}$</td>
<td>5.15$^{A}$</td>
<td>5.04$^{A}$</td>
<td>5.25$^{A}$</td>
<td>4.93$^{A}$</td>
<td>5.23</td>
</tr>
<tr>
<td>OA</td>
<td>5.16$^{A}$</td>
<td>6.10$^{AB}$</td>
<td>5.69$^{AB}$</td>
<td>5.11$^{A}$</td>
<td>5.07$^{A}$</td>
<td>6.04$^{AB}$</td>
<td>5.46$^{AB}$</td>
<td>5.67</td>
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<tr>
<td>LAE</td>
<td>4.97$^{A}$</td>
<td>6.36$^{AB}$</td>
<td>5.64$^{AB}$</td>
<td>5.77$^{AB}$</td>
<td>5.55$^{A}$</td>
<td>5.63$^{AB}$</td>
<td>4.79$^{A}$</td>
<td>5.28</td>
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<tr>
<td>PPTT</td>
<td>5.47$^{AB}$</td>
<td>6.10$^{AB}$</td>
<td>5.02$^{A}$</td>
<td>5.19$^{A}$</td>
<td>4.64$^{A}$</td>
<td>5.69$^{AB}$</td>
<td>4.95$^{A}$</td>
<td>5.48</td>
</tr>
<tr>
<td>90MX</td>
<td>7.08$^B$</td>
<td>7.59$^{B}$</td>
<td>6.95$^{B}$</td>
<td>7.56$^{B}$</td>
<td>7.37$^{B}$</td>
<td>7.34$^{B}$</td>
<td>7.14$^{B}$</td>
<td>6.89</td>
</tr>
<tr>
<td>DV</td>
<td>5.26$^A$</td>
<td>6.23$^{AB}$</td>
<td>5.78$^{AB}$</td>
<td>5.48$^{A}$</td>
<td>5.89$^{AB}$</td>
<td>6.01$^{AB}$</td>
<td>6.09$^{AB}$</td>
<td>5.61</td>
</tr>
<tr>
<td>LV1X</td>
<td>7.10$^B$</td>
<td>6.46$^{AB}$</td>
<td>6.16$^{AB}$</td>
<td>5.68$^{A}$</td>
<td>6.00$^{AB}$</td>
<td>5.94$^{AB}$</td>
<td>6.04$^{AB}$</td>
<td>5.94</td>
</tr>
<tr>
<td>SE$^c$</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

$^a$ Values are least squares means. Within a column, means with different superscripts (A through B) are significantly different ($P < 0.05$).

$^b$ Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

$^c$ Standard error of the differences of least squares means.
FIGURE 2. Effect of treatment on $a^*$ values of naturally cured RTE ham stored at $4 \pm 1 \, ^\circ C$

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
**TABLE 5. Effect of treatment on \(b^*\) values of naturally cured RTE ham stored at 4 ± 1°C**

<table>
<thead>
<tr>
<th>Treatment(^b)</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>3.65(^{AD})</td>
</tr>
<tr>
<td>HHP400</td>
<td>1.83(^B)</td>
</tr>
<tr>
<td>HHP600</td>
<td>3.00(^{AB})</td>
</tr>
<tr>
<td>OA</td>
<td>2.64(^{AB})</td>
</tr>
<tr>
<td>LAE</td>
<td>3.11(^{ABD})</td>
</tr>
<tr>
<td>PPTT</td>
<td>3.29(^{ABD})</td>
</tr>
<tr>
<td>90MX</td>
<td>4.64(^{DC})</td>
</tr>
<tr>
<td>DV</td>
<td>2.88(^{AB})</td>
</tr>
<tr>
<td>LV1X</td>
<td>5.72(^C)</td>
</tr>
<tr>
<td>SE(^c)</td>
<td>0.49</td>
</tr>
</tbody>
</table>

\(^a\) Values are least squares means. Within a column, means with different superscripts (A through D) are significantly different \((P < 0.05)\).

\(^b\) Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

\(^c\) Standard error of the differences of least squares means.
FIGURE 3. *Effect of treatment on b* values of naturally cured RTE ham stored at 4 ± 1 °C*

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
TABLE 6. Effect of treatment on residual nitrite concentrations of naturally cured RTE ham stored at 4 ± 1°C<sup>a</sup>

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41.67&lt;sup&gt;AB,Z&lt;/sup&gt;</td>
<td>37.56&lt;sup&gt;XYZ&lt;/sup&gt;</td>
<td>34.39&lt;sup&gt;XY&lt;/sup&gt;</td>
<td>32.42&lt;sup&gt;XX&lt;/sup&gt;</td>
<td>22.21&lt;sup&gt;AB,W&lt;/sup&gt;</td>
<td>22.31&lt;sup&gt;AD,W&lt;/sup&gt;</td>
<td>18.12&lt;sup&gt;AB,W&lt;/sup&gt;</td>
<td>21.15&lt;sup&gt;AC,W&lt;/sup&gt;</td>
</tr>
<tr>
<td>HHP400</td>
<td>40.18&lt;sup&gt;AB,Z&lt;/sup&gt;</td>
<td>36.98&lt;sup&gt;AB,YZ&lt;/sup&gt;</td>
<td>33.35&lt;sup&gt;AXY&lt;/sup&gt;</td>
<td>31.07&lt;sup&gt;AX&lt;/sup&gt;</td>
<td>25.06&lt;sup&gt;A,W&lt;/sup&gt;</td>
<td>24.02&lt;sup&gt;A,VW&lt;/sup&gt;</td>
<td>20.29&lt;sup&gt;AC,V&lt;/sup&gt;</td>
<td>20.03&lt;sup&gt;AC,V&lt;/sup&gt;</td>
</tr>
<tr>
<td>HHP600</td>
<td>40.13&lt;sup&gt;AB,Z&lt;/sup&gt;</td>
<td>35.43&lt;sup&gt;ABD,Y&lt;/sup&gt;</td>
<td>32.87&lt;sup&gt;AD,XY&lt;/sup&gt;</td>
<td>30.05&lt;sup&gt;AD,X&lt;/sup&gt;</td>
<td>24.28&lt;sup&gt;A,W&lt;/sup&gt;</td>
<td>24.30&lt;sup&gt;A,W&lt;/sup&gt;</td>
<td>19.87&lt;sup&gt;AC,W&lt;/sup&gt;</td>
<td>20.37&lt;sup&gt;AC,W&lt;/sup&gt;</td>
</tr>
<tr>
<td>OA</td>
<td>36.82&lt;sup&gt;BCD,Z&lt;/sup&gt;</td>
<td>32.59&lt;sup&gt;BD,Z&lt;/sup&gt;</td>
<td>27.95&lt;sup&gt;XY&lt;/sup&gt;</td>
<td>23.99&lt;sup&gt;B,X&lt;/sup&gt;</td>
<td>17.59&lt;sup&gt;BD,W&lt;/sup&gt;</td>
<td>17.12&lt;sup&gt;B,W&lt;/sup&gt;</td>
<td>14.51&lt;sup&gt;B,VW&lt;/sup&gt;</td>
<td>12.23&lt;sup&gt;BD,V&lt;/sup&gt;</td>
</tr>
<tr>
<td>LAE</td>
<td>38.88&lt;sup&gt;AB,YZ&lt;/sup&gt;</td>
<td>39.88&lt;sup&gt;A,Z&lt;/sup&gt;</td>
<td>34.65&lt;sup&gt;AXY&lt;/sup&gt;</td>
<td>32.57&lt;sup&gt;AX&lt;/sup&gt;</td>
<td>25.25&lt;sup&gt;A,W&lt;/sup&gt;</td>
<td>26.07&lt;sup&gt;A,W&lt;/sup&gt;</td>
<td>24.10&lt;sup&gt;C,W&lt;/sup&gt;</td>
<td>22.97&lt;sup&gt;A,W&lt;/sup&gt;</td>
</tr>
<tr>
<td>PPTT</td>
<td>39.09&lt;sup&gt;AD,Z&lt;/sup&gt;</td>
<td>36.72&lt;sup&gt;AB,YZ&lt;/sup&gt;</td>
<td>34.31&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>32.31&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>23.49&lt;sup&gt;AX,W&lt;/sup&gt;</td>
<td>25.12&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>19.09&lt;sup&gt;AB,VW&lt;/sup&gt;</td>
<td>17.53&lt;sup&gt;CE,V&lt;/sup&gt;</td>
</tr>
<tr>
<td>90MX</td>
<td>33.13&lt;sup&gt;C,Z&lt;/sup&gt;</td>
<td>26.99&lt;sup&gt;C,Y&lt;/sup&gt;</td>
<td>22.29&lt;sup&gt;C,X&lt;/sup&gt;</td>
<td>18.39&lt;sup&gt;C,X&lt;/sup&gt;</td>
<td>11.26&lt;sup&gt;C,W&lt;/sup&gt;</td>
<td>11.28&lt;sup&gt;C,W&lt;/sup&gt;</td>
<td>9.24&lt;sup&gt;D,W&lt;/sup&gt;</td>
<td>8.77&lt;sup&gt;D,W&lt;/sup&gt;</td>
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<tr>
<td>DV</td>
<td>36.38&lt;sup&gt;BCD,Z&lt;/sup&gt;</td>
<td>31.81&lt;sup&gt;D,YZ&lt;/sup&gt;</td>
<td>28.34&lt;sup&gt;BD,XY&lt;/sup&gt;</td>
<td>25.74&lt;sup&gt;BD,X&lt;/sup&gt;</td>
<td>17.41&lt;sup&gt;D,W&lt;/sup&gt;</td>
<td>17.29&lt;sup&gt;B,W&lt;/sup&gt;</td>
<td>16.03&lt;sup&gt;AB,W&lt;/sup&gt;</td>
<td>13.81&lt;sup&gt;BDE,W&lt;/sup&gt;</td>
</tr>
<tr>
<td>LV1X</td>
<td>36.34&lt;sup&gt;BCD,Z&lt;/sup&gt;</td>
<td>31.45&lt;sup&gt;CD,Y&lt;/sup&gt;</td>
<td>27.32&lt;sup&gt;XY&lt;/sup&gt;</td>
<td>23.44&lt;sup&gt;B,X&lt;/sup&gt;</td>
<td>16.62&lt;sup&gt;D,W&lt;/sup&gt;</td>
<td>18.09&lt;sup&gt;BD,W&lt;/sup&gt;</td>
<td>15.79&lt;sup&gt;AB,W&lt;/sup&gt;</td>
<td>14.45&lt;sup&gt;BE,W&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through E) are significantly different ($P < 0.05$). Within a row, means with different superscripts (V through Z) are significantly different ($P < 0.05$).

<sup>b</sup> Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

<sup>c</sup> Standard error of the differences of least squares means.
FIGURE 4. Effect of treatment on residual nitrite concentration of naturally cured RTE ham stored at 4 ± 1 °C

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
TABLE 7. Effect of treatment on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHP400</td>
<td>0.44^B.Z</td>
<td>0.34^C.Z</td>
<td>0.47^B.Z</td>
<td>1.32^B,YZ</td>
<td>2.36^B.Y</td>
<td>3.72^B.X</td>
<td>4.47^B.X</td>
<td>6.45^A.W</td>
</tr>
<tr>
<td>HHP600</td>
<td>ND^d</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>OA</td>
<td>0.92^B.Z</td>
<td>1.07^BC,YZ</td>
<td>2.21^C,Y</td>
<td>3.88^C,X</td>
<td>6.10^C.W</td>
<td>7.53^A.V</td>
<td>7.45^A.V</td>
<td>7.43^A.V</td>
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<td>7.17^A.W</td>
<td>7.68^A.W</td>
<td>7.52^A.W</td>
<td>7.04^A.W</td>
<td>6.79^A.W</td>
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<td>2.79^A.Z</td>
<td>3.54^AE.Z</td>
<td>6.03^AD,Y</td>
<td>7.32^A.X</td>
<td>7.87^A.X</td>
<td>7.99^A.X</td>
<td>7.80^A.X</td>
<td>7.67^A.X</td>
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<td>DV</td>
<td>2.70^A</td>
<td>2.72^AD</td>
<td>2.69^C</td>
<td>2.57^BD</td>
<td>2.80^B</td>
<td>2.78^B</td>
<td>2.91^C</td>
<td>2.79^B</td>
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<tr>
<td>LV1X</td>
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<td>2.76^AD.Z</td>
<td>2.79^C.Z</td>
<td>2.66^C.D.Z</td>
<td>2.94^B.XZ</td>
<td>3.29^B.YZ</td>
<td>4.25^B.Y</td>
<td>4.06^C.XY</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Values are least squares means. Within a column, means with different superscripts (A through E) are significantly different (P < 0.05). Within a row, means with different superscripts (V through Z) are significantly different (P < 0.05).

^b Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

^c Not detected.

^d Standard error of the differences of least squares means.
FIGURE 5. Effect of treatment on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
TABLE 8. Effect of treatment on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 42</th>
<th>Day 56</th>
<th>Day 70</th>
<th>Day 84</th>
<th>Day 98</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHP400</td>
<td>0.49^B,Z</td>
<td>0.73^B,Z</td>
<td>0.94^B,Z</td>
<td>1.56^B,YZ</td>
<td>2.42^B,Y</td>
<td>3.74^B,X</td>
<td>4.37^B,X</td>
<td>6.35^A,W</td>
</tr>
<tr>
<td>HHP600</td>
<td>ND^c</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LAE</td>
<td>0.49^B,Z</td>
<td>1.54^BC,Z</td>
<td>5.06^D,Y</td>
<td>7.11^A,X</td>
<td>7.64^A,X</td>
<td>7.52^A,X</td>
<td>7.00^A,X</td>
<td>6.76^A,X</td>
</tr>
<tr>
<td>90MX</td>
<td>2.85^A,Z</td>
<td>3.54^AD,Z</td>
<td>5.97^AD,Y</td>
<td>7.27^A,X</td>
<td>7.87^A,X</td>
<td>8.00^A,X</td>
<td>7.74^A,X</td>
<td>7.01^A,XY</td>
</tr>
<tr>
<td>DV</td>
<td>2.51^A</td>
<td>2.74^AC</td>
<td>2.70^C</td>
<td>2.52^B</td>
<td>2.77^B</td>
<td>2.64^B</td>
<td>2.84^C</td>
<td>2.63^B</td>
</tr>
<tr>
<td>LV1X</td>
<td>2.82^A,Z</td>
<td>2.79^A,Z</td>
<td>2.45^C,Z</td>
<td>2.46^B,Z</td>
<td>2.97^B,Z</td>
<td>3.35^B,Z</td>
<td>4.16^B,Y</td>
<td>4.07^C,Y</td>
</tr>
<tr>
<td>SE^d</td>
<td>0.41</td>
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</tr>
</tbody>
</table>

^a Values are least squares means. Within a column, means with different superscripts (A through D) are significantly different (P < 0.05). Within a row, means with different superscripts (U through Z) are significantly different (P < 0.05).

^b Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; HHP600, high hydrostatic pressure, 600 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

^c Not detected.

^d Standard error of the differences of least squares means.
FIGURE 6. Effect of treatment on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C.

Treatments: Control, naturally cured control; HHP400, high hydrostatic pressure, 400 MPa; OA, Octa-Gone; LAE, Protect-M; PPTT, post-packaging thermal treatment; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.
CHAPTER 6. INVESTIGATING THE CONTROL OF *LISTERIA MONOCYTOGENES* ON NATURALLY CURED READY-TO-EAT HAM USING NATURAL ANTIMICROBIAL INGREDIENTS IN COMBINATION WITH POST-LETHALITY INTERVENTIONS

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**Abstract**

Ready-to-Eat (RTE) meat and poultry products manufactured with natural or organic methods are at greater risk for *Listeria monocytogenes* growth, if contaminated, than their conventional counterparts due to the required absence of preservatives and antimicrobials. Thus, the objective of this study was to investigate the use of commercially available natural antimicrobials in combination with post-lethality interventions in the control of *L. monocytogenes* growth and recovery on naturally cured RTE ham. Antimicrobials evaluated were cranberry powder (90MX), vinegar (DV), and vinegar and lemon juice concentrate (LV1X). Post-lethality interventions studied were high hydrostatic pressure at 400 MPa (HHP), lauric arginate (LAE), octanoic acid (OA), and post-packaging thermal treatment (PPTT). Viable *L. monocytogenes* on modified Oxford (MOX) and thin agar layer (TAL) media were monitored through 98 days of storage at 4 ± 1°C. Counts on MOX and TAL did not differ. HHP, OA, and LAE
significantly reduced initial viable *L. monocytogenes* numbers compared to the Control and regardless of the antimicrobial ingredient used as part of the formulation while PPTT did not. On day 1 and compared to the Control, viable *L. monocytogenes* reductions ranged from 0.34 to 2.67 log CFU per g. Only when used in combination with DV and LV1X did HHP, OA, and LAE exhibit sustained suppression of *L. monocytogenes* recovery and growth throughout the refrigerated storage of the products. As a result, the use of natural antimicrobial ingredients such as DV and LV1X in combination with post-lethality interventions such as HHP, LAE, and OA represents a multi-hurdle approach that could be instituted by manufacturers of organic and natural processed meat and poultry products in their *L. monocytogenes* control plans.

**Introduction**

The popularity of natural and organic foods has increased and led to noticeable market growth of these food categories over the last few decades (26, 34). Natural and organic meat products, in particular, have accounted for a significant part of that growth. In 2011 in the United States, for example, meat, fish, and poultry represented the fastest growing of the eight organic food categories after experiencing a 13% increase in sales compared to the previous year (19). This increase in sales is expected to continue in spite of the fact that price premiums associated with organic products have been estimated to range between 10-40% (34) and those of organic meat and poultry products sometimes reach ≥200% (5). Stringent regulations that govern the production of natural and organic foods have rendered the use of certain ingredients illegal. For instance, in the manufacture of natural and organic processed meat products such as boneless ham and frankfurters, the direct addition of nitrite or nitrate, curing ingredients used in the
manufacture of such products, is not permitted. Additionally, lactate and diacetate, antimicrobials commonly found in ready-to-eat (RTE) meat and poultry products and proven to have inhibitory effects on pathogens such as *Listeria monocytogenes*, are not permitted in the manufacture of natural or organic meat products. Thus, RTE meat and poultry products manufactured under uncured, natural, or organic methods and requirements are at a greater risk for growth of *L. monocytogenes* if post-lethality contamination occurs than their conventional counterparts (23, 28).

The use of natural antimicrobials and “clean label” technologies or post-lethality interventions in the manufacture of these types of meat products has received attention from researchers and processors alike (23, 24, 26, 27, 29). The United States Department of Agriculture Food Safety Inspection Services (USDA FSIS) defines a post-lethality treatment as “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” (32). The use of post-lethality interventions to address the potential presence of *L. monocytogenes* in uncured, no-nitrate-or-nitrite-added, RTE natural or organic meat and poultry products is an area of interest because some of these technologies are allowed for use in these categories of products. High hydrostatic pressure processing (HHP), for example, is an example of a post-lethality intervention as it generally takes place after the product has gone through the lethality or cooking step (32). Other examples of post-lethality interventions include sprays or solutions that can be applied to the finished product such as lauric arginate (lauramide arginine ethyl ester or LAE) and octanoic acid (sometimes referred to as caprylic acid or OA) as well as post-packaging thermal
treatment or pasteurization. The USDA FSIS lists lauric arginate as a safe and suitable ingredient for the production of meat and poultry products and allows for up to 44 mg/kg (± a 20% tolerance) by weight of the product to be applied to the inside of a package as a processing aid (31). When used at this level, lauric arginate is considered a processing aid, would not have to be declared on the label of the product, and could be used in the manufacture of uncured, no-nitrate-or-nitrite-added, RTE natural or organic meat and poultry products. Similarly, the USDA FSIS also allows for octanoic acid to be used as a processing aid if applied to the surface of an RTE meat and poultry product at a rate not to exceed 400 mg/kg octanoic acid by weight of the final product (31). Octanoic acid is a saturated (C\textsubscript{8}:0) fatty acid (pK\textsubscript{a} 4.89) naturally found in coconut oil and bovine milk (11).

Although promising from an initial \textit{L. monocytogenes} lethality standpoint, the bacteriostatic effects of lauric arginate and octanoic acid have not been extensively researched and should receive more attention from the scientific community.

Some emphasis has also been placed on the investigation of natural sources of antimicrobials that could potentially replace chemical preservatives and synthetic antimicrobial ingredients as a means to address \textit{L. monocytogenes} in the highly restrictive natural and organic categories (29, 35, 36). These compounds often possess Generally Recognized as Safe (GRAS) status. However, differences in antimicrobial potency observed in natural compounds may result in part from inconsistencies of commercial samples. Another important factor to consider is the food matrix itself, as it has been shown that the antilisterial properties of natural antimicrobials can vary as a result of the fat content of the food (13). Thus, the antilisterial properties of natural antimicrobial
ingredients used in RTE meat and poultry products are likely to vary based on product characteristics such as fat content, protein content, pH, aw, and other ingredients added.

Previous work in our laboratory demonstrated that, although post-lethality interventions such as HHP, OA, and LAE can deliver an initial lethality and natural antimicrobials such as vinegar and vinegar and lemon juice concentrate may have a bacteriostatic effect, concerns still exist over the potential recovery and growth of sublethally injured and uninjured L. monocytogenes over the storage life of naturally cured RTE ham and frankfurters. Such concerns create a clear need for a combination of antimicrobial hurdles to be investigated and, eventually, implemented in order to fully address L. monocytogenes in RTE meat and poultry products. Investigating the use of commercially available natural antimicrobial ingredients in combination with post-lethality interventions that are currently allowed for use under the highly restrictive natural and organic meat and poultry manufacturing practices as a means to inhibit the recovery and growth of L. monocytogenes in naturally cured RTE ham was, therefore, the focus of our work.

Materials and Methods

Manufacture of Hams

Thirteen ham treatments (twelve experimental and one control treatment) were manufactured to evaluate the inhibition of L. monocytogenes recovery and growth by natural antimicrobial ingredients used in combination with post-lethality interventions. Hams were produced at the Iowa State University Meat Laboratory with inside ham muscles, using formulations found in Table 1. The ham muscles were obtained from a
local processor and frozen prior to use to ensure uniformity of raw materials. The ham muscles were tempered to -2°C and then were coarse ground through a plate with 9.53-mm-diameter holes (Biro MFG Co., Marblehead, OH). Nonmeat ingredients (Table 1) were added and mixed with ground ham muscles at 26 rpm for 2 min using a double action mixer (Leland Southwest, Fort Worth, TX). Pre-converted celery powder (VegStable 504, Florida Food Products, Inc., Eustis, FL) was used as the natural source of nitrite. Based on analysis, VegStable 504 is 1.5% (wt/wt) nitrite. All products were formulated to contain 50 mg/kg ingoing natural nitrite. Mixed samples were then reground using a plate with 6.35-mm-diameter holes and stuffed into a 50-mm-diameter impermeable plastic casing (Nalobar APM 45, Kalle USA, Gurnee, IL) using a rotary vane vacuum-filling machine (RS 1040 C, Risco USA Corp., South Eaton, MA). Thorough rinsing with cold water of all of the equipment utilized was conducted after each ham formulation was manufactured so as to avoid cross-contamination between product formulations. All treatments were then placed in a single-truck smokehouse (Maurer, AG, Reichenau, Germany) and heated to an internal temperature of 71.1°C. The hams were then placed in a 0°C cooler overnight to stabilize. The next day, which marked day 0 of the experiment, the hams were sliced into approximately 12.0-mm-thick slices using a hand slicer (SE 12 D, Bizerba, Piscataway, NJ), placed into barrier bags (B2470, Cryovac Sealed Air Corporation, Duncan, SC) with an oxygen transmission rate of 3-6 cc at 4°C (m², 24 hrs atm @ 4°C, 0% RH) and a water vapor transmission rate of 0.5-0.6 g at 38°C (100% RH, 0.6 m², 24 hrs), and vacuum sealed (UV 2100, Multivac, Inc., Kansas City, MO). Hams for phsycochemical analyses were placed in boxes and transferred to a holding cooler in the Iowa State University Meat Laboratory and stored at
4 ± 1°C until analyses were conducted. Hams for microbial analyses were placed in boxes with vacuum packaged ice, transferred to the Iowa State University Food Safety Research Laboratory in the Food Science and Human Nutrition Department for subsequent inoculation, and stored at 4 ± 1°C for the duration of the experiment. Two independent replications were produced.

Mean Weight and Surface Area Calculations

On day 0, a total of five randomly selected slices of ham from the Control, 90MX (Treatments 1-4), DV (Treatments 5-8), and LV1X (Treatments 9-12) ham formulations (Table 1) were weighed and measured \((n = 20\) per replication) so as to obtain representative mean weights and surface area measurements. The surface area \((\text{cm}^2)\) of the ham slices was modeled by the equation of the surface area of a cylinder: \(\text{area} = 2\pi r^2 + 2\pi rh\), where \(\pi = 3.142\), \(r = \text{radius}\), and \(h = \text{height}\). Average weight and surface area measurements would then be used to calculate log CFU per g and octanoic acid (OA) and lauric arginate (LAE) volumes per slice to be used in the study, respectively.

Proximate Analysis

Proximate analysis was conducted for moisture, fat, and protein of homogenized Control, 90MX, DV, and LV1X formulations (Table 1) on day 0 using AOAC methods 950.46, 960.63, and 992.15, respectively \((1, 2, 3)\). Samples were prepared in duplicate for each ham formulation.
pH

Product pH was measured by placing a pH probe (FC20, Hanna Instruments, Woonsocket, RI) into homogenized (KFP715 food processor, Kitchenaid, St. Joseph, MI) samples from Control, 90MX, DV, and LV1X formulations (Table 1) that were prepared by first blending the ground ham with distilled, de-ionized water in a 1:9 ratio, and then measuring the pH with a pH/ion meter (Accumet 925 pH/ion meter, Fisher Scientific). Calibration was conducted using phosphate buffers of pH 4.0, 7.0, and 10.0. Duplicate readings were taken for each product formulation on day 0.

Water Activity

Available moisture was determined using a water activity meter (AquaLab 4TE, Decagon Devices Inc., Pullman, WA). Samples were cut into small pieces, placed in disposable sample cups, covered, and allowed to equilibrate to room temperature (5-10 min). Measurements were obtained on day 0 and were performed in duplicate for Control, 90MX, DV, and LV1X formulations (Table 1). Calibration was performed using 1.00 and 0.76 sodium chloride water activity standards.

Residual Nitrite Analysis

Residual nitrite concentration was determined utilizing AOAC method 973.31 (4). Samples from each treatment were frozen at -20 ± 1°C on day 0 and evaluated at a later date in duplicate.
Natural Antimicrobial Ingredients

Three commercially available natural antimicrobial ingredients were evaluated in this study; 1.0% cranberry powder (90MX; Ocean Spray International, Middleboro, MA), 1.0% vinegar (DV; WTI Ingredients, Inc., Jefferson, GA), and 2.5% vinegar and lemon juice concentrate (LV1X; WTI Ingredients, Inc., Jefferson, GA) (wt/wt). Each ingredient was added at levels recommended by the respective supplier (Table 1). The pH of 10% solutions (w/v) of the 90MX, DV, and LV1X ingredients were 3.89, 5.87, and 5.57, respectively.

Preparation of Inoculum

*L. monocytogenes* strains Scott A NADC 2045 serotype 4b, H7969 serotype 4b, H7962 serotype 4b, H7596 serotype 4b, and H7762 serotype 4b were obtained from the Iowa State University Food Safety Research Laboratory in the Food Science and Human Nutrition Department. Each strain was cultured separately in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) (Difco, Becton Dickinson, Sparks, MD) for 24 h at 35°C. A minimum of two consecutive 24-h transfers of each strain to fresh TSBYE (35°C) were performed prior to each experiment. Aliquots (6.0-ml) from each of the five strains were then transferred into a sterile centrifuge tube. The bacterial cells were harvested by centrifugation (10 min at 10,000 rpm and 4°C) in a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT). The supernatant was discarded and the pelleted cells were resuspended in 30.0 ml of sterile buffered peptone water (BPW) (Difco, Becton Dickinson). The total concentration of the five-strain cocktail was approximately $10^9$ colony forming units (CFU) per ml based on the aerobic
plate counts of the washed cell suspension. Two serial dilutions (100-fold each) of the cell suspension were prepared in BPW to give a final inoculum concentration of $10^5$ CFU per ml. This diluted five-strain cocktail was used to inoculate samples of ham.

**Sample Inoculation**

While in the Food Safety Research Laboratory, each packaged sample was reopened and the surface of the product was aseptically inoculated with a 0.2-ml aliquot of the diluted five-strain cocktail. The cell concentration at inoculation was approximately $10^3$ CFU per gram. The bags were then vacuum sealed using a model A300/52 vacuum packaging machine (Multivac, Inc.) and stored at 4 ± 1°C for the duration of the experiment.

**Post-Lethality Interventions**

Four post-lethal interventions were evaluated in this study; high hydrostatic pressure (HHP), octanoic acid (OA), laurie arginate (LAE), and post-packaging thermal treatment (PPTT). Ham slices from each formulation were randomly assigned to these post-lethal interventions. All post-lethal interventions were applied to the product within two hours after inoculation on day 0 of the study.

HHP was evaluated under the following sets of parameters; 400 MPa, 4 min dwell time at 12 ± 2°C initial fluid temperature. Hams were transported on ice to the Food Safety Research Laboratory for inoculation and then to the High Pressure Processing Laboratory at the Iowa State University Food Science and Human Nutrition Department and subjected to HHP treatment using a FOOD-LAB 900 Plunger Press system (Standsted Fluid Power Ltd., Standsted, UK). The pressurization fluid was a 50.0%
propylene glycol (GWT Koilguard; GWT Global Water Technology, Inc., Indianapolis, IN) and 50.0% water solution (v/v). The average rate of pressurization was 350 MPa per min and depressurization occurred within 7 s.

OA (Octa-Gone; EcoLab, Inc., Eagan, MN) was applied according to the supplier’s recommendations. According to the manufacturer, Octa-Gone contains approximately 3.6% octanoic acid (v/v). A 23.4% Octa-Gone and 76.6% water solution (v/v) was prepared by mixing Octa-Gone with sterile de-ionized water at 4 ± 1°C. Based on average surface area measurements obtained per replication as previously described, the OA solution was aseptically dispensed into the bag containing the ham slice (0.0186 ml per cm²) and vacuum sealed. Vacuum sealing evenly distributed the solution throughout the bag. The pH of the working solution of Octa-Gone was 3.01.

LAE (Protect-M; Purac America, Lincolnshire, IL) was applied according to the supplier’s recommendations. According to the manufacturer, Protect-M contains approximately 10.0% lauric arginate (v/v). A 2.5% Protect-M and 97.5% water solution (v/v) was prepared by mixing Protect-M with sterile de-ionized water at 4 ± 1°C. Based on average surface area measurements obtained per replication as previously described, the LAE solution was aseptically dispensed into the bag containing the ham slice (0.007192 ml per cm²) and vacuum sealed. Vacuum sealing evenly distributed the solution throughout the bag. The pH of the working solution of Protect-M was 3.92.

PPTT was conducted by immersing packages of ham in water at 71.0 ± 1.0°C for 30 s using a water bath (Isotemp-228, Fisher Scientific). Seven packages were immersed as a group so as not to affect water temperature by more than 1.0°C. Water temperature was
monitored throughout the process. Packages were held in heated water for the prescribed length of time and then placed on ice immediately after to chill before placement in refrigerated storage.

**Microbial Analysis**

Microbial analysis of ham samples for viable *L. monocytogenes* was conducted on days 1, 14, 28, 42, 56, 70, 84, and 98. On the appropriate day, two packages for each treatment were removed from the holding cooler, opened aseptically, and their contents placed inside a sterile Whirl-Pak stomacher bag (Nasco, Ft. Atkinson, WI). Sample preparation was performed by adding 50.0 ml of sterile BPW to each bag, closing the bag so as to form a “pillow,” and then shaking the sample for approximately 30 s. The wash solution from each ham sample was then serially diluted (10-fold) in BPW to obtain predetermined dilutions of the samples according to the sampling day. An aliquot of 1.0 ml (for $10^0$ dilution, divided into three ~0.33-ml aliquots plated on three separate plates) or 0.1 ml of the appropriate dilution was surfaced plated on modified Oxford medium base (MOX) supplemented with modified Oxford antimicrobial supplement (Difco, Becton Dickinson). The dry ingredients used to manufacture the MOX were 42.5 g of Columbia agar base (Difco, Becton Dickinson), 15.0 g of lithium chloride (Difco, Becton Dickinson), 1.0 g of esculin hydrate (Sigma-Aldrich, St. Louis, MO), and 0.5 g of ferric ammonium citrate (Difco, Becton Dickinson) per liter of de-ionized water. Additionally, an aliquot of 1.0 ml (for $10^0$ dilution, divided into three ~0.33-ml aliquots plated on three separate plates) or 0.1 ml of the appropriate dilution was surfaced plated on thin agar layer medium base (TAL) that was made according to Kang and Fung (12) with some modifications. MOX was made as previously described. Then, never more than 48 hr
before sampling was to be conducted, MOX plates to be made into TAL were aseptically overlaid with 7.0 ml of sterile tryptic soy agar (Difco, Becton Dickinson) held at 55°C to facilitate the even distribution of the molten agar. Each sample was plated in duplicate. Plates used for microbial analyses were sterile and 55 mm in diameter (Fisher Scientific, Waltham, MA). All inoculated agar plates were incubated in an inverted position at 35°C for 48 hr, after which time they were removed from the incubator and colonies typical of \( L. \text{monocytogenes} \) were enumerated. The counts (CFU per ml) were averaged and then converted to log CFU per g using the average weight of the sliced ham from the two replications of the experiment (\( n = 40 \)). The detection limit of our sampling protocols was \( \geq 0.30 \) log CFU per g based on a sample weight of 25.0 g.

**Statistical Analysis**

The overall design of the experiment was a factorial design. The generalized linear mixed models (GLMMIX) procedure of Statistical Analysis System (version 9.3, SAS Institute Inc., Cary, NC) was used for statistical analysis. \( L. \text{monocytogenes} \) growth data were analyzed for treatment effects within day. Day and treatment x day interactions were also analyzed. The effects of each post-lethality intervention were analyzed separately for each natural antimicrobial ingredient studied. Likewise, the effects of each natural antimicrobial ingredient were analyzed separately for each post-lethality intervention studied. Where significant effects (\( P < 0.05 \)) were found, pair-wise comparisons between the least squares means were computed for each day using Tukey’s honestly significant difference adjustment.
Results and Discussion

Mean Surface Area and Weight Results

The mean weight of the ham slices was 24.57 ± 0.64 g, while the mean diameter, height, and surface area were 4.72 ± 0.06 cm, 1.31 ± 0.01 cm, and 54.51 ± 1.13 cm², respectively (data not shown and n = 40 for all measurements). These mean ham slice dimensions resulted in LAE and OA treatment volumes of 0.39 and 1.01 ml per package, respectively, after dosages of each compound were calculated according to the respective manufacturer’s recommendations as previously described. These dosages resulted in LAE and OA treatment concentrations of 39.82 and 343.03 mg/kg, respectively.

Physicochemical Traits

Physicochemical characteristics of the hams can be found in Table 2. All treatments exhibited significantly lower $a_w$ values than the Control treatment ($P < 0.05$). The DV and LV1X treatments, in turn, resulted in significantly lower $a_w$ values when compared to the 90MX treatment ($P < 0.05$). Final product pH was also affected by natural antimicrobial compound added. The pH of the Control treatment was not significantly different from that of the DV treatment ($P > 0.05$), but did significantly differ from both the LV1X and the 90MX treatments ($P < 0.05$). These differences in pH may stem from the presence of acidic compounds in the natural antimicrobial compounds utilized. Cranberry has been reported to contain phenolic acids and exhibit a high titratable acidity (14). Xi and others obtained similar results when using different ingoing levels of cranberry powder in a cooked meat model system (36) and in frankfurters (35). Similarly, the vinegar and vinegar and lemon juice concentrate used in this study are also likely
reservoirs of phenolic and other acidic compounds, such as acetic and citric acid, expected to cause the observed lower pH in products made with those ingredients. No significant differences in protein % and moisture % were found between the treatments ($P > 0.05$). Fat %, however, was significantly lower in the 90MX treatment compared to both the DV and LV1X treatments ($P < 0.05$). These slight differences in the proximate composition of the ham formulations were not expected to influence the results of this study.

The residual nitrite concentration found in the 90MX treatment was found to be lower ($P < 0.05$) than that of the Control and DV treatments. No significant differences between all other treatments were detected ($P > 0.05$). Although all ham formulations were manufactured with 50 mg/kg natural nitrite on an ingoing basis, the highest residual nitrite concentration observed in all of the treatments on day 0 of the study was 36.01 mg/kg (Control treatment). This indicates that part of the ingoing nitrite was depleted in curing and other reactions that took place during product manufacture. Honikel (9) reported that as much as 65% of the ingoing nitrite can be depleted during product manufacture. Similarly, Xi and others (35) reported that as much as 75% of the ingoing nitrite can be depleted during the manufacture of frankfurters. Factors such as product pH, cooking temperature, and reducing agents used have been shown to affect residual nitrite concentrations in meat systems (7). Sebranek (25) indicated that decreases in pH as small as 0.2 units during product manufacture can result in a doubling of the rate at which curing reactions occur. Thus, the significant ($P < 0.05$) decrease in pH brought about by the natural antimicrobial ingredients used in this study, especially cranberry powder, was expected to influence residual nitrite concentrations. These results indicate that the acidic
nature of the natural antimicrobials investigated in this study inversely affects residual nitrite concentrations in RTE ham under the conditions of our study.

**Viable *Listeria monocytogenes***

The growth mediums used, MOX and TAL, did not significantly differ \((P > 0.05)\) within treatment on any given day, indicating that, under the conditions of this study, the use of the TAL technique offers limited advantages compared to using a traditional medium such as MOX. Thus, the discussion about viable *L. monocytogenes* numbers as affected by treatment is limited to those results obtained using MOX.

The effects of using cranberry powder in combination with post-lethality interventions on viable *L. monocytogenes* numbers on MOX (Table 3 and Figure 1) and TAL (Table 10 and Figure 8) media were monitored throughout the duration of the study. Results showed that the Control treatment experienced a significant \((P < 0.05)\) increase in viable *L. monocytogenes* numbers by day 14. Similarly, the PPTT and 90MX treatment also exhibited a significant \((P < 0.05)\) increase in viable *L. monocytogenes* numbers after day 14 of storage, results which indicate that the combination of PPTT and cranberry powder did not offer inhibitory or bacteriostatic effects on the growth of *L. monocytogenes* under the conditions of this study. In contrast, no significant increase in viable *L. monocytogenes* numbers was observed on day 14 in the HHP, OA, or LAE treatments \((P > 0.05)\). However, by day 84 of the study all treatments had experienced significant \((P < 0.05)\) increases in *L. monocytogenes* numbers. These results indicated that, when combined with the selected post-lethality interventions and under the conditions of this study, the addition of 1.0% cranberry powder as part of the product
formulation did not offer an inhibitory effect on the recovery and growth of *L. monocytogenes*. Previous work in our laboratory demonstrated that adding 1.0% cranberry powder (wt/wt) did not exert inhibitory effects on the growth of *L. monocytogenes* on naturally cured RTE ham and frankfurters manufactured and stored under similar conditions. Similarly, Lin and others (16) concluded that cranberry extract alone was not inhibitory of *L. monocytogenes* growth upon refrigerated storage of both inoculated fish and beef slices. Additionally, results by Xi and others (36) indicated that cranberry powder, also when used at a level of 1% (wt/wt), did not inhibit the growth of *L. monocytogenes* completely.

No significant increases in viable *L. monocytogenes* numbers were observed throughout the duration of the study when DV was combined with PPTT, HHP, OA, or LAE (*P* > 0.05), which indicates that this ingredient, when combined with the selected post-lethality interventions and under the conditions of this study, exerts inhibitory effects on the recovery and growth of *L. monocytogenes* (Table 4 and Figure 2 for results obtained using MOX and Table 11 and Figure 9 for results obtained using TAL). In fact, when combined with PPTT, which did not achieve a significant reduction in initial viable *L. monocytogenes* numbers (*P* > 0.05), DV was able to inhibit the growth of *L. monocytogenes*. Previous work in our laboratory demonstrated that adding 1.0% vinegar (wt/wt) exerted inhibitory effects on the growth of *L. monocytogenes* on naturally cured RTE ham and frankfurters manufactured and stored under similar conditions. However, additional research on the subject is needed as literature on these effects, which are likely to vary based on product characteristics, is limited.
The effects of using vinegar and lemon juice concentrate in combination with post-lethality interventions on viable \textit{L. monocytogenes} numbers on MOX (Table 5 and Figure 3) and TAL (Table 12 and Figure 10) media were monitored throughout the duration of the study. No significant increases in viable \textit{L. monocytogenes} numbers were observed throughout the duration of the study in the treatments where LV1X was combined with HHP, OA, and LAE ($P > 0.05$). The LV1X and PPTT treatment, on the other hand, did experience a significant ($P < 0.05$) increase in \textit{L. monocytogenes} numbers, but this increase occurred on day 98 of the study when compared to day 1. These results allow us to conclude that, under the conditions of this study, vinegar and lemon juice concentrate exerts bacteriostatic effects on the recovery and growth of \textit{L. monocytogenes} when combined with HHP, OA, and LAE. Previous work in our laboratory demonstrated that adding 2.5\% vinegar and lemon juice concentrate (wt/wt) exhibited similar inhibitory effects on the growth of \textit{L. monocytogenes} on naturally cured RTE ham and frankfurters manufactured and stored under similar conditions. Research on similar buffered vinegar and buffered vinegar and lemon juice concentrate products and their inhibitory effects on \textit{C. perfringens} in ground turkey roast (33) and roast beef (15) concluded that these ingredients exhibit inhibitory properties against that microorganism. However, additional research on the subject is needed as literature on these effects, which are likely to vary based on product characteristics and microorganism of concern, is limited.

HHP treatment used in combination with all of the natural antimicrobial ingredients studied resulted in significant ($P < 0.05$) reductions in viable \textit{L. monocytogenes} numbers on day 1 when compared to the Control treatment (Table 6 and Figure 4 for results obtained using MOX and Table 13 and Figure 11 for results obtained using TAL). More
specifically, HHP treatment resulted in 2.25, 1.99, and 1.67 log CFU per g reductions ($P < 0.05$) on day 1 when combined with cranberry powder, vinegar and lemon juice concentrate, and vinegar, respectively, and compared to the Control treatment. The differences in log CFU per g reductions observed on day 1 in the different treatments subjected to HHP, however, were not significant ($P > 0.05$), indicating that the three antimicrobial ingredients used did not influence the bactericidal properties of the HHP treatment utilized and under the conditions of this study. These results demonstrate the bactericidal properties of HHP against *L. monocytogenes*. However, only when combined with vinegar or vinegar and lemon juice concentrate was the initial reduction in viable *L. monocytogenes* numbers sustained throughout the duration of the study.

Damage to the cell membrane seems to be the main mode of action for HHP as the damage this technology causes to bacterial cell membranes can be extensive and often results in cell death (10, 18). Changes in membrane permeability, scarring around the cell wall, separation of the cell wall from the membrane, protein denaturation, as well as damage to transport systems have been reported in HHP treated microbial populations (20, 22). Thus, it is likely that the observed bacteriostatic effect observed in the HHP treatments that combined the use of this technology with ingredients such as vinegar or vinegar and lemon juice concentrate was a result of the migration of growth inhibitory compounds present in these ingredients into the bacterial cells. As a result, the use of HHP in combination with vinegar or vinegar and lemon juice concentrate, under the conditions of this study, represents a promising multiple-hurdle approach at not only addressing the potential presence of *L. monocytogenes* in processed meats, but also at
inhibiting the potential recovery and growth of those cells that remain viable over the refrigerated storage of the products.

Combining OA with the natural antimicrobial ingredients evaluated in this study (Table 7 and Figure 5 for results obtained using MOX and Table 14 and Figure 12 for results obtained using TAL) yielded similar patterns to those obtained when combining HHP with said ingredients in terms of viable _L. monocytogenes_ numbers observed throughout the study. Significant (_P_ < 0.05) reductions in viable _L. monocytogenes_ numbers were observed when OA was combined with all of the natural antimicrobial ingredients evaluated after day 1 and compared to the Control treatment. More specifically, on day 1 of the study and compared to the Control treatment, log CFU per g reductions were 2.67, 2.52, and 2.33 when OA was combined with 90MX, DV, and LV1X, respectively. The differences in log CFU per g reductions observed on day 1 in the different treatments subjected to OA, however, were not significant (_P_ > 0.05), indicating that the three antimicrobial ingredients used did not influence the bactericidal properties of the OA treatment utilized and under the conditions of this study. Burnett and others (6) concluded that octanoic acid solutions acidified to pH 2.0 or 4.0 and applied to RTE meat and poultry resulted in _L. monocytogenes_ log reductions ranging from 0.85 to 2.89 log CFU per sample in the different RTE products following 24 ± 4 h of storage at 5°C. It has been reported that the main mechanism by which medium and short chain fatty acids achieve microbial inactivation is through the diffusion of undisociated acids across the bacterial cells and the subsequent intracellular acidification (30). Thus, it is likely that the bactericidal effects of OA on _L. monocytogenes_ follow that mechanism.
Sustained inhibition of *L. monocytogenes* recovery and growth was exhibited by treatments that combined OA with DV or LV1X (*P* < 0.05) but not with 90MX (*P* > 0.05). Previous work in our laboratory showed that OA, when applied by itself to naturally cured frankfurters and RTE ham using similar protocols, exerted an initial bactericidal effect on *L. monocytogenes* but failed to inhibit the organism’s recovery and growth over the refrigerated life of the products. Thus, the use of OA in combination with vinegar or vinegar and lemon juice concentrate, under the conditions of this study, represents a promising multiple-hurdle approach at not only addressing the potential presence of *L. monocytogenes* in processed meats, but also at inhibiting the potential recovery and growth of those cells that remain viable over the refrigerated storage of the products.

The effects of using lauric arginate in combination with natural antimicrobial ingredients on viable *L. monocytogenes* numbers on MOX (Table 8 and Figure 6) and TAL (Table 15 and Figure 13) were monitored throughout the duration of the experiment. On day 1 of the study, LAE in combination with DV, 90MX, and LV1X resulted in 2.67, 2.37, and 2.16 log CFU per g reductions, respectively, in viable *L. monocytogenes* numbers (*P* < 0.05). These reductions were not different (*P* > 0.05) based on antimicrobial ingredients used as part of the product formulation. These results demonstrate that lauric arginate, under the conditions of this study, exerts bactericidal effects on *L. monocytogenes*. Similar to patterns observed when combining HHP and OA with the specified antimicrobial ingredients, sustained inhibition of the recovery and growth of *L. monocytogenes* was only observed when LAE was combined with the DV or LV1X ingredients. When LAE was used in combination with the 90MX ingredient, on
the other hand, significant \((P < 0.05)\) increases in viable \(L.\ monocytogenes\) numbers were observed after day 14 of the study. These findings are in agreement with those of Porto-Fett and others \((21)\), as these authors discovered that only when used in combination with lactate or diacetate will lauric arginate exert a bacteriostatic effect on the pathogen under storage temperatures of \(4^\circ\)C for 120 days. Similar results were obtained by Luchansky and others \((22)\) when they researched the effects of lauric arginate on the growth of \(L.\ monocytogenes\) on hams. Thus, combining a LAE post-lethality intervention with vinegar or vinegar and lemon juice concentrate, much like combining HHP and OA post-lethality interventions with those same natural antimicrobial ingredients as described in this study, represents another promising multiple-hurdle approach at not only addressing the potential presence of \(L.\ monocytogenes\) in processed meats, but also at inhibiting the potential recovery and growth of those cells that remain viable over the refrigerated storage of the products.

On day 1 of the study, no significant reduction in viable \(L.\ monocytogenes\) numbers was observed in any of the products that had PPTT applied to them \((P > 0.05)\) when compared to the Control treatment (Table 9 and Figure 7 for results obtained using MOX and Table 16 and Figure 14 for results obtained using TAL). These results suggest that, under the parameters and conditions implemented in this study, the use of PPTT did not exert a bactericidal effect on \(L.\ monocytogenes\). Results of our study contrast those obtained by Chen and others \((8)\), as these authors concluded that a post-packaging thermal treatment of \(71 \pm 1^\circ\)C for 30 sec would result in a 1.4 log CFU per g reduction in \(L.\ monocytogenes\) numbers on 1-link packages of frankfurters when using a 3.4 log CFU per g initial inoculation level. Differing product characteristics such as salt concentration,
pH, $a_w$, among others, may have accounted for the discrepancies in results. Furthermore, compared to viable numbers of *L. monocytogenes* observed on day 1 of the study in the respective PPTT treatments, significant ($P < 0.05$) inhibition of the growth of *L. monocytogenes* throughout the entire duration of the study was only observed in products formulated with DV. These results highlight the bacteriostatic effect of this ingredient on *L. monocytogenes* under the conditions of this study.

In conclusion, as evidenced by our results, the use of high hydrostatic pressure, octanoic acid, or lauric arginate post-lethality interventions in combination with vinegar or vinegar and lemon juice concentrate, under the conditions of this study, represent promising multiple-hurdle approaches for not only addressing the potential presence of *L. monocytogenes* in naturally cured RTE ham, but also at inhibiting the potential recovery and growth of those cells that remain viable over the refrigerated storage of the products. The combinations of these hurdles represent effective options that could be instituted by manufacturers of organic and natural processed meat and poultry products in their *L. monocytogenes* control plans. Due to the fact that limited literature exists on the use of these natural antimicrobial ingredients in combination with the aforementioned post-lethality interventions, nevertheless, further research should be conducted on their inhibitory properties against *L. monocytogenes* in processed meats.

**Acknowledgements**

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Kalle USA. Special thanks to Devin Maurer and Daniel Fortin for the invaluable contributions made throughout the conduction of these experiments.
References


germination and outgrowth by buffered vinegar and lemon juice concentrate during chilling of ground turkey roast containing minimal ingredients. *J. Food Prot.* 73:470–476.


### TABLE 1. Naturally cured RTE ham formulations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ham (kg)</th>
<th>Water (kg)</th>
<th>Salt (kg)</th>
<th>Sugar (kg)</th>
<th>Pre-converted Celery Powder&lt;sup&gt;a&lt;/sup&gt; (g)</th>
<th>Antimicrobial A&lt;sup&gt;b&lt;/sup&gt; (g)</th>
<th>Antimicrobial B&lt;sup&gt;c&lt;/sup&gt; (g)</th>
<th>Antimicrobial C&lt;sup&gt;d&lt;/sup&gt; (g)</th>
<th>Post-Lethality Intervention</th>
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<td>3.66</td>
<td>0.50</td>
<td>0.30</td>
<td>74.84</td>
<td>-</td>
<td>-</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td>PTTT&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Vegstable 504 (Natural Nitrite; Florida Food Products, Inc., Eustis, FL).

<sup>b</sup> 90MX (Cranberry Powder; Ocean Spray International, Middleboro, MA).

<sup>c</sup> DV (Vinegar; WTI Ingredients, Inc., Jefferson, GA).

<sup>d</sup> LV1X (Vinegar and Lemon Juice Concentrate; WTI Ingredients, Inc., Jefferson, GA).

<sup>e</sup> High Hydrostatic Pressure (400 MPa, 4 min dwell time at 12 ± 2°C).

<sup>f</sup> Octa-Gone (Octanoic Acid; EcoLab, Inc., Eagan, MN).

<sup>g</sup> Protect-M (Lauric Arginate; Purac America, Lincolnshire, IL).

<sup>h</sup> Post-Packaging Thermal Treatment (71.0 ± 1°C for 30 s).
TABLE 2. Effect of natural antimicrobial ingredients on physicochemical properties of naturally cured RTE ham

<table>
<thead>
<tr>
<th>Treatment</th>
<th>a&lt;sub&gt;w&lt;/sub&gt;</th>
<th>pH</th>
<th>Fat %</th>
<th>Moisture %</th>
<th>Protein %</th>
<th>Residual Nitrite (mg/kg)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.9819&lt;sup&gt;A&lt;/sup&gt;</td>
<td>6.35&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.96&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>75.84</td>
<td>18.09</td>
<td>36.01&lt;sup&gt;BC&lt;/sup&gt;</td>
</tr>
<tr>
<td>90MX</td>
<td>0.9793&lt;sup&gt;B&lt;/sup&gt;</td>
<td>6.05&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.58&lt;sup&gt;A&lt;/sup&gt;</td>
<td>75.82</td>
<td>17.95</td>
<td>31.32&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>DV</td>
<td>0.9759&lt;sup&gt;C&lt;/sup&gt;</td>
<td>6.24&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>2.26&lt;sup&gt;B&lt;/sup&gt;</td>
<td>75.30</td>
<td>17.88</td>
<td>35.36&lt;sup&gt;BC&lt;/sup&gt;</td>
</tr>
<tr>
<td>LV1X</td>
<td>0.9772&lt;sup&gt;C&lt;/sup&gt;</td>
<td>6.18&lt;sup&gt;AC&lt;/sup&gt;</td>
<td>2.32&lt;sup&gt;B&lt;/sup&gt;</td>
<td>74.93</td>
<td>18.02</td>
<td>33.56&lt;sup&gt;AC&lt;/sup&gt;</td>
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<td>0.29</td>
<td>0.20</td>
<td>1.11</td>
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<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different (P < 0.05).

<sup>b</sup> Control, naturally cured control; 90MX, cranberry powder; DV, vinegar; LV1X, vinegar and lemon juice concentrate.

<sup>c</sup> Standard error of the differences of least squares means.
**TABLE 3. Effect of cranberry powder in combination with post-lethality interventions on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>2.75&lt;sup&gt;B,Z&lt;/sup&gt;</td>
<td>5.22&lt;sup&gt;D,Y&lt;/sup&gt;</td>
<td>6.67&lt;sup&gt;D,X&lt;/sup&gt;</td>
<td>7.52&lt;sup&gt;H,X&lt;/sup&gt;</td>
<td>7.29&lt;sup&gt;B,X&lt;/sup&gt;</td>
<td>7.14&lt;sup&gt;B,X&lt;/sup&gt;</td>
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<td>7.20&lt;sup&gt;H,X&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0.50&lt;sup&gt;A,Z&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;A,Z&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;A,Z&lt;/sup&gt;</td>
<td>ND</td>
<td>0.61&lt;sup&gt;A,Z&lt;/sup&gt;</td>
<td>3.02&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>4.75&lt;sup&gt;A,X&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.08&lt;sup&gt;A,Z&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;B,Z&lt;/sup&gt;</td>
<td>0.68&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>1.36&lt;sup&gt;C,Y&lt;/sup&gt;</td>
<td>2.54&lt;sup&gt;B,X&lt;/sup&gt;</td>
<td>3.60&lt;sup&gt;C,W&lt;/sup&gt;</td>
<td>4.71&lt;sup&gt;C,V&lt;/sup&gt;</td>
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<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through D) are significantly different ($P < 0.05$). Within a row, means with different superscripts (U through Z) are significantly different ($P < 0.05$).

<sup>b</sup> Control, naturally cured control; 1, 90MX and HHP; 2, 90MX and OA; 3, 90MX and LAE; 4, 90MX and PPTT.

<sup>c</sup> Not detected.

<sup>d</sup> Standard error of the differences of least squares means.
FIGURE 1. Effect of cranberry powder in combination with post-lethality interventions on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C

Treatments: Control, naturally cured control; 1, 90MX and HHP; 2, 90MX and OA; 3, 90MX and LAE; 4, 90MX and PPTT.
TABLE 4. *Effect of vinegar in combination with post-lethality interventions on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C* ^a^  

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 42</th>
<th>Day 56</th>
<th>Day 70</th>
<th>Day 84</th>
<th>Day 98</th>
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<tr>
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<td>2.75^CZ</td>
<td>5.22^CY</td>
<td>6.67^CX</td>
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<tr>
<td>6</td>
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^a^ Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different (*P* < 0.05). Within a row, means with different superscripts (W through Z) are significantly different (*P* < 0.05).

^b^ Control, naturally cured control; 5, DV and HHP; 6, DV and OA; 7, DV and LAE; 8, DV and PPTT.

^c^ Not detected.

^d^ Standard error of the differences of least squares means.
FIGURE 2. Effect of vinegar in combination with post-lethality interventions on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C.

Treatments: Control, naturally cured control; 5, DV and HHP; 6, DV and OA; 7, DV and LAE; 8, DV and PPTT.
TABLE 5. Effect of vinegar and lemon juice concentrate in combination with post-lethality interventions on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C\(^a\)

<table>
<thead>
<tr>
<th>Treatment(^b)</th>
<th>Day 1</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 42</th>
<th>Day 56</th>
<th>Day 70</th>
<th>Day 84</th>
<th>Day 98</th>
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<tr>
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<td>2.75(^{B, Z})</td>
<td>5.22(^{C, Y})</td>
<td>6.67(^{C, X})</td>
<td>7.52(^{C, X})</td>
<td>7.29(^{C, X})</td>
<td>7.14(^{C, X})</td>
<td>7.07(^{C, X})</td>
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<td>9</td>
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<td>0.33(^{A})</td>
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<td>0.25(^{A})</td>
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<td>2.35(^{H, L})</td>
<td>2.40(^{H, L})</td>
<td>2.58(^{H, L})</td>
<td>2.86(^{H, YZ})</td>
<td>3.23(^{H, YZ})</td>
<td>3.41(^{H, YZ})</td>
<td>4.01(^{H, Y})</td>
</tr>
<tr>
<td>SE(^d)</td>
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<td></td>
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\(^a\) Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different (\(P < 0.05\)). Within a row, means with different superscripts (X through Z) are significantly different (\(P < 0.05\)).

\(^b\) Control, naturally cured control; 9, LV1X and HHP; 10, LV1X and OA; 11, LV1X and LAE; 12, LV1X and PPTT.

\(^c\) Not detected.

\(^d\) Standard error of the differences of least squares means.
FIGURE 3. Effect of vinegar and lemon juice concentrate in combination with post-lethality interventions on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C

Treatments: Control, naturally cured control; 9, LV1X and HHP; 10, LV1X and OA; 11, LV1X and LAE; 12, LV1X and PPTT.
TABLE 6. Effect of high hydrostatic pressure treatment in combination with natural antimicrobials on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C<sup>a</sup>

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Day 1</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 42</th>
<th>Day 56</th>
<th>Day 70</th>
<th>Day 84</th>
<th>Day 98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.75&lt;sup&gt;A,Z&lt;/sup&gt;</td>
<td>5.22&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>6.67&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.52&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.29&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.14&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.07&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.20&lt;sup&gt;C,X&lt;/sup&gt;</td>
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<td>1</td>
<td>0.50&lt;sup&gt;B,Z&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;B,Z&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;B,Z&lt;/sup&gt;</td>
<td>ND</td>
<td>0.61&lt;sup&gt;B,Z&lt;/sup&gt;</td>
<td>3.02&lt;sup&gt;B,Y&lt;/sup&gt;</td>
<td>4.68&lt;sup&gt;A,X&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>1.08&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.30&lt;sup&gt;B&lt;/sup&gt;</td>
<td>ND</td>
<td>0.08&lt;sup&gt;B&lt;/sup&gt;</td>
<td>ND</td>
<td>0.23&lt;sup&gt;B&lt;/sup&gt;</td>
<td>ND</td>
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<td>0.33&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.08&lt;sup&gt;B&lt;/sup&gt;</td>
<td>ND</td>
<td>0.15&lt;sup&gt;B&lt;/sup&gt;</td>
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<td>ND</td>
<td>0.65&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>SE&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different ($P < 0.05$). Within a row, means with different superscripts (X through Z) are significantly different ($P < 0.05$).

<sup>b</sup> Control, naturally cured control; 1, HHP and 90MX; 5, HHP and DV; 9, HHP and LV1X.

<sup>c</sup> Not detected.

<sup>d</sup> Standard error of the differences of least squares means.
FIGURE 4. Effect of high hydrostatic pressure treatment in combination with natural antimicrobials on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C.

Treatments: Control, naturally cured control; 1, HHP and 90MX; 5, HHP and DV; 9, HHP and LV1X.
TABLE 7. Effect of octanoic acid treatment in combination with natural antimicrobials on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C<sup>a</sup>

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>2.75&lt;sup&gt;H,Z&lt;/sup&gt;</td>
<td>5.22&lt;sup&gt;H,Y&lt;/sup&gt;</td>
<td>6.67&lt;sup&gt;H,X&lt;/sup&gt;</td>
<td>7.52&lt;sup&gt;C,X&lt;/sup&gt;</td>
<td>7.29&lt;sup&gt;C,X&lt;/sup&gt;</td>
<td>7.14&lt;sup&gt;C,X&lt;/sup&gt;</td>
<td>7.07&lt;sup&gt;C,X&lt;/sup&gt;</td>
<td>7.20&lt;sup&gt;C,X&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>0.08&lt;sup&gt;A,Z&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;A,Z&lt;/sup&gt;</td>
<td>0.68&lt;sup&gt;A,YZ&lt;/sup&gt;</td>
<td>1.36&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>2.54&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>3.60&lt;sup&gt;A,X&lt;/sup&gt;</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>0.23&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.27&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.38&lt;sup&gt;H&lt;/sup&gt;</td>
<td>0.50&lt;sup&gt;H&lt;/sup&gt;</td>
<td>0.44&lt;sup&gt;H&lt;/sup&gt;</td>
<td>0.53&lt;sup&gt;H&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;H&lt;/sup&gt;</td>
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</tr>
<tr>
<td>10</td>
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<td>0.08&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.35&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.25&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.54&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;B&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>SE&lt;sup&gt;d&lt;/sup&gt;</td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different (P < 0.05). Within a row, means with different superscripts (V through Z) are significantly different (P < 0.05).

<sup>b</sup> Control, naturally cured control; 2, OA and 90MX; 6, OA and DV; 10, OA and LV1X.

<sup>c</sup> Not detected.

<sup>d</sup> Standard error of the differences of least squares means.
FIGURE 5. Effect of octanoic acid treatment in combination with natural antimicrobials on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C.

Treatments: Control, naturally cured control; 2, OA and 90MX; 6, OA and DV; 10, OA and LV1X.
TABLE 8. Effect of lauric arginate treatment in combination with natural antimicrobials on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C\textsuperscript{a}

<table>
<thead>
<tr>
<th>Treatment\textsuperscript{b}</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.75\textsuperscript{A,Z}</td>
<td>5.22\textsuperscript{C,Y}</td>
<td>6.67\textsuperscript{C,X}</td>
<td>7.52\textsuperscript{C,W}</td>
<td>7.29\textsuperscript{A,WX}</td>
<td>7.14\textsuperscript{A,WX}</td>
<td>7.07\textsuperscript{A,WX}</td>
<td>7.20\textsuperscript{A,WX}</td>
</tr>
<tr>
<td>3</td>
<td>0.38\textsuperscript{B,Z}</td>
<td>1.31\textsuperscript{A,Y}</td>
<td>3.28\textsuperscript{A,X}</td>
<td>6.71\textsuperscript{A,W}</td>
<td>7.61\textsuperscript{A,V}</td>
<td>7.71\textsuperscript{A,V}</td>
<td>7.64\textsuperscript{A,V}</td>
<td>7.54\textsuperscript{A,V}</td>
</tr>
<tr>
<td>7</td>
<td>0.08\textsuperscript{B}</td>
<td>0.35\textsuperscript{B}</td>
<td>0.23\textsuperscript{B}</td>
<td>0.15\textsuperscript{B}</td>
<td>0.08\textsuperscript{B}</td>
<td>0.08\textsuperscript{B}</td>
<td>0.20\textsuperscript{B}</td>
<td>ND \textsuperscript{c}</td>
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<td>11</td>
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<td>0.38\textsuperscript{B}</td>
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<td>ND</td>
<td>0.38\textsuperscript{B}</td>
<td>ND</td>
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<td>0.40\textsuperscript{B}</td>
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<tr>
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<td>0.25</td>
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\textsuperscript{a} Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different (\(P < 0.05\)). Within a row, means with different superscripts (V through Z) are significantly different (\(P < 0.05\)).

\textsuperscript{b} Control, naturally cured control; 3, LAE and 90MX; 7, LAE and DV; 11, LAE and LV1X.

\textsuperscript{c} Not detected.

\textsuperscript{d} Standard error of the differences of least squares means.
FIGURE 6. Effect of lauric arginate treatment in combination with natural antimicrobials on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C

Treatments: Control, naturally cured control; 3, LAE and 90MX; 7, LAE and DV; 11, LAE and LV1X.
<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
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</thead>
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<td>5.22&lt;sup&gt;A,Y&lt;/sup&gt;</td>
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<td>7.14&lt;sup&gt;A,X&lt;/sup&gt;</td>
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<td>2.33&lt;sup&gt;H&lt;/sup&gt;</td>
<td>2.30&lt;sup&gt;H&lt;/sup&gt;</td>
<td>2.37&lt;sup&gt;H&lt;/sup&gt;</td>
<td>2.16&lt;sup&gt;H&lt;/sup&gt;</td>
<td>2.13&lt;sup&gt;H&lt;/sup&gt;</td>
<td>2.47&lt;sup&gt;H&lt;/sup&gt;</td>
<td>2.31&lt;sup&gt;H&lt;/sup&gt;</td>
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<td>3.41&lt;sup&gt;C,XY&lt;/sup&gt;</td>
<td>4.01&lt;sup&gt;C,X&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>c</sup> Standard error of the differences of least squares means.

<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different ($P < 0.05$). Within a row, means with different superscripts (X through Z) are significantly different ($P < 0.05$).

<sup>b</sup> Control, naturally cured control; 4, PPTT and 90MX; 8, PPTT and DV; 12, PPTT and LV1X.

TABLE 9. Effect of post-packaging thermal treatment in combination with natural antimicrobials on viable *Listeria monocytogenes* (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C.
FIGURE 7. Effect of post-packaging thermal treatment in combination with natural antimicrobials on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C.

Treatments: Control, naturally cured control; 4, PPTT and 90MX; 8, PPTT and DV; 12, PPTT and LV1X.
TABLE 10. Effect of cranberry powder in combination with post-lethality interventions on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C$^a$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 42</th>
<th>Day 56</th>
<th>Day 70</th>
<th>Day 84</th>
<th>Day 98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.72$^{A,Z}$</td>
<td>5.20$^{A,Y}$</td>
<td>6.72$^{A,X}$</td>
<td>7.56$^{A,X}$</td>
<td>7.30$^{A,X}$</td>
<td>7.13$^{A,X}$</td>
<td>7.04$^{A,X}$</td>
<td>7.21$^{A,X}$</td>
</tr>
<tr>
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<td>0.23$^{B,D,Z}$</td>
<td>0.27$^{B,Z}$</td>
<td>0.15$^{B,Z}$</td>
<td>0.15$^{B,Z}$</td>
<td>0.87$^{B,Z}$</td>
<td>2.99$^{B,Y}$</td>
<td>4.72$^{B,X}$</td>
</tr>
<tr>
<td>2</td>
<td>0.31$^{B,Z}$</td>
<td>0.08$^{D,Z}$</td>
<td>0.68$^{B,YZ}$</td>
<td>1.38$^{D,Y}$</td>
<td>2.56$^{C,X}$</td>
<td>3.61$^{C,W}$</td>
<td>4.71$^{C,V}$</td>
<td>6.21$^{C,U}$</td>
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<tr>
<td>3</td>
<td>0.30$^{B,Z}$</td>
<td>1.02$^{B,Z}$</td>
<td>3.25$^{C,Y}$</td>
<td>6.64$^{A,X}$</td>
<td>7.58$^{A,WX}$</td>
<td>7.68$^{A,W}$</td>
<td>7.84$^{A,W}$</td>
<td>7.51$^{A,WX}$</td>
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<tr>
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<td>3.35$^{C,Z}$</td>
<td>5.00$^{D,Y}$</td>
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<td>7.52$^{A,X}$</td>
<td>7.58$^{A,X}$</td>
<td>7.51$^{A,X}$</td>
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<td></td>
</tr>
</tbody>
</table>

$^a$ Values are least squares means. Within a column, means with different superscripts (A through D) are significantly different ($P < 0.05$). Within a row, means with different superscripts (U through Z) are significantly different ($P < 0.05$).

$^b$ Control, naturally cured control; 1, 90MX and HHP; 2, 90MX and OA; 3, 90MX and LAE; 4, 90MX and PPTT.

$^c$ Standard error of the differences of least squares means.
FIGURE 8. Effect of cranberry powder in combination with post-lethality interventions on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C

Treatments: Control, naturally cured control; 1, 90MX and HHP; 2, 90MX and OA; 3, 90MX and LAE; 4, 90MX and PPTT.
**TABLE 11. Effect of vinegar in combination with post-lethality interventions on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C**

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>5.20&lt;sup&gt;A,Y&lt;/sup&gt;</td>
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<tr>
<td>5</td>
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<td>0.23&lt;sup&gt;B,Y&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
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<td></td>
</tr>
<tr>
<td>6</td>
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<td>0.30&lt;sup&gt;C,YZ&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;B,YZ&lt;/sup&gt;</td>
<td>0.08&lt;sup&gt;B,Z&lt;/sup&gt;</td>
<td>0.30&lt;sup&gt;B,YZ&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;B,YZ&lt;/sup&gt;</td>
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<tr>
<td>7</td>
<td>ND</td>
<td>0.42&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.20&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.08&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.08&lt;sup&gt;B&lt;/sup&gt;</td>
<td>ND</td>
<td>0.08&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.08&lt;sup&gt;B&lt;/sup&gt;</td>
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<td>8</td>
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<td>2.45&lt;sup&gt;C&lt;/sup&gt;</td>
<td>2.27&lt;sup&gt;C&lt;/sup&gt;</td>
<td>2.19&lt;sup&gt;C&lt;/sup&gt;</td>
<td>2.49&lt;sup&gt;D&lt;/sup&gt;</td>
<td>2.14&lt;sup&gt;C&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>SE&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through D) are significantly different (P < 0.05). Within a row, means with different superscripts (W through Z) are significantly different (P < 0.05).

<sup>b</sup> Control, naturally cured control; 5, DV and HHP; 6, DV and OA; 7, DV and LAE; 8, DV and PPTT.

<sup>c</sup> Not detected.

<sup>d</sup> Standard error of the differences of least squares means.
FIGURE 9. Effect of vinegar in combination with post-lethality interventions on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C.

Treatments: Control, naturally cured control; 5, DV and HHP; 6, DV and OA; 7, DV and LAE; 8, DV and PPTT.
TABLE 12. Effect of vinegar and lemon juice concentrate in combination with post-lethality interventions on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°Ca

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>1.26B,Z</td>
<td>1.10B,YZ</td>
<td>ND</td>
<td>ND</td>
<td>0.08B,XY</td>
<td>0.50B,XZ</td>
<td>0.23B,XY</td>
<td>0.70B,XZ</td>
</tr>
<tr>
<td>11</td>
<td>0.23C,YZ</td>
<td>0.08C,Z</td>
<td>ND</td>
<td>0.31B,YZ</td>
<td>0.35B,YZ</td>
<td>0.15B,YZ</td>
<td>1.16C,Y</td>
<td>0.33B,YZ</td>
</tr>
<tr>
<td>10</td>
<td>0.33C</td>
<td>0.20BC</td>
<td>0.46B</td>
<td>0.27B</td>
<td>0.42B</td>
<td>ND</td>
<td>0.23B</td>
<td>ND</td>
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<tr>
<td>12</td>
<td>2.45A,YZ</td>
<td>2.41D,Z</td>
<td>2.49C,YZ</td>
<td>2.58C,YZ</td>
<td>2.82C,YZ</td>
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<td>3.46D,XY</td>
<td>4.06C,X</td>
</tr>
<tr>
<td>SEd</td>
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</table>

a Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different ($P < 0.05$). Within a row, means with different superscripts (X through Z) are significantly different ($P < 0.05$).

b Control, naturally cured control; 9, LV1X and HHP; 10, LV1X and OA; 11, LV1X and LAE; 12, LV1X and PPTT.

c Not detected.

d Standard error of the differences of least squares means.
FIGURE 10. Effect of vinegar and lemon juice concentrate in combination with post-lethality interventions on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C.

Treatments: Control, naturally cured control; 9, LV1X and HHP; 10, LV1X and OA; 11, LV1X and LAE; 12, LV1X and PPTT.
TABLE 13. Effect of high hydrostatic pressure treatment in combination with natural antimicrobials on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C<sup>a</sup>

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
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</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
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<td>5.20&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>6.72&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.56&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.30&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.13&lt;sup&gt;A,X&lt;/sup&gt;</td>
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</tr>
<tr>
<td>1</td>
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<td>0.27&lt;sup&gt;B,Z&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;B,Z&lt;/sup&gt;</td>
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</tr>
<tr>
<td>5</td>
<td>1.26&lt;sup&gt;B,Z&lt;/sup&gt;</td>
<td>1.16&lt;sup&gt;C,YZ&lt;/sup&gt;</td>
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<td>0.27&lt;sup&gt;B,YZ&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;B,Y&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;B,Y&lt;/sup&gt;</td>
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<tr>
<td>9</td>
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<td>1.10&lt;sup&gt;C,XZ&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>0.08&lt;sup&gt;B,Y&lt;/sup&gt;</td>
<td>0.50&lt;sup&gt;B,YZ&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;C,XY&lt;/sup&gt;</td>
<td>0.70&lt;sup&gt;C,YZ&lt;/sup&gt;</td>
</tr>
<tr>
<td>SE&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.33</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different ($P < 0.05$). Within a row, means with different superscripts (X through Z) are significantly different ($P < 0.05$).

<sup>b</sup> Control, naturally cured control; 1, HHP and 90MX; 5, HHP and DV; 9, HHP and LV1X.

<sup>c</sup> Not detected.

<sup>d</sup> Standard error of the differences of least squares means.
FIGURE 11. Effect of high hydrostatic pressure treatment in combination with natural antimicrobials on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C.
TABLE 14. Effect of octanoic acid treatment in combination with natural antimicrobials on viable *Listeria monocytogenes* (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C*<sup>a</sup>*

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.72&lt;sup&gt;A,Z&lt;/sup&gt;</td>
<td>5.20&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>6.72&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.56&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.30&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.13&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.04&lt;sup&gt;A,X&lt;/sup&gt;</td>
<td>7.21&lt;sup&gt;A,X&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>0.31&lt;sup&gt;B,Z&lt;/sup&gt;</td>
<td>0.08&lt;sup&gt;B,Z&lt;/sup&gt;</td>
<td>0.68&lt;sup&gt;B,YZ&lt;/sup&gt;</td>
<td>1.38&lt;sup&gt;B,Y&lt;/sup&gt;</td>
<td>2.56&lt;sup&gt;B,X&lt;/sup&gt;</td>
<td>3.61&lt;sup&gt;B,W&lt;/sup&gt;</td>
<td>4.71&lt;sup&gt;B,V&lt;/sup&gt;</td>
<td>6.21&lt;sup&gt;B,U&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>0.38&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.30&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.08&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.30&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.79&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.40&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>0.33&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.20&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.46&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.27&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.42&lt;sup&gt;C&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;C&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
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<td>0.32</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different (*P* < 0.05). Within a row, means with different superscripts (U through Z) are significantly different (*P* < 0.05).

<sup>b</sup> Control, naturally cured control; 2, OA and 90MX; 6, OA and DV; 10, OA and LV1X.

<sup>c</sup> Not detected.

<sup>d</sup> Standard error of the differences of least squares means.
FIGURE 12. Effect of octanoic acid treatment in combination with natural antimicrobials on viable *Listeria monocytogenes* (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C.

Treatments: Control, naturally cured control; 2, OA and 90MX; 6, OA and DV; 10, OA and LV1X.
### TABLE 15. Effect of lauric arginate treatment in combination with natural antimicrobials on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 42</th>
<th>Day 56</th>
<th>Day 70</th>
<th>Day 84</th>
<th>Day 98</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
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<td>1.02B, Z</td>
<td>3.25B, Y</td>
<td>6.64B, X</td>
<td>7.58A, W</td>
<td>7.68A, W</td>
<td>7.84B, W</td>
<td>7.51A, W</td>
</tr>
<tr>
<td>7</td>
<td>NDc</td>
<td>0.42BC</td>
<td>0.20C</td>
<td>0.08C</td>
<td>0.08B</td>
<td>ND</td>
<td>0.08C</td>
<td>0.08B</td>
</tr>
<tr>
<td>11</td>
<td>0.23B, Z</td>
<td>0.08C, Z</td>
<td>ND</td>
<td>0.31C, Z</td>
<td>0.35B, Z</td>
<td>0.15B, Z</td>
<td>1.16D, Y</td>
<td>0.33B, Z</td>
</tr>
</tbody>
</table>

SEd 0.25

a Values are least squares means. Within a column, means with different superscripts (A through D) are significantly different (P < 0.05). Within a row, means with different superscripts (W through Z) are significantly different (P < 0.05).

b Control, naturally cured control; 3, LAE and 90MX; 7, LAE and DV; 11, LAE and LV1X.

c Not detected.

d Standard error of the differences of least squares means.
FIGURE 13. Effect of lauric arginate treatment in combination with natural antimicrobials on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C.

Treatments: Control, naturally cured control; 3, LAE and 90MX; 7, LAE and DV; 11, LAE and LV1X.
TABLE 16. Effect of post-packing thermal treatment in combination with natural antimicrobials on viable \( \text{Listeria monocytogenes} \) (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.72</td>
<td>5.20</td>
<td>6.72</td>
<td>7.56</td>
<td>7.30</td>
<td>7.13</td>
<td>7.04</td>
<td>7.21</td>
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<td>6.75</td>
<td>7.52</td>
<td>7.58</td>
<td>7.51</td>
<td>7.55</td>
</tr>
<tr>
<td>8</td>
<td>2.35</td>
<td>2.43</td>
<td>2.29</td>
<td>2.45</td>
<td>2.27</td>
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<td>2.41</td>
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<td>3.46</td>
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<tr>
<td>SE</td>
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<td></td>
</tr>
</tbody>
</table>

\(^a\) Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different \((P < 0.05)\). Within a row, means with different superscripts (W through Z) are significantly different \((P < 0.05)\).

\(^b\) Control, naturally cured control; 4, PPTT and 90MX; 8, PPTT and DV; 12, PPTT and LV1X.

\(^c\) Standard error of the differences of least squares means.
FIGURE 14. Effect of post-packaging thermal treatment in combination with natural antimicrobials on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C

Treatments: Control, naturally cured control; 4, PPTT and 90MX; 8, PPTT and DV; 12, PPTT and LV1X.
CHAPTER 7. EFFECTS OF VARYING CONCENTRATIONS OF NITRITE FROM A VEGETABLE SOURCE AND HIGH HYDROSTATIC PRESSURE ON THE RECOVERY AND GROWTH OF LISTERIA MONOCYTOGENES ON NATURALLY CURED READY-TO-EAT HAM

A paper to be submitted to the Journal of Food Protection

Nicolas A. Lavieri, Joseph G. Sebranek, Joseph C. Cordray, James S. Dickson, Ashley M. Horsch, Stephanie Jung, David K. Manu, and Aubrey F. Mendonça

Abstract

It has been previously suggested that sodium nitrite exerts an inhibitory effect on the growth of Listeria monocytogenes. Thus, the objective of this study was to investigate the effects of varying levels of nitrite from a vegetable source and high hydrostatic pressure (HHP) on the recovery and growth of L. monocytogenes on naturally cured ready-to-eat (RTE) ham. A pre-converted celery powder was used as the vegetable source of nitrite. Target ingoing concentrations of natural nitrite investigated were 0, 50, and 100 mg/Kg. HHP treatments evaluated were 400 MPa for 4 min, 600 MPa for 1 min, and 600 MPa for 4 min at 12 ± 2°C. Viable L. monocytogenes numbers were monitored on modified Oxford (MOX) and thin agar layer (TAL) media through 98 days of storage at 4 ± 1°C. Counts on MOX and TAL did not differ. The 600 MPa for 4 min HHP treatment resulted in L. monocytogenes levels below the detection limit of our sampling protocols throughout storage of the products regardless of ingoing natural nitrite concentration. The
combination of HHP at 400 MPa for 4 min or 600 MPa for 1 min with natural nitrite achieved a temporary inhibition of viable *L. monocytogenes*. Formulations that did not contain natural nitrite, whether they were treated with HHP or not, tended to allow for faster growth of *L. monocytogenes* than those that did. Our results indicate that nitrite from a vegetable source may extend the lag phase of this microorganism but only for a limited period of time. HHP treatments seemed to enhance the inhibitory effects of natural nitrite on *L. monocytogenes* growth. Thus, a combination of hurdles such as natural nitrite concentration and HHP may have an additive effect on *L. monocytogenes* growth inhibition.

**Introduction**

Although it is undetermined when nitrate and/or nitrite were first used to cure meat, it was not until the later part of the nineteenth century that studies showed that nitrite, rather than nitrate, was the key ingredient in curing processes (22, 23). In 1901, Haldane (11) demonstrated by adding nitrite to hemoglobin, thereby forming nitrosylhemoglobin, and heating this mixture that the pigment responsible for the characteristic color of cooked cured meats was nitrosylhemochromogen. A later study conducted by Hoagland (12) concluded that reduction of nitrate in saltpeter to nitrite, nitrous acid (HNO₂) and nitric oxide (NO), thanks to the action of bacteria or enzymes, or a combination of both, was necessary for nitrosylhemoglobin to form. This robust scientific knowledge of the science behind meat curing reactions led to the more widespread use of nitrite, rather than nitrate, in the production of cured meats. Consequently, the meat industry has derived unquantifiable benefits from the use of nitrite. Increased food safety, improved flavor and lipid stability, and an overall increased shelf-life of cured meat products are a few of the
advantages we have come to expect from cured meat products (28, 31). Thus, it is safe to say that the use of nitrite in cured meat and poultry production has led to the existence of products whose specific flavors, colors, and textures cannot be reproduced by using any other ingredient (23, 28, 31).

Although the color and flavor stability benefits derived from using nitrite are clear, of greater significance are its inhibitory properties against Clostridium botulinum and other microorganisms (14). The growth of other members of the Clostridium genus (i.e., C. butyricum, C. tyrobutyricum, C. sporogenes, and C. perfringens) is also known to be affected by nitrite (14). Furthermore, the inhibitory effects of nitrite against Listeria monocytogenes have been studied but are not as well understood (16). Upon evaluating the effects of sodium nitrite concentrations ranging from 0-1,000 mg per ml of Tryptose Phosphate Broth on the growth kinetics of L. monocytogenes Scott A, Buchanan and Phillips (5) were able to conclude that sodium nitrite represents an important parameter that plays a role in the survival and growth of this pathogen. Similarly, Pelroy and others (24) observed that 190-200 mg/kg sodium nitrite exerted a bacteriostatic effect on L. monocytogenes inoculated onto slices of cold-smoked salmon and that other factors such as packaging atmosphere, storage temperature, and sodium chloride concentration also played a role. Other studies have also suggested that increasing sodium nitrite concentration increased the lag phase of the L. monocytogenes logarithmic growth curve and, as a result, decreased the organism’s growth rate within a given time period (5, 6, 7, 8, 10, 17, 38).

Nitrate or nitrite, given their classification as chemical preservatives, are prohibited from use in either natural or organic processed meat and/or poultry products (37). Given
that there are no direct substitutes for nitrite, regardless of whether it is added to the product directly or derived from the addition of nitrate and its subsequent reduction, the production of natural and organic processed meat products whose quality and safety properties and characteristics resemble those of their conventionally cured counterparts has represented a challenge to the meat industry. Because the quality and safety benefits derived from meat curing are unquestionable, the indirect addition of nitrate or nitrite to natural and organic processed meat products, sometimes referred to as “natural curing,” represents a new technology that has garnered interested from processors, consumers and scientists alike (29, 31).

Some fruits and vegetables are known to contain relatively high levels of nitrate. Potatoes, lettuce, melons, cabbage, celery, spinach, beets, carrots, cauliflower, and broccoli are only a few examples of such vegetables and fruits (39). However, due to concerns over the flavor and/or color compatibility or clash that may stem from using some of these as sources of nitrate or nitrite in the production of natural and organic processed meat products, more emphasis has been placed on celery (Apium graveolens var. dulce) than on any other vegetable or fruit. Analysis conducted by Sindelar and others (32) showed that a commercially available celery juice powder contained 27,462 mg/Kg, or approximately 2.75%, nitrate. The use of natural sources of nitrate and nitrate-reducing starter cultures, and the ensuing need for an incubation step for the reduction of nitrate to nitrite, in the production of natural or organic processed meat products result in increased production times. This scenario is not very compatible with today’s high throughput production systems and consumers’ increased demand for these categories of products. Thus, manufacturers of celery powders have begun to add nitrate-reducing
starter cultures such as *Staphylococcus carnosus* directly to the celery purees before the drying step and, as a result, have started to market “pre-converted” nitrite versions of celery powders. Once dried or slightly condensed, pre-converted celery powders or juices will contain 10,000-15,000 mg/Kg, or 1.0-1.5%, nitrite. Recommended usage levels differ depending on not only the product but also the manufacturer of the celery powder or juice and range from 0.2-1.0% based on green (raw) meat weight. Using a pre-converted celery powder in which the active ingredient is nitrite instead of nitrate will effectively eliminate the need for a nitrate reduction step and, therefore, result in decreased production times.

Ready-to-eat (RTE) meat and poultry products manufactured under uncured, natural, or organic methods and requirements are at a greater risk for growth of *L. monocytogenes* if post-lethality contamination occurs than their conventional counterparts mainly due to the required absence of preservatives and antimicrobials traditionally used in the manufacture of conventional products (26, 34). As a result, the use of “clean label” technologies or post-lethality interventions in the manufacture of these types of meat products has received attention from researchers and processors alike (26, 27, 29, 33, 35). High hydrostatic pressure processing (HHP), for example, is considered a post-lethality intervention as it generally takes place after the product has gone through the lethality or cooking step (36). This technology has been shown to achieve reductions in *L. monocytogenes* numbers in RTE meat and other food products (9, 13, 18, 19, 20, 30). Given that the inhibitory effects of nitrite against *L. monocytogenes* have been studied but are still not well understood and that the use of nitrite from vegetable sources represents a relatively new technology in the production of natural and organic processed meat and
poultry products, it was the objective of this study to investigate the effects of varying levels of nitrite from a vegetable source and HHP on the recovery and growth of *L. monocytogenes* on naturally cured RTE ham.

**Materials and Methods**

**Manufacture of Hams**

Two independent experiments were conducted. Experiment 1, outlined in Table 1, was designed to investigate the effects of 0, 50, and 100 mg/kg ingoing natural nitrite and HHP at either 400 or 600 MPa for 4 min dwell time at 12 ± 2°C on *L. monocytogenes*. Experiment 2, outlined in Table 5, was designed to investigate the effects of 0, 50, and 100 mg/kg ingoing natural nitrite and HHP at 600 MPa for 1 min dwell time at 12 ± 2°C *L. monocytogenes*. Naturally cured RTE boneless hams were produced at the Iowa State University Meat Laboratory with inside ham muscles, using formulations found in Table 1 and Table 5 for experiments 1 and 2, respectively. The ham muscles were obtained from a local processor and frozen prior to use to ensure uniformity of raw materials. The ham muscles were tempered to -2°C and then were coarse ground through a plate with 9.53-mm-diameter holes (Biro MFG Co., Marblehead, OH). Nonmeat ingredients (Table 1 and Table 5) were added and mixed with ground ham muscles at 26 rpm for 2 min using a double action mixer (Leland Southwest, Fort Worth, TX). Pre-converted celery powder (VegStable 504, Florida Food Products, Inc., Eustis, FL) was used as the natural source of nitrite and is approximately 1.5% nitrite (wt/wt), according to the manufacturer. Mixed samples were then reground using a plate with 6.35-mm-diameter holes and stuffed into a 50-mm-diameter impermeable plastic casing (Nalobar APM 45, Kalle
USA, Gurnee, IL) using a rotary vane vacuum-filling machine (RS 1040 C, Risco USA Corp., South Eaton, MA). Thorough rinsing with cold water of all of the equipment utilized was conducted after each ham formulation was manufactured so as to avoid cross-contamination between product formulations. All treatments were then placed in a single-truck smokehouse (Maurer, AG, Reichenau, Germany) and heated to an internal temperature of 71.1°C. The hams were then placed in a 0°C cooler overnight to stabilize. The next day, which marked day 0 of the experiment, the hams were sliced into approximately 12.0-mm-thick slices using a hand slicer (SE 12 D, Bizerba, Piscataway, NJ), placed into barrier bags (B2470, Cryovac Sealed Air Corporation, Duncan, SC) with an oxygen transmission rate of 3-6 cc at 4°C (m², 24 hrs atm @ 4°C, 0% RH) and a water vapor transmission rate of 0.5-0.6 g at 38°C (100% RH, 0.6 m², 24 hrs), and vacuum sealed (UV 2100, Multivac, Inc., Kansas City, MO). Hams for analytical analyses were placed in boxes and transferred to a holding cooler in the Iowa State University Meat Laboratory and stored at 4 ± 1°C until analyses were conducted. Hams for microbial analyses were placed in boxes with vacuum packaged ice, transferred to the Iowa State University Food Safety Research Laboratory in the Food Science and Human Nutrition Department for subsequent inoculation, and stored at 4 ± 1°C for the duration of the experiment. Two independent replications were produced for each experiment.

Mean Weight Calculations

On day 0, a total of five randomly selected slices of ham from the 0, 50, and 100 mg/kg natural nitrite ham formulations (Table 1 and Table 5) were weighed (n = 15 per replication per experiment) so as to obtain representative average weights measurements.
Average weight measurements from both replications of each study (n = 30) would then be used to calculate log CFU per g.

**Proximate Analysis**

Proximate analysis was conducted for moisture, fat, and protein of homogenized 0, 50, and 100 mg/kg natural nitrite formulations (Table 1 and Table 5) on day 0 using AOAC methods 950.46, 960.63, and 992.15, respectively (1, 2, 3). Samples were prepared in duplicate for each ham formulation.

**pH**

Product pH was measured by placing a pH probe (FC20, Hanna Instruments, Woonsocket, RI) into homogenized (KFP715 food processor, Kitchenaid, St. Joseph, MI) samples from 0, 50, and 100 mg/kg natural nitrite formulations (Table 1 and Table 5) that were prepared by first blending the ground ham with distilled, de-ionized water in a 1:9 ratio, and then measuring the pH with a pH/ion meter (Accumet 925 pH/ion meter, Fisher Scientific). Calibration was conducted using phosphate buffers of pH 4.0, 7.0, and 10.0. Duplicate readings were taken for each product formulation on day 0.

**Water Activity**

Available moisture was determined using a water activity meter (AquaLab 4TE, Decagon Devices Inc., Pullman, WA). Samples were cut into small pieces, placed in disposable sample cups, covered, and allowed to equilibrate to room temperature (5-10 min). Measurements were obtained on day 0 and were performed in duplicate for 0, 50,
and 100 mg/kg natural nitrite formulations (Table 1 and Table 5). Calibration was performed using 1.00 and 0.76 sodium chloride water activity standards.

**Residual Nitrite Analysis**

Residual nitrite concentration was determined utilizing AOAC method 973.31 (4). Samples from each the 0, 50, and 100 mg/kg natural nitrite formulations (Table 1 and Table 5) were frozen at -20 ± 1°C on day 0 and evaluated at a later date in duplicate.

**Preparation of Inoculum**

*L. monocytogenes* strains Scott A NADC 2045 serotype 4b, H7969 serotype 4b, H7962 serotype 4b, H7596 serotype 4b, and H7762 serotype 4b were obtained from the Iowa State University Food Safety Research Laboratory in the Food Science and Human Nutrition Department. Each strain was cultured separately in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) (Difco, Becton Dickinson, Sparks, MD) for 24 h at 35°C. A minimum of two consecutive 24-h transfers of each strain to fresh TSBYE (35°C) were performed prior to each experiment. Aliquots (6.0-ml) from each of the five strains were then transferred into a sterile centrifuge tube. The bacterial cells were harvested by centrifugation (10 min at 10,000 rpm and 4°C) in a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT). The supernatant was discarded and the pelleted cells were resuspended in 30.0 ml of sterile buffered peptone water (BPW) (Difco, Becton Dickinson). The total concentration of the five-strain cocktail was approximately $10^9$ colony forming units (CFU) per ml based on the aerobic plate counts of the washed cell suspension. Two serial dilutions (100-fold each) of the
cell suspension were prepared in BPW to give a final inoculum concentration of $10^5$ CFU per ml. This diluted five-strain cocktail was used to inoculate samples of ham.

**Sample Inoculation**

While in the Food Safety Research Laboratory, each packaged sample was reopened and the surface of the product was aseptically inoculated with a 0.2-ml aliquot of the diluted five-strain cocktail. The cell concentration at inoculation was approximately $10^3$ CFU per gram. The bags were then vacuum sealed using a model A300/52 vacuum packaging machine (Multivac, Inc.) and stored at 4 ± 1°C for the duration of the experiment.

**High Hydrostatic Pressure Treatment**

HHP was evaluated under two different sets of parameters for experiment 1; 400 MPa, 4 min dwell time at 12 ± 2°C initial fluid temperature or 600 MPa, 4 min dwell time at 12 ± 2°C initial fluid temperature. For experiment 2, HHP was evaluated at 600 MPa, 1 min dwell time at 12 ± 2°C initial fluid temperature. Hams were transported on ice to the Food Safety Research Laboratory for inoculation as previously described and then to the High Pressure Processing Laboratory at the Iowa State University Food Science and Human Nutrition Department and subjected to the appropriate HHP treatment using a FOOD-LAB 900 Plunger Press system (Standsted Fluid Power Ltd., Standsted, UK). The pressurization fluid was a 50.0% propylene glycol (GWT Koilguard; GWT Global Water Technology, Inc., Indianapolis, IN) and 50.0% water solution (v/v). The average rate of pressurization was 350 MPa per min and depressurization occurred within 7 s. HHP treatment was applied to products within two hours after inoculation.
Microbial Analysis

Microbial analysis of ham samples for viable *L. monocytogenes* was conducted on days 1, 14, 28, 42, 56, 70, 84, and 98. On the appropriate day, two packages for each treatment were removed from the holding cooler, opened aseptically, and their contents placed inside a sterile Whirl-Pak stomacher bag (Nasco, Ft. Atkinson, WI). Sample preparation was performed by adding 50.0 ml of sterile BPW to each bag, closing the bag so as to form a “pillow,” and then shaking the sample for approximately 30 s. The wash solution from each ham sample was then serially diluted (10-fold) in BPW to obtain pre-determined dilutions of the samples according to the sampling day. An aliquot of 1.0 ml (for $10^0$ dilution, divided into three ~0.33-ml aliquots plated on three separate plates) or 0.1 ml of the appropriate dilution was surfaced plated on modified Oxford medium base (MOX) supplemented with modified Oxford antimicrobial supplement (Difco, Becton Dickinson). The dry ingredients used to manufacture the MOX were 42.5 g of Columbia agar base (Difco, Becton Dickinson), 15.0 g of lithium chloride (Difco, Becton Dickinson), 1.0 g of esculin hydrate (Sigma-Aldrich, St. Louis, MO), and 0.5 g of ferric ammonium citrate (Difco, Becton Dickinson) per liter of de-ionized water. Additionally, 0.1 ml of the appropriate dilution was surfaced plated on thin agar layer medium base (TAL) that was made according to Kang and Fung (15) with some modifications. MOX was made as previously described. Then, never more than 48 hr before sampling was to be conducted, MOX plates to be made into TAL were aseptically overlaid with 7.0 ml of sterile tryptic soy agar (Difco, Becton Dickinson) held at 55°C to facilitate the even distribution of the molten agar. Each sample was plated in duplicate. Plates used for microbial analyses were sterile and 55 mm in diameter (Fisher Scientific, Waltham, MA).
All inoculated agar plates were incubated in an inverted position at 35°C for 48 hr, after which time they were removed from the incubator and colonies typical of *L. monocytogenes* were enumerated. The counts (CFU per ml) were averaged and then converted to log CFU per g using the average weight of the sliced ham from the two replications of the experiment \((n = 30)\). The detection limit of our sampling protocols was \(\geq 0.30\) log CFU per g based on a sample weight of 25.0 g.

**Statistical Analysis**

The overall design of the experiment was a factorial design. The generalized linear mixed models (GLIMMIX) procedure of Statistical Analysis System (version 9.3, SAS Institute Inc., Cary, NC) was used for statistical analysis. Viable *L. monocytogenes* data were analyzed for treatment effects within day. Day and treatment x day interactions were also analyzed. Where significant effects \((P < 0.05)\) were found, pair-wise comparisons between the least squares means were computed for each day using Tukey’s honestly significant difference adjustment.

**Results**

**Mean Weight Results**

For experiment 1, the mean weight of the ham slices \((n = 30)\) was 25.27 g with a standard deviation of \(\pm 0.98\) g (data not shown). For experiment 2, the mean weight of the ham slices \((n = 30)\) was 24.50 with a standard deviation of \(\pm 0.62\) g (data not shown).
Physicochemical Traits

Physicochemical characteristics of the naturally cured RTE hams manufactured for experiment 1 can be found in Table 2. The proximate composition of the hams was not affected by the ingoing concentration of natural nitrite as no significant differences were found in fat, moisture, and protein % ($P > 0.05$). pH of the hams was also unaffected by ingoing concentration of natural nitrite ($P > 0.05$). The $a_w$ and day 0 residual nitrite concentrations of the different formulations, on the other hand, were affected by ingoing concentration of natural nitrite. The 100 mg/kg ingoing natural nitrite formulation had a lower $a_w$ than the 0 mg/kg ingoing natural nitrite formulation ($P < 0.05$). As expected, the residual nitrite concentration of the different formulations varied based on ingoing level of natural nitrite ($P < 0.05$), with the 100 mg/kg natural nitrite formulation exhibiting the highest residual nitrite concentration (83.13 mg/kg) and the 0 mg/kg ingoing natural nitrite formulation exhibiting the lowest (4.78 mg/kg). Similar effects of ingoing natural nitrite level on day 0 residual nitrite concentrations were observed in products manufactured as part of experiment 2 (Table 5). However, in said experiment, $a_w$ was not significantly affected by ingoing natural nitrite concentration ($P > 0.05$). These slight differences in $a_w$ were not expected to affect the results of these experiments.

Experiment 1 Viable *L. monocytogenes* Results

Viable *L. monocytogenes* numbers on MOX (Table 3 and Figure 1) and TAL (Table 4 and Figure 2) media were monitored throughout the duration of the study. The growth mediums used did not significantly differ ($P > 0.05$) within treatment on any given day, indicating that, under the conditions of this study, the use of the TAL technique offers
limited advantages compared to using a traditional medium such as MOX. Thus, the discussion about viable *L. monocytogenes* numbers as affected by treatment is based on results obtained using MOX.

The HHP600 treatment employed in experiment 1 resulted in viable *L. monocytogenes* numbers below the detection limit of our sampling protocols throughout the duration of the study regardless of ingoing natural nitrite concentration. These results indicate that, under the conditions of this study, the HHP600 treatment exerted bactericidal effects on *L. monocytogenes* and represents an effective post-lethality intervention against this microorganism. The HHP400 treatment, on the other hand, only resulted in viable *L. monocytogenes* numbers below the detection limit of our sampling protocols in treatment 2 on day 1. Furthermore, on day 1, a significant (*P* < 0.05) reduction in viable *L. monocytogenes* numbers as a result of the HHP400 treatment was observed in the 50 mg/kg ingoing natural nitrite treatments but not in the 100 mg/kg ingoing natural nitrite treatment (*P* > 0.05).

Ingoing natural nitrite concentration was found to have a significant effect on viable *L. monocytogenes* (*P* < 0.05). While treatment 1 showed a significant (*P* < 0.05) increase in viable *L. monocytogenes* numbers by day 14 compared to day 1, treatments 4 and 7 did not. Moreover, treatment 7 exhibited significantly lower viable *L. monocytogenes* levels than treatment 1 on all sampling days except day 1 (*P* < 0.05). Although the numbers of viable *L. monocytogenes* found in treatment 4 remained below those found in treatment 1 from day 14 forward, this difference was only significant (*P* < 0.05) on days 28 and 84 of the study. The results indicate that, under the conditions of this study, an ingoing
concentration of 100 mg/kg natural nitrite exerts a slight inhibitory effect on the growth of *L. monocytogenes*.

The interaction of ingoing natural nitrite concentration with the HHP400 treatment was also found to be significant (*P* < 0.05). Treatment 2 showed a significant increase in viable *L. monocytogenes* numbers, compared to day 1 numbers, by day 42 of the study (*P* < 0.05). Treatments 5 and 9, on the other hand, exhibited significantly higher (*P* < 0.05) viable *L. monocytogenes* levels compared to their respective day 1 values by day 70 and 98 of the study, respectively. Moreover, viable *L. monocytogenes* numbers found in treatment 8 were significantly lower than those found in treatment 2 from day 42 of the study forward (*P* < 0.05). Similarly, viable *L. monocytogenes* numbers found in treatment 5 were significantly lower than those found in treatment 2 from day 42 through day 84 of the study (*P* < 0.05). These results suggest that, under the conditions of this study, the natural nitrite ingredient used has bacteriostatic effects on *L. monocytogenes* and that its inhibitory effects are enhanced when combined with the HHP400 treatment used.

**Experiment 2 Viable *L. monocytogenes* Results**

Viable *L. monocytogenes* numbers on MOX (Table 7 and Figure 3) and TAL (Table 8 and Figure 4) media were monitored throughout the duration of experiment 2. As in experiment 1, the growth mediums used did not significantly differ (*P* > 0.05) within treatment on any given day, indicating that, under the conditions of this experiment, the use of the TAL technique offered limited advantages. Thus, the discussion about viable *L. monocytogenes* is based on results obtained using MOX.
Treatment 17 exhibited significantly lower viable *L. monocytogenes* numbers than treatment 13 on days 14 and 28 of the experiment (*P* < 0.05). However, from day 42 forth, no significant differences between these two treatments existed (*P* > 0.05). Furthermore, no significant differences in viable *L. monocytogenes* were found between treatments 13 and 15 (*P* > 0.05). These results allow us to conclude that an ingoing natural nitrite concentration of 100 mg/kg only had a temporary inhibitory effect on the growth of *L. monocytogenes* whereas 50 mg/kg ingoing natural nitrite was not sufficient to exert significant inhibition of this microorganism.

The HHP treatment employed in experiment 2, which consisted of 600 MPa for 1 min at a starting fluid temperature of 12 ± 2 °C, resulted in viable *L. monocytogenes* numbers below the detection limit of our sampling protocols until day 28 of the study regardless of ingoing natural nitrite concentration, indicating that HHP, under the conditions of this experiment, has bactericidal effects on *L. monocytogenes*. However, those *L. monocytogenes* cells that survived the HHP treatment they were subjected to showed differing growth patterns based on ingoing natural nitrite concentration. For example, treatment 14 experienced a significant (*P* < 0.05) increase in viable *L. monocytogenes* numbers on day 70 compared to day 28. Viable bacterial numbers in this treatment continued to increase by day 84 (*P* < 0.05). Significant increases in viable bacterial levels in treatment 16 were not observed until day 98 of the study with respect to day 28 (*P* < 0.05). Interestingly, viable *L. monocytogenes* levels observed in treatment 18 did not significantly change from day 28 of the experiment forward (*P* > 0.05) and were lower (*P* < 0.05) than those observed in treatment 14 on days 84 and 98 of the study. These results indicate that, under the conditions of this study, the inhibitory effects of natural nitrite on
the growth of *L. monocytogenes* are enhanced when combined with the 600 MPa for 1 min HHP treatment used. Given that the bacterial cell membrane is widely assumed to be the main site of damage as a result of HHP treatment (13, 20, 21, 25), it is likely that nitrite or any of its metabolites that exert inhibitory effects on the growth of *L. monocytogenes* more readily affected those cells that survived HHP treatment.

**Discussion**

Our experiments demonstrated that HHP, depending on the parameters used, has varying bactericidal effects on viable *L. monocytogenes*. The 600 MPa for 4 min HHP treatment utilized in experiment 1 resulted in viable *L. monocytogenes* numbers below the detection limit of our sampling protocols throughout the entire duration of the study. These results agree with those obtained by Myers and others (20), as these authors found that an HHP treatment of 600 MPa for 3 min and 17°C resulted in a 3.85-4.35 log CFU per g reduction in *L. monocytogenes* numbers on RTE meat products. Similarly, Myers and others (19) also concluded that HHP treatment consisting of 600 MPa for 3 min and 17°C resulted in a 3.9-4.3 log CFU per g reduction in *L. monocytogenes* numbers on RTE sliced ham. Thus, the implementation of an HHP treatment that mimics these parameters and the conditions of our studies may represent a useful tool for meat processors to use as part of their *L. monocytogenes* control plans. However, variations in the effectiveness of this HHP treatment as a result of inoculation level, food matrix composition, and other intrinsic and extrinsic factors are likely.

The 400 MPa for 4 min HHP treatment utilized in experiment 1, on the other hand, only achieved partial inactivation of *L. monocytogenes* and those cells that survived were
able to grow upon refrigerated storage of the product. Similar results were reported by Myers and others (19). These authors reported that after 400 MPa HHP treatment of RTE sliced ham for 3 min at 17°C, which resulted in less than a 1 log CFU per g reduction in *L. monocytogenes* numbers, the pathogen was able to grow to numbers above inoculation levels upon storage under refrigeration. Thus, a 400 MPa HHP treatment seeking to achieve complete inhibition of *L. monocytogenes* when using an inoculation level of 3 log CFU per g may need to be extended in duration to do so.

Similarly, the 600 MPa for 1 min HHP treatment employed as part of experiment 2 resulted in viable *L. monocytogenes* numbers below the detection limit of our sampling protocols through 28 days of refrigerated storage. Surviving *L. monocytogenes* were able to grow after day 28 of the study, however. Although advantageous from a production efficiency standpoint, shortening the 600 MPa HHP treatment from 4 min to 1 min is not enough to completely inactivate viable *L. monocytogenes* under the conditions of our studies.

Some authors have suggested that sodium nitrite concentration increases the lag phase of the *L. monocytogenes* logarithmic growth curve and, as a result, decreases the organism’s growth rate within a given time period (5, 6, 7, 8, 10, 17, 38). Our results partially agree with this statement as 100 mg/kg ingoing natural nitrite generally resulted in lower growth rates exhibited by viable *L. monocytogenes* compared to our 0 mg/kg ingoing natural nitrite control, whereas 50 mg/kg ingoing natural nitrite generally did not. Myers and others (19, 20) reached similar conclusions after investigating the effects of nitrite from both a traditional and a vegetable source and HHP on the growth of *L. monocytogenes* in RTE processed meat products. Formulations that did not contain
natural nitrite, whether they were treated with HHP or not, tended to allow for faster growth of *L. monocytogenes*, indicating that nitrite from a vegetable source may extend the lag phase of this microorganism but only for a limited period of time. Thus, a combination of hurdles such as nitrite concentration and HHP may have an additive effect on *L. monocytogenes* growth inhibition.

**Acknowledgements**

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References


### TABLE 1. Naturally cured RTE ham formulations used for experiment 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ham (kg)</th>
<th>Water (kg)</th>
<th>Salt (kg)</th>
<th>Sugar (kg)</th>
<th>Pre-converted Celery Powder&lt;sup&gt;a&lt;/sup&gt; (g)</th>
<th>Calculated Ingoing Natural Nitrite Concentration (mg/Kg)</th>
<th>HHP Intervention</th>
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<tr>
<td>1</td>
<td>18.14</td>
<td>3.66</td>
<td>0.50</td>
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<td>18.14</td>
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</table>

<sup>a</sup> Vegetable 504 (Natural Nitrite; Florida Food Products, Inc., Eustis, FL).

<sup>b</sup> High Hydrostatic Pressure (400 MPa, 4 min dwell time at 12 ± 2°C).

<sup>c</sup> High Hydrostatic Pressure (600 MPa, 4 min dwell time at 12 ± 2°C).
**TABLE 2. Effect of ingoing natural nitrite concentration on physicochemical properties of naturally cured RTE ham for experiment 1**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>$a_w$</th>
<th>pH</th>
<th>Fat %</th>
<th>Moisture %</th>
<th>Protein %</th>
<th>Residual Nitrite Concentration (mg/kg)</th>
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<tr>
<td>0 mg/kg</td>
<td>0.9672$^A$</td>
<td>6.06</td>
<td>1.73</td>
<td>76.18</td>
<td>19.00</td>
<td>4.78$^A$</td>
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<td>50 mg/kg</td>
<td>0.9633$^{AB}$</td>
<td>6.14</td>
<td>1.64</td>
<td>76.30</td>
<td>18.71</td>
<td>41.90$^B$</td>
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<tr>
<td>100 mg/kg</td>
<td>0.9604$^B$</td>
<td>6.18</td>
<td>1.67</td>
<td>76.06</td>
<td>18.77</td>
<td>83.13$^C$</td>
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<tr>
<td>SE$^b$</td>
<td>0.001</td>
<td>0.04</td>
<td>0.28</td>
<td>0.39</td>
<td>0.32</td>
<td>0.71</td>
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</table>

$^a$ Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different ($P < 0.05$).

$^b$ Standard error of the differences of least squares means.
TABLE 3. Effect of ingoing natural nitrite concentration alone and in combination with high hydrostatic pressure on viable *Listeria monocytogenes* (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C<sup>a</sup>

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Day</th>
<th>1</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
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<td></td>
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<tr>
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<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.71&lt;sup&gt;BZ&lt;/sup&gt;</td>
<td>1.45&lt;sup&gt;BZ&lt;/sup&gt;</td>
<td>4.00&lt;sup&gt;BY&lt;/sup&gt;</td>
<td>7.26&lt;sup&gt;ACX&lt;/sup&gt;</td>
<td>8.08&lt;sup&gt;Ax&lt;/sup&gt;</td>
<td>7.94&lt;sup&gt;AX&lt;/sup&gt;</td>
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<tr>
<td>4</td>
<td>2.76&lt;sup&gt;ZA&lt;/sup&gt;</td>
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<td>1.62&lt;sup&gt;BYZ&lt;/sup&gt;</td>
<td>2.78&lt;sup&gt;CY&lt;/sup&gt;</td>
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<td>5.93&lt;sup&gt;WYX&lt;/sup&gt;</td>
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<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through D) are significantly different (*P* < 0.05). Within a row, means with different superscripts (W through Z) are significantly different (*P* < 0.05).

<sup>b</sup> 1, 0 mg/kg natural nitrite; 2, 0 mg/kg natural nitrite and high hydrostatic pressure (400 MPa, 4 min dwell time); 3, 0 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 4 min dwell time); 4, 50 mg/kg natural nitrite; 5, 50 mg/kg natural nitrite and high hydrostatic pressure (400 MPa, 4 min dwell time); 6, 50 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 4 min dwell time); 7, 100 mg/kg natural nitrite; 8, 100 mg/kg natural nitrite and high hydrostatic pressure (400 MPa, 4 min dwell time); 9, 100 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 4 min dwell time).

<sup>c</sup> Not detected.

<sup>d</sup> Standard error of the differences of least squares means.
FIGURE 1. Effect of ingoing natural nitrite concentration alone and in combination with high hydrostatic pressure on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C

Treatments: 1, 0 mg/kg natural nitrite; 2, 0 mg/kg natural nitrite and high hydrostatic pressure (400 MPa, 4 min dwell time); 4, 50 mg/kg natural nitrite; 5, 50 mg/kg natural nitrite and high hydrostatic pressure (400 MPa, 4 min dwell time); 7, 100 mg/kg natural nitrite; 8, 100 mg/kg natural nitrite and high hydrostatic pressure (400 MPa, 4 min dwell time)
TABLE 4. Effect of ingoing natural nitrite concentration alone and in combination with high hydrostatic pressure on viable *Listeria monocytogenes* (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C

<table>
<thead>
<tr>
<th>Day</th>
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<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
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<td>8.22&lt;sup&gt;A&lt;/sup&gt;,&lt;sup&gt;X&lt;/sup&gt;</td>
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<td>1.27&lt;sup&gt;B&lt;/sup&gt;,&lt;sup&gt;Z&lt;/sup&gt;</td>
<td>4.05&lt;sup&gt;B&lt;/sup&gt;,&lt;sup&gt;Y&lt;/sup&gt;</td>
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<td>6.48&lt;sup&gt;B&lt;/sup&gt;,&lt;sup&gt;Y&lt;/sup&gt;</td>
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<td>5.87&lt;sup&gt;D&lt;/sup&gt;,&lt;sup&gt;XY&lt;/sup&gt;</td>
<td>6.39&lt;sup&gt;C&lt;/sup&gt;,&lt;sup&gt;X&lt;/sup&gt;</td>
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<tr>
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<td>1.33&lt;sup&gt;B&lt;/sup&gt;,&lt;sup&gt;Z&lt;/sup&gt;</td>
<td>1.41&lt;sup&gt;C&lt;/sup&gt;,&lt;sup&gt;Z&lt;/sup&gt;</td>
<td>1.15&lt;sup&gt;B&lt;/sup&gt;,&lt;sup&gt;Z&lt;/sup&gt;</td>
<td>0.99&lt;sup&gt;E&lt;/sup&gt;,&lt;sup&gt;Z&lt;/sup&gt;</td>
<td>1.90&lt;sup&gt;C&lt;/sup&gt;,&lt;sup&gt;Z&lt;/sup&gt;</td>
<td>4.28&lt;sup&gt;C&lt;/sup&gt;,&lt;sup&gt;Y&lt;/sup&gt;</td>
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<td>ND</td>
<td>ND</td>
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<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through E) are significantly different ($P < 0.05$). Within a row, means with different superscripts (W through Z) are significantly different ($P < 0.05$).

<sup>b</sup> 1, 0 mg/kg natural nitrite; 2, 0 mg/kg natural nitrite and high hydrostatic pressure (400 MPa, 4 min dwell time); 3, 0 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 4 min dwell time); 4, 50 mg/kg natural nitrite; 5, 50 mg/kg natural nitrite and high hydrostatic pressure (400 MPa, 4 min dwell time); 6, 50 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 4 min dwell time); 7, 100 mg/kg natural nitrite; 8, 100 mg/kg natural nitrite and high hydrostatic pressure (400 MPa, 4 min dwell time); 9, 100 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 4 min dwell time).

<sup>c</sup> Not detected.

<sup>d</sup> Standard error of the differences of least squares means.
FIGURE 2. Effect of ingoing natural nitrite concentration alone and in combination with high hydrostatic pressure on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C

Treatments: 1, 0 mg/kg natural nitrite; 2, 0 mg/kg natural nitrite and high hydrostatic pressure (400 MPa, 4 min dwell time); 4, 50 mg/kg natural nitrite; 5, 50 mg/kg natural nitrite and high hydrostatic pressure (400 MPa), 4 min dwell time; 7, 100 mg/kg natural nitrite; 8, 100 mg/kg natural nitrite and high hydrostatic pressure (400 MPa, 4 min dwell time)
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<th>Salt (kg)</th>
<th>Sugar (kg)</th>
<th>Pre-converted Celery Powder$^a$ (g)</th>
<th>Calculated Ingoing Natural Nitrite Concentration (mg/kg)</th>
<th>HHP Intervention</th>
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$^a$ Vegstable 504 (Natural Nitrite; Florida Food Products, Inc., Eustis, FL).

$^b$ High Hydrostatic Pressure (600 MPa, 1 min dwell time at 12 ± 2°C).
TABLE 6. Effect of ingoing natural nitrite concentration on physicochemical properties of naturally cured RTE ham for experiment 2\textsuperscript{a}

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<th>Formulation</th>
<th>a\textsubscript{w}</th>
<th>pH</th>
<th>Fat %</th>
<th>Moisture %</th>
<th>Protein %</th>
<th>Residual Nitrite Concentration (mg/kg)</th>
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<td>1.96</td>
<td>75.84</td>
<td>18.09</td>
<td>36.05\textsuperscript{B}</td>
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<tr>
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<td>6.39</td>
<td>1.95</td>
<td>75.81</td>
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<td>72.55\textsuperscript{C}</td>
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<td>0.17</td>
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\textsuperscript{a} Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different (\(P < 0.05\)).

\textsuperscript{b} Standard error of the differences of least squares means.
**TABLE 7. Effect of ingoing natural nitrite concentration alone and in combination with high hydrostatic pressure on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C**

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
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<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
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<th>84</th>
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<tbody>
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<td>8.18&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>8.09&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>7.98&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>7.82&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>7.68&lt;sup&gt;A,Y&lt;/sup&gt;</td>
<td>7.48&lt;sup&gt;A,Y&lt;/sup&gt;</td>
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<tr>
<td>14</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>0.15&lt;sup&gt;B,Z&lt;/sup&gt;</td>
<td>0.08&lt;sup&gt;B,Z&lt;/sup&gt;</td>
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<sup>a</sup> Values are least squares means. Within a column, means with different superscripts (A through C) are significantly different (P < 0.05). Within a row, means with different superscripts (W through Z) are significantly different (P < 0.05).

<sup>b</sup> 13, 0 mg/kg natural nitrite; 14, 0 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 1 min dwell time); 15, 50 mg/kg natural nitrite; 16, 50 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 1 min dwell time); 17, 100 mg/kg natural nitrite; 18, 100 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 1 min dwell time).

<sup>c</sup> Not detected.

<sup>d</sup> Standard error of the differences of least squares means.
FIGURE 3. Effect of ingoing natural nitrite concentration alone and in combination with high hydrostatic pressure on viable Listeria monocytogenes (log CFU per gram) on modified Oxford medium on naturally cured RTE ham stored at 4 ± 1°C.

Treatments: 13, 0 mg/kg natural nitrite; 14, 0 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 1 min dwell time); 15, 50 mg/kg natural nitrite; 16, 50 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 1 min dwell time); 17, 100 mg/kg natural nitrite; 18, 100 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 1 min dwell time).
TABLE 8. Effect of ingoing natural nitrite concentration alone and in combination with high hydrostatic pressure on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C

<table>
<thead>
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<th>Day</th>
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<th>14</th>
<th>28</th>
<th>42</th>
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<td>3.92</td>
<td>5.65</td>
<td>6.34</td>
<td>7.26</td>
<td>7.17</td>
<td>7.12</td>
<td>7.34</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>ND</td>
<td>ND</td>
<td>1.23</td>
<td>1.88</td>
<td>1.19</td>
<td>0.82</td>
<td>1.02</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.82</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are least squares means. Within a column, means with different superscripts (A through D) are significantly different ($P < 0.05$). Within a row, means with different superscripts (X through Z) are significantly different ($P < 0.05$).

13, 0 mg/kg natural nitrite; 14, 0 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 1 min dwell time); 15, 50 mg/kg natural nitrite; 16, 50 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 1 min dwell time); 17, 100 mg/kg natural nitrite; 18, 100 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 1 min dwell time).

Not detected.

Standard error of the differences of least squares means.
FIGURE 4. Effect of ingoing natural nitrite concentration alone and in combination with high hydrostatic pressure on viable Listeria monocytogenes (log CFU per gram) on thin agar layer medium on naturally cured RTE ham stored at 4 ± 1°C

Treatments: 13, 0 mg/kg natural nitrite; 14, 0 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 1 min dwell time); 15, 50 mg/kg natural nitrite; 16, 50 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 1 min dwell time); 17, 100 mg/kg natural nitrite; 18, 100 mg/kg natural nitrite and high hydrostatic pressure (600 MPa, 1 min dwell time)
CHAPTER 8. GENERAL CONCLUSIONS

The objective of investigating the use of natural antimicrobial ingredients and post-lethality interventions, first by themselves and then in combination, that are currently allowed for use under the highly restrictive natural and organic meat and poultry products manufacturing practices was to determine if these interventions represented effective means to inhibit the recovery and growth of *L. monocytogenes* on naturally cured RTE processed meat products. After evaluating each ingredient and post-lethality intervention separately, it was demonstrated that the vinegar and the vinegar and lemon juice concentrate ingredients investigated exerted strong bacteriostatic effects on *L. monocytogenes* whereas cranberry powder did not. However, these ingredients did not reduce initial numbers of viable *L. monocytogenes* inoculated onto the products. Of the post-lethality interventions evaluated, high hydrostatic pressure, octanoic acid, and lauric arginate exhibited significant initial bactericidal effects on but did not prevent the recovery and growth of *L. monocytogenes* upon refrigerated storage of the products. Furthermore, post-packaging thermal treatment (71°C for 30 s) did not affect initial viable *L. monocytogenes* numbers and did not curb the growth of the microorganism upon refrigerated storage of the products. The aforementioned conclusions apply to both naturally cured frankfurters and RTE ham, indicating that differences between these types of products did not influence the antilisterial properties of the ingredients and post-lethality interventions evaluated.

After evaluating the effects of combining each natural antimicrobial ingredient with each post-lethality intervention, results indicated that both initial bactericidal and sustained bacteriostatic effects were exerted on *L. monocytogenes* by combining the
vinegar and the vinegar and lemon juice concentrate ingredients with the high hydrostatic pressure, octanoic acid, and the lauric arginate post-lethality interventions, but not when cranberry powder or post-packaging thermal treatment were employed. Thus, a reduction in initial numbers of viable *L. monocytogenes* and suppression of the growth those cells that may survive post-lethality interventions can be achieved in the production of natural and organic processed meat products by combining the use of certain natural antimicrobial ingredients and post-lethality interventions.

The combination of natural antimicrobial ingredients with post-lethality interventions represents an effective approach to addressing *L. monocytogenes* in natural and organic processed meat products. These hurdles could be instituted by manufacturers of organic and natural processed meat and poultry products in their *L. monocytogenes* control plans. Due to the fact that limited literature exists on the use of the aforementioned natural antimicrobial ingredients in combination with post-lethality interventions, nevertheless, further research should be conducted on their inhibitory properties against *L. monocytogenes* in processed meats. Additionally, the effects of these natural antimicrobial ingredients and post-lethality interventions on the sensory characteristics of natural and organic processed meat and poultry products should also be investigated.
**APPENDIX 1: FLORIDA FOOD PRODUCTS VEGSTABLE™ 504 PRODUCT INFORMATION**

---

**FLORIDA FOOD PRODUCTS, INC. CERTIFICATE OF ANALYSIS**

<table>
<thead>
<tr>
<th>Test</th>
<th>LTP</th>
<th>Specification</th>
<th>Lot Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PHYSICAL TEST:</strong></td>
<td></td>
<td></td>
<td>50411D01F</td>
</tr>
<tr>
<td>Appearance</td>
<td>025</td>
<td>Tan Brown</td>
<td></td>
</tr>
<tr>
<td>Dispersion Rate</td>
<td>013</td>
<td>&lt; 1 min.</td>
<td>Yes</td>
</tr>
<tr>
<td>Moisture Content</td>
<td>005</td>
<td>&lt; 5%</td>
<td>1.1%</td>
</tr>
<tr>
<td><strong>CHEMICAL TEST:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH (6.0% solution @ 25°C)</td>
<td>003</td>
<td>8.5-10</td>
<td>9.5</td>
</tr>
<tr>
<td><strong>MICROBIOLOGICAL TEST:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic Plate Count</td>
<td>017</td>
<td>&lt; 20,000 cfu/gm</td>
<td>420</td>
</tr>
<tr>
<td>Yeast &amp; Mold</td>
<td>018</td>
<td>&lt; 100 cfu/gm</td>
<td>&lt;100 / &lt;100</td>
</tr>
<tr>
<td>E.coli (Detection Limit &gt; 10 cfu/gm)</td>
<td>009</td>
<td>None Detected</td>
<td>None Detected</td>
</tr>
<tr>
<td>Total Coliform (Detection Limit &gt; 10 cfu/gm)</td>
<td>009</td>
<td>None Detected</td>
<td>None Detected</td>
</tr>
</tbody>
</table>

---

Growers and Processors of Food and Cosmetic Ingredients
2231 W. CR 44 • P.O Box 1300 • Eustis, Florida, USA 32727-1300 • Phone (352) 357-4141
Visit our Web Site at: www.floridafood.com

Revised: 2/13/08
Product Specifications and Information

PRODUCT NAME - Veg Stable™ 504

FFP PRODUCT CODE - 504

INGREDIENT DECLARATION - Celery Powder (or Natural Flavors), Sea Salt and Silicon Dioxide (anti-caking).

USE - Meats, natural curing processes

DESCRIPTION - Veg Stable™ 504 is a water soluble dried powder consisting of celery powder and sea salt. Veg Stable™ 504 is high in naturally occurring nitrates that are standardized with sea salt.

GENERAL SPECIFICATIONS

<table>
<thead>
<tr>
<th></th>
<th>Veg Stable™ 504</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPEARANCE</td>
<td>Tan to Brown free flowing powder</td>
</tr>
<tr>
<td>MOISTURE</td>
<td>≤ 5%</td>
</tr>
<tr>
<td>pH (5% Solution)</td>
<td>8.5 - 10</td>
</tr>
<tr>
<td>TOTAL PLATE COUNT</td>
<td>≤ 20,000 cfu/gm</td>
</tr>
<tr>
<td>YEAST AND MOLD</td>
<td>≤ 100 cfu/gm</td>
</tr>
<tr>
<td>TOTAL COLIFORMS</td>
<td>Negative</td>
</tr>
<tr>
<td>PRESERVATIVES</td>
<td>None</td>
</tr>
</tbody>
</table>

SPECIAL INSTRUCTIONS - Store in ambient temperatures (< 70°F). Avoid temperature extremes.

PACKAGING - Available in 44.1 lbs. (20 kg) vacuum-sealed foil bag-n-box.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross</td>
<td>47.2 lbs. (21.4 kg)</td>
</tr>
<tr>
<td>Net</td>
<td>44.1 lbs (20kg)</td>
</tr>
</tbody>
</table>

SHELF STABILITY AND STORAGE - Keep container tightly sealed when not in use. Store in cool, dry area not exceeding 90°F. If stored at or below 70°F, the recommended shelf life is two years.

SUGGESTED USAGE - 0.1% to 0.4% of finished weight.

The technical information and suggestions for use contained herein are believed to be reliable, but they are not to be construed as warranties and no patent liability can be assumed. Specifications are subject to change based on raw material variations.
### APPENDIX 2: OCEAN SPRAY INTERNATIONAL CRANBERRY 90MX

#### PRODUCT INFORMATION

**Production Date:** 03/10/2010  
**Best before:** 10 Mar 2013

**CERTIFICATE OF ANALYSIS**  
**UPC 94513-000**  
**90MX CRANBERRY POWDER – 50 lb.**

<table>
<thead>
<tr>
<th>LOT # 031010B</th>
<th><strong>Specification</strong></th>
<th><strong>Test Result</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Drums #: 1783-1962</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test Name</td>
<td>% Moisture</td>
<td>2 - 5%</td>
</tr>
<tr>
<td></td>
<td>pH (1% solution)</td>
<td>3.50 - 4.50</td>
</tr>
<tr>
<td></td>
<td>Titratable Acidity (as citric acid)</td>
<td>8.5 minimum</td>
</tr>
<tr>
<td></td>
<td>Solubility (5g/100ml H2O)</td>
<td>Good -- No Lumps</td>
</tr>
<tr>
<td></td>
<td>Bulk Density (Loose)</td>
<td>0.450 - 0.600 g/cc</td>
</tr>
<tr>
<td></td>
<td>Color</td>
<td>Compares to Standard</td>
</tr>
<tr>
<td></td>
<td>Flavor &amp; Odor</td>
<td>Compares to Standard</td>
</tr>
<tr>
<td></td>
<td>Granulation (30 Mesh)</td>
<td>100% Through</td>
</tr>
<tr>
<td></td>
<td>Granulation (100 Mesh)</td>
<td>95% Through</td>
</tr>
</tbody>
</table>

#### Microbiology Results

| | **Specification** | **Test Result** |
| | Yeast (C.F.U. per gram) | <100 | <10 |
| | Mold (C.F.U. per gram) | <100 | <10 |
| | Aerobic Plate Count (C.F.U. per gram) | <1,000 | <250 |
| | Escherichia coli | Negative in 25g | Neg |
| | Salmonella | Negative in 25g | Neg |
| | Staph. aureus (coagulase positive) | Negative in 25g | Neg |

* Shelf life is three years from production date.

No solvents were used in the manufacturing of this material.

Quality Operating Guidelines
Customer Ingredient Specifications

TITLE: CRANBERRY 90MX POWDER
DATE: 09 FEB 09
SUPERScedes: 04 MAY 07

PRODUCT DESCRIPTION:
The powder is fine, free-flowing and rosy red in color. Processing consists of spray drying cranberry concentrate with magnesium hydroxide as the carrier and tri-calcium phosphate as an anti-caking agent. The powder contains approximately 90% cranberry solids. Cranberry 90MX Powder is prepared from the juice of sound, mature berries of the commonly cultivated cranberry plant (Genus/species – Vaccinium macrocarpon). Processing conforms to all provisions of the Food, Drug and Cosmetic Act.

<table>
<thead>
<tr>
<th>SPECIFICATIONS</th>
<th>TOLERANCE</th>
<th>METHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Moisture</td>
<td>2% - 5%</td>
<td>Computrac</td>
</tr>
<tr>
<td>pH</td>
<td>3.50 – 4.50</td>
<td>pH meter (10% solution)</td>
</tr>
<tr>
<td>% Titratable Acidity (as citric acid)</td>
<td>8.5% w/w minimum</td>
<td>Titration</td>
</tr>
<tr>
<td>% Organic Acids</td>
<td>30% minimum</td>
<td>HPLC-AOAC Method</td>
</tr>
<tr>
<td>% Proanthocyanidins (PAC's)</td>
<td>*Contact your Broker / Agent for comparative values.</td>
<td></td>
</tr>
<tr>
<td>Solubility</td>
<td>Good</td>
<td>5g powder in 100 ml 50°F (10°C) water. Powder will completely dissolve in 2 minutes.</td>
</tr>
<tr>
<td>Bulk Density</td>
<td>0.450 – 0.600 g/cc</td>
<td>Tare 200-ml cup. Funnel is product and level with a straight edge. Divide weight by volume to obtain density.</td>
</tr>
<tr>
<td>Appearance</td>
<td>Fine, free-flowing and rosy red in color.</td>
<td>Visual</td>
</tr>
<tr>
<td>Flavor and Aroma (of solution)</td>
<td>Clean, typical cranberry flavors with no burned or off notes.</td>
<td>Sensory</td>
</tr>
<tr>
<td>Screen Analysis</td>
<td>Through 30 mesh 100%</td>
<td>Rotap with hammer.</td>
</tr>
</tbody>
</table>

Ocean Spray Cranberries Ingredient Technology Group
One Ocean Spray Drive, Lakeville/Middleboro, MA 02349
Telephone: 508-946-7227  Fax: 508-946-4994
CRANBERRY 90MX POWDER
UPC 94513

| MICROBIOLOGICAL (per g or mL): Analysis represents an average sampling. |
|---------------------------------|-----------------|
| Yeast                           | ≤ 100 CFU per gram |
| Mold                            | ≤ 100 CFU per gram |
| Aerobic Plate Count             | ≤ 1,000 CFU per gram |
| *Escherichia coli*              | Negative in 25 grams |
| *Salmonella*                    | Negative in 25 grams |

PACKAGING

94513 – Double poly-lined fiberboard drum with tamper-evident tape. One desiccant pouch is placed between the two sealed bags and another desiccant bag is on top of the outer sealed bag. Contains 50 lbs. (22.7 kg) powder net.

LABELING

All containers shall bear the following information: name of product including UPC, ingredients, net contents, manufacture date (year/ Julian date), best before date (day/month/year) and lot number, Triangle K (for Kosher), Ocean Spray® name and address.

INGREDIENTS

Cranberry Juice Concentrate, Magnesium Hydroxide, Tricalcium Phosphate [anti-caking agent]

STORAGE RECOMMENDATIONS

Recommend conditioned shipping and storage of 50°-70°F (10°-21°C), 50% relative humidity. Protect from moisture.

EXPECTED SHELF-LIFE

At least three years when protected from moisture and excessive heat.

OCEAN SPRAY® WARRANTS ONLY THAT THE CRANBERRY 90MX POWDER, 94513 SOLD SHALL CONFORM TO THESE SPECIFICATIONS. ALL OTHER WARRANTIES, EXPRESSED OR IMPLIED, INCLUDING ANY WARRANTIES OF MERCHANTABILITY OR FITNESS, ARE EXCLUDED.
APPENDIX 3: WTI INGREDIENTS, INC. DV PRODUCT INFORMATION

MATERIAL SAFETY DATA SHEET

IDENTITY (As used on Label and List)

**DV**

**Section I - Contact Information**

<table>
<thead>
<tr>
<th>Manufacturer's Name</th>
<th>Emergency Telephone Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>World Technology Ingredients, Inc.</td>
<td>706-387-5150</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Address (Number, Street, City, State and ZIP Code)</th>
<th>Telephone Number for Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>281 Martin Luther King Ave. Jefferson, Georgia 30549</td>
<td>706-387-5150</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Date Prepared</th>
<th>Preparer</th>
</tr>
</thead>
<tbody>
<tr>
<td>January 5, 2011</td>
<td>Jenni Ralph</td>
</tr>
</tbody>
</table>

**Section II - Hazardous Ingredients/Identity Information**

<table>
<thead>
<tr>
<th>Other Limits</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Hazardous Components</th>
<th>CAS Number</th>
<th>OSHA PEL</th>
<th>ACGIH TLV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Vinegar</td>
<td>CAS 8028-52-2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Section III - Physical/Chemical Characteristics**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiling Point</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Specific Gravity (H₂O = 1)</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Vapor Pressure (mm Hg)</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Melting Point</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Vapor Density (AIR = 1)</td>
<td>Evaporation Rate (Butyl Acetate = 1)</td>
</tr>
<tr>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Solubility in Water</td>
<td>Highly soluble in water</td>
</tr>
<tr>
<td>Appearance and Color</td>
<td>Beige and white powder</td>
</tr>
<tr>
<td>Odor</td>
<td>Slight vinegar</td>
</tr>
</tbody>
</table>

**Section IV - Fire and Explosion Hazard Data**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flash Point (Method Used)</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Flammable Limits in Air, %</td>
<td>Not applicable</td>
</tr>
<tr>
<td>LEL</td>
<td>Not applicable</td>
</tr>
<tr>
<td>UEL</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Extinguishing Media</td>
<td>Hyd, CO₂, Foam, Dry Chemical</td>
</tr>
<tr>
<td>Special Fire Fighting Procedures</td>
<td>None</td>
</tr>
</tbody>
</table>

**Unusual Fire and Explosion Hazards**

None
### Section V - Reactivity Data

<table>
<thead>
<tr>
<th>Stability</th>
<th>Conditions to Avoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstable</td>
<td>Prolonged storage at elevated temperatures</td>
</tr>
<tr>
<td>Stable</td>
<td>X</td>
</tr>
</tbody>
</table>

**Incompatibility (Materials to Avoid)**

**Hazardous Decomposition or Byproducts**

*None*

**Hazardous Polymerization**

<table>
<thead>
<tr>
<th>May Occur</th>
<th>Conditions to Avoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Will not Occur</td>
<td>X</td>
</tr>
</tbody>
</table>

### Section VI - Health Hazard Data

**Route(s) of Entry:**

- Inhalation?
- Skin?
- Ingestion?

*May cause irritation if contact with eyes. No known adverse effects when in contact with skin. Inhalation may cause slight irritation. Ingestion causes no known symptoms or irritation.*

**Health Hazards (Acute and Chronic)**

*None*

**Carcinogenicity:**

- NIP?
- IARC Monographs?
- OSHA Regulated?

*None, Not applicable, Not applicable, No*

**Signs and Symptoms of Exposure**

*See above*

**Medical Conditions / Generally Aggravated by Exposure**

*None reported, handle with reasonable care*

**Emergency and First Aid Procedures**

- **If Inhaled:** Remove to fresh air.
- **Eye contact:** Immediately flush eyes with water for 15 minutes. Get immediate medical attention.
- **If swallowed:** Drink lots of water.

### Section VII - Precautions for Safe Handling and Use

**Steps to Be Taken in Case Material is Released or Spilled**

*Material should be mopped/vacuumed up for disposal. Comply with all applicable governmental regulations on spill reporting, handling, and disposal waste.*

**Waste Disposal Method**

*Product is biodegradable and therefore can be released to the environment when adequately diluted with water.

**Precautions to Be Taken in Handling and Storage**

- Wear safety glasses and rubber gloves when handling. Wash thoroughly after handling.
- Avoid direct contact with eyes, skin, and clothing. Avoid temperature extremes store at ambient temperatures less than 85°F.

**Other Precautions**

*This product is intended for use in food, animal feed, drug, or cosmetic manufacture and it has been produced and packaged in accordance with good manufacturing practices.*
### Section VIII - Control Measures

<table>
<thead>
<tr>
<th>Respiratory Protection (Specify Type)</th>
<th>Not applicable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventilation</td>
<td></td>
</tr>
<tr>
<td>Local Exhaust</td>
<td>Special None</td>
</tr>
<tr>
<td>Mechanical (General)</td>
<td>Other</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protective Gloves</th>
<th>Eye Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impervious gloves while in continuous contact</td>
<td>Safety glasses</td>
</tr>
</tbody>
</table>

Other Protective Clothing or Equipment

- Wear long sleeves to reduce exposed skin area.

Work/Hygienic Practices

- Not applicable

OSHA 174, Sept. 1995

MSDS DV Page 3
### CERTIFICATE OF ANALYSIS

**Formula:** DV  
**Lot No.:** 0911190301  
**Produced on:** April 01, 2011  
**PO Number:** Sample

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5% solution</td>
<td>5.87</td>
<td>5.60-6.20</td>
</tr>
</tbody>
</table>
# APPENDIX 4: WTI INGREDIENTS, INC. MOSTATIN LV1X PRODUCT INFORMATION

---

**MATERIAL SAFETY DATA SHEET**

**Mostatin™ LV1X**

<table>
<thead>
<tr>
<th>Section I</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Manufacturer's Name</strong></td>
<td>World Technology Ingredients, Inc.</td>
</tr>
<tr>
<td><strong>Address (Number, Street, City, State and ZIP Code)</strong></td>
<td>281 Martin Luther King Ave., Jefferson, Georgia 30549</td>
</tr>
<tr>
<td><strong>Emergency Telephone Number</strong></td>
<td>706-387-5150</td>
</tr>
<tr>
<td><strong>Telephone Number for Information</strong></td>
<td>706-387-5150</td>
</tr>
<tr>
<td><strong>Date Prepared</strong></td>
<td>March 13, 2008</td>
</tr>
<tr>
<td><strong>Preparer</strong></td>
<td>Rory McClintock</td>
</tr>
</tbody>
</table>

**Section II - Hazardous Ingredients/Identity Information**

- **Other Limits:**
  - **Hazardous Components** (Specify chemical identity, common name(s), OSHA PEL, ACGIH TLV, Recommended % (optional))
  - Distilled Vinegar: CAS 8028-52-2

**Section III - Physical/Chemical Characteristics**

- **Boiling Point:** 110 - 116°C
- **Specific Gravity (H₂O = 1):** 1.51
- **Vapor Pressure (mm Hg):** N/A
- **Melting Point:** N/A
- **Vapor Density (AIR = 1):** N/A
- **Evaporation Rate (Butyl Acetate = 1):** N/A
- **Solubility in Water:** Highly soluble in cold water
- **Appearance and Odor:**
  - Appearance: Yellowish brown opaque liquid
  - Odor: Slight Lemon and vinegar

**Section IV - Fire and Explosion Hazard Data**

- **Flash Point (Method Used):** N/A
- **Flammable Limits in Air:** N/A
- **LEL:** N/A
- **UEL:** N/A
- **Extinguishing Media:** H₂O, CO₂, Foam, Dry Chemical
- **Special Fire Fighting Procedures:** None
- **Unusual Fire and Explosion Hazards:** None
### Section V - Reactivity Data

<table>
<thead>
<tr>
<th>Stability</th>
<th>Unstable</th>
<th>Stable</th>
<th>Conditions to Avoid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Prolonged storage at elevated temperatures</strong></td>
</tr>
</tbody>
</table>

**Incompatibility (Materials to Avoid)**

**Hazardous Decomposition or Byproducts**

None

**Hazardous Polymerization**

<table>
<thead>
<tr>
<th>May Occur</th>
<th>Conditions to Avoid</th>
<th>Will not Occur</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>X</strong></td>
</tr>
</tbody>
</table>

### Section VI - Health Hazard Data

**Route(s) of Entry**

- **Inhalation?**
- **Skin?**
- **Ingestion?**

- May cause irritation if contact with eyes. No known adverse effects when in contact with skin. Inhalation may cause slight irritation.
- Ingestion causes no known symptoms or irritation.

**Health Hazards (Acute and Chronic)**

None

**Carcinogenicity**

- None

**NTP Monographs?**

N/A

**IARC Monographs?**

N/A

**OSHA Regulated?**

No

**Signs and Symptoms of Exposure**

See above

**Medical Conditions Generally Aggravated by Exposure**

None reported, handle with reasonable care

**Emergency and First Aid Procedures**

- **If inhaled:** Remove to fresh air.
- **Eye contact:** Immediately flush eyes with running water for 15 minutes. Get immediate medical attention.
- **If swallowed:** Drink lots of water.

### Section VII - Precautions for Safe Handling and Use

**Steps to Be Taken in Case Material is Released or Spilled**

- Material should be mopped up and vacuumed up for disposal. Comply with all applicable governmental regulations on spill reporting, handling, and disposal waste.

**Waste Disposal Method**

- Product is biodegradable and therefore can be released to the environment when adequately diluted with water

**Precautions to be Taken in Handling and Storing**

- Wear safety glasses and rubber gloves when handling. Wash thoroughly after handling.
- Avoid direct contact with eyes, skin, and clothing. Avoid temperature extremes.
- Store at ambient temperatures less than 85°F.

**Other Precautions**

- This product is intended for use in food, animal feed, drug, or cosmetic manufacture and it has been produced and packaged in accordance with good manufacturing practices.
### Section VIII - Control Measures

<table>
<thead>
<tr>
<th>Respiratory Protection (Specify Type)</th>
<th>Not applicable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventilation</td>
<td>Local Exhaust</td>
</tr>
<tr>
<td></td>
<td>Special None</td>
</tr>
<tr>
<td>Mechanical (General)</td>
<td>Other</td>
</tr>
<tr>
<td>Protective Gloves</td>
<td>Impervious gloves while in continuous contact</td>
</tr>
<tr>
<td></td>
<td>Eye Protection</td>
</tr>
<tr>
<td>Other Protective Clothing of Equipment</td>
<td>Safety glasses</td>
</tr>
<tr>
<td>Work/Hygiene Practices</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>

Other Protective Clothing of Equipment:
- Wear long sleeves to reduce exposed skin area.

OSHA 174, Sept. 1995
CERTIFICATE OF ANALYSIS

Formula: MOstatin LV1X
Lot No: 0451190302
Produced on: February 14, 2011
PO Number: Sample

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Conforms</td>
<td>Yellowish Brown Opaque Liq</td>
</tr>
<tr>
<td>Density g/cc</td>
<td>1.25</td>
<td>1.04-1.04</td>
</tr>
<tr>
<td>Odor</td>
<td>Conforms</td>
<td>Slight Citrus</td>
</tr>
<tr>
<td>pH 10% solution</td>
<td>5.66</td>
<td>5.55-5.85</td>
</tr>
</tbody>
</table>
APPENDIX 5: ECOLAB, INC. OCTA-GONE PRODUCT INFORMATION

Material Safety Data Sheet
OCTA-GONE

1. Product and company identification

<table>
<thead>
<tr>
<th>Trade name of product</th>
<th>OCTA-GONE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product use</td>
<td>Food additive.</td>
</tr>
<tr>
<td>Product dilution information</td>
<td>Up to 34.56 oz/gal in water</td>
</tr>
</tbody>
</table>

Supplier's Information

<table>
<thead>
<tr>
<th>Code</th>
<th>902900-01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of issue</td>
<td>08-March-2011</td>
</tr>
</tbody>
</table>

EMERGENCY HEALTH INFORMATION: 1-800-328-0026
Outside United States and Canada CALL 1-651-222-5352 (In USA)

2. Hazards identification

<table>
<thead>
<tr>
<th>Physical state</th>
<th>Product AS SOLD</th>
<th>Liquid.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emergency overview</td>
<td>CAUTION !</td>
<td>MAY CAUSE EYE IRRITATION. Avoid contact with eyes. Wash thoroughly after handling.</td>
</tr>
</tbody>
</table>

Potential acute health effects

<table>
<thead>
<tr>
<th>Eyes</th>
<th>Moderately irritating to eyes.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Slightly irritating to the skin.</td>
</tr>
<tr>
<td>Inhalation</td>
<td>No known significant effects or critical hazards.</td>
</tr>
<tr>
<td>Ingestion</td>
<td>No known significant effects or critical hazards.</td>
</tr>
</tbody>
</table>

Product AT USE DILUTION

| Product AT USE DILUTION | CAUTION ! | MAY CAUSE EYE IRRITATION. Avoid contact with eyes. Wash thoroughly after handling. |

3. Composition/information on ingredients

<table>
<thead>
<tr>
<th>Name</th>
<th>CAS number</th>
<th>% by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>CITRIC ACID</td>
<td>77-92-9</td>
<td>1 - 5</td>
</tr>
<tr>
<td>CAPRYLIC ACID</td>
<td>124-07-2</td>
<td>1 - 5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>CAS number</th>
<th>% by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>CITRIC ACID</td>
<td>77-92-9</td>
<td>1 - 5</td>
</tr>
<tr>
<td>CAPRYLIC ACID</td>
<td>124-07-2</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>
4. First aid measures

<table>
<thead>
<tr>
<th>Condition</th>
<th>Product AS SOLD</th>
<th>Product AT USE DILUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye contact</td>
<td>In case of contact, immediately flush eyes with plenty of water. Remove contact lenses and flush again. Get medical attention if irritation persists.</td>
<td>Rinse with water for a few minutes.</td>
</tr>
<tr>
<td>Skin contact</td>
<td>Rinse with water for a few minutes.</td>
<td>No known effect after skin contact. Rinse with water for a few minutes.</td>
</tr>
<tr>
<td>Inhalation</td>
<td>No special measures required. Treat symptomatically.</td>
<td>No special measures required. Treat symptomatically.</td>
</tr>
<tr>
<td>Ingestion</td>
<td>Get medical attention if symptoms occur.</td>
<td>Get medical attention if symptoms occur.</td>
</tr>
</tbody>
</table>

5. Fire-fighting measures

- **Product AS SOLD**: Decomposition products may include the following materials:
  - Carbon dioxide
  - Carbon monoxide
  - Metal oxide/oxides

- **Fire-fighting media and instructions**: Use an extinguishing agent suitable for the surrounding fire. Dike area of fire to prevent runoff. In a fire or if heated, a pressure increase will occur and the container may burst.

- **Special protective equipment for fire-fighters**: Fire-fighters should wear appropriate protective equipment and self-contained breathing apparatus (SCBA) with a full face-piece operated in positive pressure mode.

6. Accidental release measures

<table>
<thead>
<tr>
<th>Condition</th>
<th>Product AS SOLD</th>
<th>Product AT USE DILUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Personal precautions</td>
<td>Use personal protective equipment as required.</td>
<td>Use personal protective equipment as required.</td>
</tr>
<tr>
<td>Environmental precautions</td>
<td>Avoid contact of large amounts of spilled material and runoff with soil and surface waterways.</td>
<td>Avoid contact of large amounts of spilled material and runoff with soil and surface waterways.</td>
</tr>
<tr>
<td>Methods for cleaning up</td>
<td>Use a water rinse for final clean-up.</td>
<td>Use a water rinse for final clean-up.</td>
</tr>
</tbody>
</table>

7. Handling and storage

- **Product AS SOLD**: Avoid contact with eyes. Do not mix with bleach or other chlorinated products - will cause chlorine gas. Wash thoroughly after handling.

- **Product AT USE DILUTION**: Avoid contact with eyes. Do not mix with bleach or other chlorinated products - will cause chlorine gas. Wash thoroughly after handling.

- **Handling**: Keep out of reach of children. Store in a closed container. Do not store below the following temperature: 0°C

- **Storage**: Keep out of reach of children. Store in a closed container.

8. Exposure controls/personal protection
8. **Exposure controls/personal protection**

<table>
<thead>
<tr>
<th>Engineering measures</th>
<th>Product AS SOLD</th>
<th>Good general ventilation should be sufficient to control worker exposure to airborne contaminants.</th>
<th>Product AT USE DILUTION</th>
<th>Good general ventilation should be sufficient to control worker exposure to airborne contaminants.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Personal protection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eyes</td>
<td>Safety eyewear should be used when there is a likelihood of exposure.</td>
<td>No protective equipment is needed under normal use conditions.</td>
<td>No protective equipment is needed under normal use conditions.</td>
<td>No protective equipment is needed under normal use conditions.</td>
</tr>
<tr>
<td>Hands</td>
<td>No protective equipment is needed under normal use conditions.</td>
<td>No protective equipment is needed under normal use conditions.</td>
<td>No protective equipment is needed under normal use conditions.</td>
<td>No protective equipment is needed under normal use conditions.</td>
</tr>
<tr>
<td>Skin</td>
<td>No protective equipment is needed under normal use conditions.</td>
<td>No protective equipment is needed under normal use conditions.</td>
<td>No protective equipment is needed under normal use conditions.</td>
<td>No protective equipment is needed under normal use conditions.</td>
</tr>
<tr>
<td>Respiratory</td>
<td>No special protection is required.</td>
<td>Expiration</td>
<td>No special protection is required.</td>
<td>Expiration</td>
</tr>
<tr>
<td>Consult local authorities for acceptable exposure limits.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

9. **Physical and chemical properties**

<table>
<thead>
<tr>
<th>Physical state</th>
<th>Product AS SOLD</th>
<th>Liquid.</th>
<th>Product AT USE DILUTION</th>
<th>Liquid. &gt; 100°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flash point</td>
<td>&gt; 100°C</td>
<td>Product does not support combustion.</td>
<td>Pale color</td>
<td>Faint odor</td>
</tr>
<tr>
<td>Color</td>
<td>Yellow [Light]</td>
<td>Faint odor</td>
<td>2.5 to 3.5</td>
<td></td>
</tr>
<tr>
<td>Odor</td>
<td>Faint odor</td>
<td>Faint odor</td>
<td>2.5 to 3.5</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>3.2 [Conc. (% w/w): 100%]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boiling/condensation point</td>
<td>&gt;100°C (=212°F)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative density</td>
<td>1.073</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solubility</td>
<td>Easily soluble in the following materials: cold water and hot water.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

10. **Stability and reactivity**

<table>
<thead>
<tr>
<th>Product AS SOLD</th>
<th>The product is stable. Under normal conditions of storage and use, hazard polymerization will not occur.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactivity</td>
<td>Reactive or incompatible with the following materials: alcohols. Do not mix with bleach or other chlorinated products - will cause chlorine gas.</td>
</tr>
<tr>
<td>Hazardous decomposition products</td>
<td>Under normal conditions of storage and use, hazardous decomposition products should not be produced.</td>
</tr>
<tr>
<td>Hazardous polymerization</td>
<td>Under normal conditions of storage and use, hazardous polymerization will not occur.</td>
</tr>
</tbody>
</table>

11. **Toxicological information**

<table>
<thead>
<tr>
<th>Potential acute health effects</th>
<th>Product AS SOLD</th>
<th>Slightly irritating to the eyes.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eyes</td>
<td>Moderately irritating to the eyes.</td>
<td>No known significant effects or critical hazards.</td>
</tr>
<tr>
<td>Skin</td>
<td>Slightly irritating to the skin.</td>
<td>No known significant effects or critical hazards.</td>
</tr>
<tr>
<td>Inhalation</td>
<td>No known significant effects or critical hazards.</td>
<td>No known significant effects or critical hazards.</td>
</tr>
<tr>
<td>Ingestion</td>
<td>No known significant effects or critical hazards.</td>
<td>No known significant effects or critical hazards.</td>
</tr>
</tbody>
</table>
12. Ecological information
Ecotoxicity: Not reported

13. Disposal considerations

<table>
<thead>
<tr>
<th>Waste disposal</th>
<th>Product AS SOLD</th>
<th>Product AT USE DILUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted product can be flushed to sanitary sewer. Discard empty container in trash.</td>
<td>Diluted product can be flushed to sanitary sewer. Discard empty container in trash.</td>
<td></td>
</tr>
</tbody>
</table>

14. Transport information
Certain shipping modes or package sizes may have exceptions from the transport regulations. The classification provided may not reflect those exceptions and may not apply to all shipping modes or package sizes.

<table>
<thead>
<tr>
<th>Product AS SOLD</th>
<th>DOT Classification</th>
</tr>
</thead>
</table>

For transport in bulk or using IMDG regulations, see shipping documents for specific transportation information.

15. Regulatory information

<table>
<thead>
<tr>
<th>Product AS SOLD</th>
<th>Product AT USE DILUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCS Classification: Irritating material</td>
<td>Not regulated.</td>
</tr>
</tbody>
</table>

U.S. Federal regulations

<table>
<thead>
<tr>
<th>TSCA 8(b) inventory</th>
<th>CAS number</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>All components are listed or exempted.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SARA 302/304/311/312 extremely hazardous substances: No listed substance
SARA 302/304 emergency planning and notification: No listed substance

SARA 313
Form R - Reporting requirements: No listed substance

California Prop. 65: No listed substance

16. Other information

<table>
<thead>
<tr>
<th>Health</th>
<th>Flammability</th>
<th>Physical hazards</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

National Fire Protection Association (U.S.A.)
### 16. Other information

<table>
<thead>
<tr>
<th>Health</th>
<th>Flammability</th>
<th>Instability/Reactivity</th>
<th>Special</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Health</th>
<th>Flammability</th>
<th>Instability/Reactivity</th>
<th>Special</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Date of issue**: 08-March-2011  
**Responsible name**: Regulatory Affairs  
1-800-352-5326  

\(\wedge\) Indicates information that has changed from previously issued version.  

**Notice to reader**

The above information is believed to be correct with respect to the formula used to manufacture the product in the country of origin. As data, standards, and regulations change, and conditions of use and handling are beyond our control, NO WARRANTY, EXPRESS OR IMPLIED, IS MADE AS TO THE COMPLETENESS OR CONTINUING ACCURACY OF THIS INFORMATION.
Octa-Gone Mix Instructions and Directions for Use

Materials Required:
Octa-Gone
Water with total alkalinity less than 300 ppm

Equipment Required:
Top-loading balance or graduated cylinder
Clean mixing vessel
Clean mixing device, i.e. a stir bar

Procedure:
1. Determine the quantity of use solution you would like to make. Our best recommended practice is an application rate of 0.12 mL/m² or approximately 20 µL/cm² of surface area of product.
2. If you are making the solution up weight/weight a 25.0% dilution is required.
   If you are making the solution volumetrically a 23.4% dilution is required.

Weight/Weight Dilution
1. After determining the mass of use solution, multiply by 25% to determine the mass of Octa-Gone required. Add this amount of Octa-Gone to a mixing vessel.
2. Add water until the total mass of use solution is achieved.

Example: For 1000 grams of use-solution:
1000 grams x 25.0% = 250 grams of Octa-Gone
1000 - 250 = 750 grams of water

Volume/Volume Dilution
1. After determining the volume of use solution, multiply by 23.5% to determine the volume of Octa-Gone required. Add this volume of Octa-Gone to a mixing vessel.
2. Add water until the total volume of use solution is achieved.

Example: For 1000 milliliters of use-solution
1000 mL x 23.4% = 234 milliliters of Octa-Gone
1000 - 234 = 766 milliliters of water (approximate)

3. After adding the water, mix solution in the vessel with a mixing device.
4. Apply to food product surface at the application rate recommended above.
5. For best results the Octa-Gone use solution should be used within 24 hours.

If you have additional questions, contact your Ecolab Inc. account representative.

Octa-Gone Mix Instructions, November 2007.doc
## APPENDIX 6: PURAC, PROTECT-M PRODUCT INFORMATION

### Preliminary SAFETY DATA SHEET

**Revision Date:** 10/10/07  
**Ref.:** SD2007-01

<table>
<thead>
<tr>
<th><strong>Product name</strong></th>
<th>Protect M</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Use of the Substance</strong></td>
<td>Anti-microbial for food products</td>
</tr>
</tbody>
</table>
| **Supplier** | PURAC America, Inc.  
111 Barclay Blvd.,  
Lincolnshire, IL 60069  
USA |
| **Telephone** | +1 847 634 6330 |
| **Fax** | +1 847 634 1992 |
| **Emergency telephone** | (909) 481 9700 |

### Chemical nature of the preparation

<table>
<thead>
<tr>
<th><strong>Synonyms</strong></th>
<th>LAE, lauric arginate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Components</strong></td>
<td>Lauric arginate</td>
</tr>
</tbody>
</table>
| **EC-No.** | 434-630-6  
**CAS-No.** | 60372-77-2  
**Weight, %** | 10-11 |
| **Hazard classification** | R41, R50. For details see chapter 15. |

### HAZARDS IDENTIFICATION

| **Classification** | XI: R41  
N: R50 |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Most important hazards</strong></td>
<td>Risk of serious damage to eyes</td>
</tr>
<tr>
<td><strong>Specific hazards</strong></td>
<td>Toxic to aquatic organisms</td>
</tr>
</tbody>
</table>

---

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Additional safety precautions and/or information can be found at www.purac.com.
4. FIRST AID MEASURES

General advice
Immediate medical attention is not required. Show this safety data sheet to the doctor in attendance.

Inhalation
Not applicable.

Skin contact
Not applicable.

Eye contact
Rinse thoroughly with plenty of water for at least 15 minutes, keeping the eyelids separated. In case of discomfort, seek advice of an ophthalmologist.

Ingestion
Even the product is not dangerous for that exposition way, in case of accidental event, do not induce vomiting and seek for hospital assistance.

5. FIRE-FIGHTING MEASURES

Suitable extinguishing media
Low risk of flammability (flash point in open cup 123°C). In case of fire, use water spray, water with foam forming agent, carbonic anhydride, universal foam, extinguishing powder or any other available extinguishing media.

Extinguishing media which must not be used for safety reasons
None.

Special protective equipment for firefighters
Use a complete fire-fighting suit or an appropriate wear in accordance with the created emergency situation, and a self-contained breathing apparatus.

Additional information
Keep containers away from dangerous areas, and cool them using water spray, if they are exposed to fire or strong heat. Do not inhale the fumes and vapours due to heating or combustion of the product.

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Use safety goggles.

Environmental precautions
Do not allow to spill into public sewage systems.

Methods for cleaning up
Mechanic barriers are recommended to avoid the spread out of the product. Pick up the product with absorbent inert material and keep it in tightly closed containers to recovery or controlled disposal. Drag remains with plenty of water.
7. HANDLING AND STORAGE

Handling

Technical measures/Precautions
No special technical protective measures required.

Safe handling advice
Use safety goggles to prevent direct splashes on the eyes. Depending on handling conditions, it will require equipments with anti-deflagrating electric systems.

Storage

Technical measures/Storage conditions
Store in dry, cool and well-ventilated places away from the sun, heat and sources of ignition or strong heat. Keep the product in its original containers tightly closed and away from incompatible material. Avoid temperatures above 30°C and below 4°C on storage.

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Respiratory protection
Special measures are not required.

Hand protection
As a protective measure, use gloves.

Eye protection
Safety goggles.

Skin protection
Special measures are not required.

Hygiene measures
Avoid eating, drinking or smoking while handling the product.
Preliminary
SAFETY DATA SHEET
2001/58/EC

Lauric arginate
Revision Date: 16/10/07
Ref: SD/2007-01

9. PHYSICAL AND CHEMICAL PROPERTIES

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Form</td>
<td>transparent or slightly opalescent liquid</td>
</tr>
<tr>
<td>Color</td>
<td>slightly yellow</td>
</tr>
<tr>
<td>Odor</td>
<td>specific, faint</td>
</tr>
<tr>
<td>pH</td>
<td>4 ± 2 @ 20°C</td>
</tr>
<tr>
<td>Boiling point/range</td>
<td>no data available</td>
</tr>
<tr>
<td>Flash point</td>
<td>approx. 122°C (open cup)</td>
</tr>
<tr>
<td>Explosion limits</td>
<td>not applicable</td>
</tr>
<tr>
<td>Density</td>
<td>no data available</td>
</tr>
<tr>
<td>Solubility in water</td>
<td>dispersible</td>
</tr>
<tr>
<td>Solubility in other solvents</td>
<td>soluble in ethanol and glycerine</td>
</tr>
<tr>
<td>Viscosity</td>
<td>&lt; 150 cPs</td>
</tr>
</tbody>
</table>

10. STABILITY AND REACTIVITY

Stability
Stable at normal conditions of pressure and temperature. No dangerous reactions are expected.

Conditions to avoid
Temperatures above 30°C and below 4°C.

Materials to avoid
Strong oxidizing agents, Alkalis.

Hazardous decomposition products
None. By total combustion carbon oxides and nitrogen oxides.
## 11. TOXICOLOGICAL INFORMATION

<table>
<thead>
<tr>
<th>Acute toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD₅₀ oral in rats: above 2000 mg/kg</td>
</tr>
<tr>
<td>LD₅₀ dermal in rats: above 2000 mg/kg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chronic toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOAEL in rats: 907 mg/kg bw/day</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Two generation reproductive study</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOAEL in rats: 1073 mg/kg bw/day</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td>The available data about the product shows no sensitizing effects by any exposition way.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other information and additional indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>No mutagenic, no clastogenic substance. Risk of serious damage to eyes.</td>
</tr>
</tbody>
</table>

## 12. ECOLOGICAL INFORMATION

<table>
<thead>
<tr>
<th>Environmental behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biodegradability: 89% degradation (29 days modified storm test). Hydrolysis rate: half-life periods of &gt; 1 year, 57.3 days and 34 hours at pH values of 4, 7 and 9, respectively.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ecotoxicity effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC₅₀ for Daphnia magna (96 h): 23.7 mg/L</td>
</tr>
<tr>
<td>LC₅₀ for Daphnia magna (48 h): 6.54 mg/L</td>
</tr>
<tr>
<td>EC₅₀ for algae (72 h): 0.461 mg/L</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Additional ecological indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption/desorption test: KOC 58.8 at 18.5 °C. According to this result, the product is not bioaccumulative.</td>
</tr>
</tbody>
</table>

## 13. DISPOSAL CONSIDERATIONS

<table>
<thead>
<tr>
<th>Product related</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incineration in authorized premises in accordance with the current legislation.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Container and packing related</th>
</tr>
</thead>
<tbody>
<tr>
<td>Once washed thoroughly they can be re-used. If not, dispose of them according to regulations in force.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Further information</th>
</tr>
</thead>
<tbody>
<tr>
<td>According to the European Waste Catalogue, Waste Codes are not product specific, but application specific. Waste codes should be assigned by the user based on the application for which the product was used.</td>
</tr>
</tbody>
</table>

## 14. TRANSPORT INFORMATION

| Not classified as dangerous in the meaning of transport regulations. |

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www.purac.com
15. REGULATORY INFORMATION

The product is classified in accordance with Annex VI to Directive 67/548/EEC.

Symbols

R- Phrases

S-Phrases

German Water Hazard Class (WGK): 1

16. OTHER INFORMATION

Test of symbols and R phrases

N : dangerous for the environment
R-50 : very toxic to aquatic organisms

Further information on the safety assessment of sodium lactate and lactic acid can be obtained in a CFTA Report of June 6th, 1997. Additional data on the calculated ecotoxicity of lactic acid and its salts and esters can be obtained in a report entitled "The ecotoxicity and biodegradability of lactic acid, alkyl lactate esters and lactic acid salts" by Bowmer et al. (Reference: Chemosphere 37: 1317-1333 (1998))

This information only concerns the above mentioned product and is not valid if the product is used with other products(s) or in any process. The information is to the best of our present knowledge correct and complete and is given in good faith but without warranty. It remains the user's own responsibility to make sure that the information is appropriate and complete for his special use of this product.

# Indicates updated section.
Certificate of Analysis

Cust Order Ref: 16283
Product: Protect-M
Order Nr: 633225
Propylene glycol, Polysorbate 20, Lauric arginate.
Lot No: 1004003174
Manufacturing Date: 08-Jun-2010
Retest Date: 07-Dec-2011

<table>
<thead>
<tr>
<th>Test</th>
<th>Units</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td></td>
<td>slightly opalescent and yellow</td>
<td></td>
</tr>
<tr>
<td>Assay LAE (Lauric arginate)</td>
<td>%</td>
<td>10.0 - 11.0</td>
<td>10.5</td>
</tr>
<tr>
<td>Refractive index (20°C)</td>
<td></td>
<td>1.43 - 1.47</td>
<td>1.45</td>
</tr>
<tr>
<td>Viscosity (20°C, n2, v=50 rpm)</td>
<td>cps</td>
<td>&lt;=1.50</td>
<td>116</td>
</tr>
<tr>
<td>pH 1%, 20°C</td>
<td></td>
<td>2 - 6</td>
<td>5.7</td>
</tr>
</tbody>
</table>

This document is generated by a validated system and therefore not signed.
Michaela Hume
QA/QC Manager
**Protect-M**

**Description**
Protect-M, based on Mirenat® is a Lauric Arginate (LAE), N⁰-lauroyl-L-arginine ethyl ester formulation. LAE is a novel antimicrobial compound derived from lauric acid, arginine and ethanol. Protect-M has a slight yellowish color.

<table>
<thead>
<tr>
<th>Specification</th>
<th>Product Form</th>
<th>Lauric arginate liquid, slight opalescent and yellowish color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay LAE</td>
<td>10.0-11.0 % w/w</td>
<td></td>
</tr>
<tr>
<td>Refractive index at 20°C</td>
<td>1.43-1.47</td>
<td></td>
</tr>
<tr>
<td>Viscosity at 20°C</td>
<td>&lt;150 cps</td>
<td></td>
</tr>
<tr>
<td>pH (1%, 20°C)</td>
<td>2-6</td>
<td></td>
</tr>
</tbody>
</table>

**Physical-chemical properties**
- Molecular formula: Lauric arginate: C₂₀H₄₂N₀₄O₂.H₂O HCL
- Chemical name: L-Arginine, N⁰-(1-oxododecyl)-, ethyl ester monohydrochloride
- Ethyl- N⁰-lauroyl-L-arginate monohydrochloride

**Registration**
- CAS number: Lauric arginate: 60372-77-2
- FDA: self affirmed GRAS (FDA no objection letter)
- USDA/FSIS: FSIS Directive 7120.1

**Intended Use**
- Recommended use level: Food
  - 0.1-0.2% Protect-M; dilution is recommended for better surface coverage
  - Max use level: 0.2% Protect-M