INVESTIGATING THE ANTIMICROBIAL EFFECT OF NITRITE AND ADJUNCT INGREDIENTS ON *LISTERIA MONOCYTOGENES* AND *CLOSTRIDIUM PERFRINGENS* IN READY-TO-EAT MEATS

by

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Animal Science)

at the

UNIVERSITY OF WISCONSIN-MADISON

2014

Date of final oral examination: 10/30/2014

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ABSTRACT OF DISSERTATION

The objective of this research was to determine critical concentrations of purified nitrite and natural nitrite from cultured celery juice powder, used in alternative curing of ready-to-eat meat and poultry products, in combination with adjunct antimicrobial ingredients for inhibition of *Listeria monocytogenes* and *Clostridium perfringens*. A preliminary study investigated *L. monocytogenes* growth during 7°C storage in pork sausage made with varying ingoing concentrations of purified nitrite and ascorbate, and showed that nitrite significantly ($P < 0.05$) slowed growth. Response surface modeling (RSM) was used to formulate pork sausage treatments with ingoing nitrite (0 to 352 ppm) and ascorbate (0 to 643 ppm) in a central composite design. To isolate residual nitrite as a factor, each treatment was split in half and inoculated at one of two time points after manufacturing, each with different residual nitrite concentration. *L. monocytogenes* lag time and growth rate were calculated from the data and analyzed to determine the antimicrobial effects of ingoing nitrite, ascorbate, and residual nitrite. Ingoing nitrite was a significant factor affecting growth rate, increased concentrations of residual nitrite significantly increased lag time, and ascorbate did not have an antimicrobial effect on *L. monocytogenes*.

Nitrite, as purified nitrite or cultured celery juice powder (PCN), and ascorbate, as purified sodium ascorbate or cherry powder (CP), were evaluated for antimicrobial effects against *C. perfringens* during 15 h cooling in deli-style turkey breast. Results demonstrated that ingredient source had no significant effect on the antimicrobial activity, and though ≤100 ppm nitrite alone was insufficient to limit outgrowth to <1 log during cooling, the combination of ≥75 ppm PCN and ≥250 ppm CP inhibited growth during 15 h chilling. Natural and clean label antimicrobials, alone and in combination with 50 ppm PCN, were investigated and showed that

the antimicrobials performed differently from each other. 1.0% fruit extract and 1.0% cultured sugar/vinegar inhibited growth to <1 log, whereas 0.7% dried vinegar and 2.0% lemon/vinegar allowed for >1 log growth; supplementing antimicrobial-containing treatments with 50 ppm PCN improved inhibition during cooling. Results show that adjunct ingredients can be used with PCN to increase the safety of alternative cured meats.

ACKNOWLEDGEMENTS

I am unsure where to begin to recognize the numerous individuals who have been so instrumental in this achievement. First and foremost, I would like to express my most sincere gratitude to my major professor and mentor, Dr. Jeff Sindelar, for your continued guidance and encouragement. It has truly been an honor to be a part of your program and pursue my degree at UW-Madison. Throughout the past 4+ years, you have challenged me to set lofty goals and provided me with the tools and support to achieve them, and I owe a great deal of my success to your guidance. I will be forever grateful for the opportunities afforded me during my time here, from contributing to extension programs and working with meat processors to traveling the country and the world teaching and learning about meat science.

I would also like to extend my appreciation to the members of my committee. To Dr. Andy Milkwoski, nearly every conversation with you provided insight for my research and contributed to my development as a scientist, and for that, I thank you. To Dr. Kathy Glass, I am grateful for both your encouragement when endeavors were successful and your reassurance and patience when they were not. To Drs. Jim Claus, Charles Kaspar, and Jim Steele, I am greatly appreciative of your expertise and willingness to offer assistance and input.

I would like to thank my fellow graduate students, both past and present, especially Robby Weyker, Megan McGough, Bill Shazer, Matt Terns, Dale Perez, Jonathan Pleitner, and Russ McMinn, for your assistance in conducting this research, your continued friendship and support. To the members of "Team Sindelar," we have certainly made some unforgettable memories, and it has truly been a pleasure working with you.

I would also like to recognize the staff and students at the Food Research Institute Applied Food Safety Laboratory, especially Max Golden and Brandon Wanless. You accepted a

"meat head" into the lab family and made the long hours in the lab utterly entertaining, to say the least.

I would also like to acknowledge the University of Wisconsin Meat Laboratory faculty, staff, and student employees for your assistance and encouragement throughout my graduate career at UW. Special thanks to Adam Bartling for your involvement and assistance in conducting this research.

Special gratitude is extended to Dr. Dennis Seman and Steve Quickert of Kraft Foods/Oscar Mayer for the countless discussions and advice that contributed to the success and industry applicability of this research.

Last, but certainly not least, for my family, my parents, Ron and Patti, and my sister, Amelia, you will never know how much strength I have drawn from knowing I've always had you three in my corner. I continue to cherish your love, support, and patience throughout this journey. I could not have done it without you.

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CHAPTER 1: GENERAL INTRODUCTION

The practice of curing meat with sodium nitrite has been used for centuries to yield products with stable color, modified flavor, extended shelf life, and perhaps most importantly, improved food safety characteristics. A substantial body of research has been built to support the impact of nitrite on the development of cured color and its antioxidant capacity to prevent lipid oxidation and ensuing development of rancid flavors. Nitrite has been identified as an antimicrobial agent against Gram-positive pathogens, but is used in ready-to-eat (RTE) meats primarily to prevent the growth of *Clostridium botulinum.* Further, nitrite has been shown to inhibit *Listeria monocytogenes,* a pathogen that can grow during refrigerated storage and has a high mortality rate in at-risk populations, and has also been considered an inhibitor of outgrowth of *Clostridium perfringens,* a concern to processed meat manufacturers during cooling of RTE products.

Although both the quality and safety benefits of nitrite use in meats have been demonstrated, a number of reports have criticized its use and provided associations between nitrite and human health risks, and such skepticism among consumers has stimulated a widespread consumer movement to reduce exposure to preservatives, including nitrite, in foods *(2, 4)*. A number of ingredients have been investigated as replacements for nitrite, though no true replacement has been identified to date. However, naturally occurring nitrate and nitrite found in vegetables, have been isolated and characterized for use as non-meat ingredients. These natural sources of nitrite can be used to produce alternative cured meat products without the direct addition of the preservative, sodium nitrite, and yield products with similar cured color and flavor to those conventionally cured with sodium nitrite. Commonly made with cultured celery

juice powder, alternative cured products can qualify for natural or organic labeling claims under United States Department of Agriculture (USDA) labeling policy. They can also be considered "clean label", a less defined class of cured meat products generally considered to contain ingredients that are easily understood by consumers. In general, consumers perceive natural, organic, and clean label products to be safer and healthier options of processed meats. Furthermore, many adjunct antimicrobials shown to be effective as additional hurdles to prevent pathogen growth in RTE meats are also preservatives, and thus eliminated as options to enhance the safety of alternative cured products.

While purified sodium nitrite is added to meat formulations in known, controlled quantities, and thus can provide consistent quality and safety impacts, the levels of nitrite contributed to alternative cured products via celery powder, on the other hand, can be quite variable. Research has shown curing via this process provides sufficient nitrite to result in products with similar color and flavor to conventionally cured products, even though ingoing nitrite concentrations have been reported to be less. As such, concerns have arisen regarding the inherent microbiological safety provided by the lower concentration of nitrite.

The safety of alternative cured products has deservingly been given significant research attention in recent years, and results have suggested that alternative cured products are not as safe as those cured with conventional sources of nitrite *(5)*. Comparisons of alternative and conventionally cured products for inhibition of *L. monocytogenes* and *C. perfringens* showed that alternative cured products allowed for greater growth of both pathogens than conventionally cured *(1, 3, 5)*. With limited options for adjunct antimicrobial ingredients to be used without interfering with natural, organic, or clean labeling claims, it is imperative to characterize the effects of nitrite, from purified and natural sources, and potential adjunct antimicrobials, on key

pathogens of concern in RTE meats. Therefore, the first objective of this research was to determine critical concentrations of nitrite, both ingoing and residual, that were most effective in controlling *L. monocytogenes.* The second objective was to demonstrate the antimicrobial equivalence of similar concentrations of nitrite from purified and natural sources to control *C. perfringens* outgrowth during chilling of RTE products. Furthermore, the third objective was to identify adjunct ingredients to supplement the antimicrobial impact of nitrite to inhibit *C. perfringens.*

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DISSERTATION ORGANIZATION

This dissertation is organized into seven chapters. The first chapter is a general introduction. The second chapter reviews literature and background information relevant to this research project, while chapters three through six are manuscripts describing research experiments conducted to investigate specific areas that contribute as components of this collective research project. Manuscripts are formatting based on the *Journal of Food Protection* style. All tables and figures are presented at the end of each respective chapter. Chapter 3 is entitled "Investigating the impact of ingoing sodium nitrite and sodium ascorbate concentrations on growth of *Listeria monocytogenes* in a cooked, cured meat system." Chapter 4 is entitled "Modeling the impact of ingoing sodium nitrite, sodium ascorbate, and residual nitrite concentrations on growth parameters of *Listeria monocytogenes* in a cooked, cured pork sausage." Chapter 5 is entitled "Comparison of curing ingredients derived from purified and natural sources on inhibition of *Clostridium perfringens* outgrowth during the cooling of deli-style turkey breast." Chapter 6 is entitled "Impact of clean label antimicrobials and nitrite derived from natural sources on outgrowth of *Clostridium perfringens* during cooling of deli-style turkey breast." The seventh chapter provides a general summary and directions for further research.

CHAPTER 2: REVIEW OF RELEVANT LITERATURE

MEAT CURING AND PRESERVATION

Origins of meat curing and preservation. To prolong the shelf life of foods gathered in times of plenty to be consumed when supply is scarce, preservatives such as salt have been used to prevent deterioration in quality and to reduce microbial spoilage of foods, especially meat products. Before nitrite was commonly added as a preservative, salt, or sodium chloride, was one of the earliest forms of preservatives used. When applied to the meat surface, salt draws moisture from the meat interior, thus reducing the amount of available water (water activity; a_w) for microbial growth *(134)*. In a review of preserved cured meats, Binkerd et al. credited some of the first practices of salt preservation of meat to people in the saline deserts of Hither Asia *(10)*. The Greeks and Romans followed similar practices, and in the *Odyssey,* written around the eighth century B.C., Homer referred to sausage made by adding salt to meat *(102)*. The natural salt available in these locations often was contaminated with nitrate impurities, which produced early forms of meat curing *(10, 18)*. The presence of potassium nitrate, also known as saltpeter, as a contaminant of salt often resulted in a unique flavor and red color when applied to meats. Eventually, people developed a liking for this distinct product, and this preference encouraged more widespread and intentional use of saltpeter. However, it wasn't until near the turn of the 20^{th} century that scientists determined it was, nitrite, a reduced form of nitrate, rather than nitrate or pure salt (sodium chloride), that was the agent responsible for the typical pink color and unique flavor of cured meats *(71)*.

At the same time, scientists began to study the chemistry behind cured meats and nitrite, further characterizing the pigment changes that resulted in the specific color of cured meat and

the conditions necessary for color development. Significant discoveries revealed the basics of cured meat chemistry. One of the first groundbreaking studies regarding curing was done by Polenske, who in 1891 showed that nitrate was reduced to nitrite and further to nitric oxide after examining sterilized and unsterilized solutions of potassium nitrate *(108)*. He credited the reduction to microorganisms present in unsterilized solution. In 1899, Lehman and Kisskalt showed that it was nitrite, not nitrate, that caused cured meat color, and shortly thereafter, Haldane uncovered the reaction between nitric oxide and meat pigment during heating. Haldane demonstrated the formation of nitrosohemoglobin when nitrite was added to hemoglobin, and its subsequent breakdown during heating to form nitrosohemochromogen and the characteristic pink color of cured meats *(50, 71)*. In 1914, Hoagland confirmed that nitrate must be reduced to nitrite to achieve meat curing, but also showed the necessity of a reducing agent to yield nitric oxide *(54)*. These examples of early works describing the use of nitrite to cure meat products became the foundation for research and discovery of the science behind meat curing.

Regulation of nitrite. With the discovery of the essential role of nitrite in curing and preserving meat products came the need for a more detailed understanding of the current meat curing practices at the time, including details about nitrite use and concentrations of nitrite necessary for effective curing. Early studies had revealed the inherent inconsistencies associated with bacterial conversion of nitrate to nitrite and highlighted the variability of the resulting nitrite levels in meat products. For example, studies by Kerr et al. characterized a drastic increase in nitrite concentration in a stored brine due to bacterial reduction of nitrate *(68)*. In that case, nitrite concentration in brine increased from 3.6 ppm to 887 ppm on day 0 and day 65, respectively, and this kind of variability would inevitably result in vastly different levels of nitrite in a meat product produced with this brine. In the same report, 54 types of cured meats

were analyzed for nitrite concentration, 14 of which contained greater than 200 ppm residual nitrite, with a wide range of 208 to 960 ppm *(68)*. These results emphasized the inconsistency of nitrite concentrations in products produced with added nitrate, the curing method used at the time, and led to the direct addition of nitrite to improve control of concentrations in product.

The first approval for a processor to directly use nitrite in addition to nitrate for curing was given in 1923 by the Bureau of Animal Industry of the USDA, the predecessor to the Food Safety Inspection Service (FSIS). However, the Bureau also closely supervised subsequent experiments to determine nitrite levels in commercially produced products. In a follow up experiment to the survey and brine testing, Kerr et al. compared nitrite cured hams (1142 lb meat cured in brine containing 2000 ppm sodium nitrite) to a control cured with nitrate for residual concentrations and quality differences *(68)*. This experiment found that the hams cured with nitrite resulted in a maximum of 200 ppm residual nitrite, with an average of 42 to 150 ppm, while those control hams cured with nitrate had a maximum of 45 ppm residual nitrite. Furthermore, despite varied residual nitrite levels, flavor and color were indistinguishable between hams cured with nitrite or nitrate *(68)*. Other studies evaluated products of parallel lots cured with either nitrate or a lesser amount of nitrite and concluded that nitrite could be used in smaller quantities and achieve similar quality characteristics, while reducing curing time *(68, 82)*. Researchers also concluded that sodium nitrite could replace the use of nitrate without compromising typical cured meat characteristics. Based on those experiments and recommendations, the USDA authorized meat processors the option of including sodium nitrite, "up to one-fourth of an ounce per hundred pounds of meat" *(68, 164)*. Nitrite limits are often discussed in terms of ppm, but these regulations were originally set based on commonly used units of measure [2 lb to 100 gal pickle for 10% pumping product (200 ppm), 1 ounce to 100 lb

dry cure mix (625 ppm), or 4 ounces to 100 lb comminuted product (156 ppm)]. Since its approval for use, nitrite has remained a strictly regulated substance by the USDA, and the maximum allowable levels have remained relatively unchanged (Table 2.1).

Table 2.1. Maximum ingoing nitrite and nitrate for meat and poultry products (ppm), based on meat weight.

	Curing Method			
Curing Agent*	Immersion	Massaged or Pumped	Comminuted	Dry Cured
Sodium nitrite	200	200	156	625
Sodium nitrate	700	700	1718	2187

*Potassium nitrite and potassium nitrate can be substituted for sodium salts within the same limits. Adapted from USDA Processing Inspectors' Calculations Handbook *(163)*.

These regulations apply to all cured products today except bacon, in which nitrate is prohibited from use in formulations, and the use of either sodium ascorbate or sodium erythorbate as a cure accelerator is required, which functions to minimize the formation of potentially carcinogenic nitrosamines. Additionally, the increased molecular weight of potassium salts of nitrite or nitrate compared to sodium salts is taken into account for bacon formulations, but not for other cured products; thus, there are differing regulations for the use of curing agents for bacon based on whether the ingredients are sodium or potassium salts. In general, immersion cured or pumped bacon is required to have 120 ppm of sodium nitrite (148 ppm of potassium nitrite), no nitrate, and 550 ppm of sodium ascorbate or sodium erythorbate *(163)*.

Throughout the last century, four main functions of nitrite in meat products have been identified: development and stabilization of cured meat color, contribution to cured meat flavor, prevention or retardation of the development of rancidity, and inhibition of spoilage and pathogenic microorganisms *(102)*. Whether added directly or derived from nitrate, nitrite's

distinctive impact on the quality and safety of cured meats is irrefutable and to date, researchers have not been successful in finding an alternative ingredient to achieve the same remarkable effects of nitrite. Today, meat curing can be defined as the combined use of salt and nitrite in meats that affect the physical and chemical composition, and often the microbiological properties as well *(17, 126)*. This process has been used to produce a variety of cured products, with very distinct characteristics relative to other meat products, based on color, flavor, texture, shelf life, and safety attributes *(126)*. Though decades of research have been conducted regarding the complex reactions associated with nitrite and meat, many of the precise mechanisms causing these specific quality and safety characteristics of cured meats are not yet fully understood.

MEAT COLOR CHEMISTRY

Basic pigment chemistry. One of the most recognizable influences of nitrite use in meat products is the change in meat color. The primary pigments in muscle and meat that are responsible for meat color are myoglobin and hemoglobin. These proteins can be found in many chemical redox forms and determine the color of fresh, cooked, and cured meats. Both serve specific and related functions in living muscle. The function of hemoglobin in a living animal is to transport oxygen from the lungs to cells in other tissues in the body, while myoglobin serves as a storage site of this oxygen once it reaches a muscle cell *(1)*. Myoglobin is comprised of a chain of 153 amino acids folded around a heme group *(112)*. In the center of the heme ring, also known as the tetrapyrrole ring, is an atom of iron which provides six binding sites, four of which are bound to nitrogen atoms that are part of the tetrapyrrole ring. The fifth site is occupied by the imidazole nitrogen of histidine, part of the globin protein component of myglobin, leaving the sixth site available to bind small molecules. The free electron pair at the sixth binding site can

bind to a variety of other molecules, such as oxygen, nitric oxide, or carbon monoxide. Depending on the presence of oxidants or reductants in the environment within the meat system, the iron atom in the heme ring may be in a ferrous (Fe^{2+}) or ferric (Fe^{3+}) state *(103)*. The oxidation state of the iron, the molecule bound to the sixth binding site, and the physical state of the protein (intact or denatured) determine the color of meat.

Fresh meat color. In fresh meat, normal color is largely dependent on the presence or absence of oxygen, along with other factors. In the absence of oxygen, as in vacuum packaged meat or uncut meat tissue, a dark purple-red color is observed, the result of the pigment form, deoxymyoglobin. This state of myoglobin exists because natural reducing conditions in the meat utilize all the oxygen present, resulting in reduced ferrous iron and leaving only water to bind with myoglobin. When oxygen is introduced, oxymyoglobin, the bright red color also referred to as "bloom", is observed. This color, normally associated with fresh meat, exists when the iron is in the ferrous state and oxygen is bound to the sixth binding site. When the natural reducing capacity of the meat has been depleted and oxygen is present, iron is oxidized to its ferric state, and can then bind water but not oxygen, forming metmyoglobin. In this metmyoglobin form, the resulting color is brown *(112)*. When fresh meat is cooked, metmyoglobin is formed as heme iron is oxidized to its ferric state, and additional heat permanently fixes the brown color by denaturing the myoglobin molecule *(104)*.

Cured meat color. The characteristic cured pink color of cured meats is likely the most distinguishable feature of nitrite addition. The complex chemistry of meat curing, specifically nitrite and chloride chemistry, has been reviewed by Sebranek et al. *(126)*. Chemical changes in cured meat are generally considered to involve nitrite, which exists in equilibrium as either the nitrite ion (NO_2^-) or the weak acid, nitrous acid (HNO_2), the latter of which results in nitrosating.

Though many prior complex reactions occur, these nitrosating compounds eventually produce nitrosylmyochromogen, the pink color associated with cured meats. Nitrite has a pK_a, or acid-dissociation constant, of 3.4, meaning at pH 3.4, approximately 50% of the acid is dissociated. Therefore, based on the Henderson-Hasselbach equation, at normal pH of meat (5.5-6.5) nearly all of the nitrite is in the dissociated ion form (NO_2^-) and very little (as little as 0.1%) is in the more reactive HNO_2 form for nitrosation *(112)*. Regardless, this small amount of nitrous acid is quite reactive and generates sufficient nitric oxide to impart cured meat color. Upon addition to meat, nitrite, a strong oxidant, oxidizes the heme portion of the myoglobin molecule, causing the formation of metmyoglobin, which as previously mentioned, appears brown. Then, the nitrite derivative nitric oxide binds to the aforementioned sixth ligand binding site of myoglobin, forming nitrosylmetmyoglobin, an unstable intermediate pigment. Reductants in the muscle tissue, whether endogenous or exogenous, allow for the reduction of the heme to its ferrous state, yielding nitrosylmyoglobin, the red color observed in raw cured meats. When heat is applied during thermal processing, the globin portion of the protein is denatured, and the pigment is converted to the stable nitrosylmyochromogen, resulting in the bright pink cured color *(17, 36, 104)*.

Nitric oxide generation in a meat system, which is critical to development of cured color, is based on a variety of factors, including the pH and presence of reductants in the environment within the meat system. During the conversion of muscle to meat, one of the major biochemical changes is the reduction in pH *(1)*. When an animal is alive, the circulatory system functions to deliver oxygen and remove waste generated via aerobic metabolism. However, upon exsanguination during the harvest process, blood flow stops, causing a shift to anaerobic metabolism and the accumulation of lactic acid as a metabolic byproduct and the postmortem pH

decline. This creates a mildly acidic environment, which can greatly increase the rate of nitric oxide formation from nitrite. Decreases in pH of the environment as little as 0.2 to 0.3 pH units can as much as double the rate of nitric oxide production *(37)*. Salt (sodium chloride), an ingredient in every cured meat formulation, has also been shown to catalyze the production of nitric oxide in meat systems *(94, 126)*. The chloride ion of sodium chloride can react with nitrous acid, forming nitrosyl chloride, a reactive nitrosating species *(94)*. However, more acidic conditions than those present in meat systems would be necessary for salt to have a significant effect on nitrosation reactions *(126)*.

Similar to the chemical reactions of cured color development, color stability over time is equally complex. Over time, discoloration or cured color fading can be caused by a combination of factors, but most often is attributed to exposure to air (oxygen) and light, especially fluorescent lighting *(103)*. A grayish-brown color can develop due to the oxygen- and light-catalyzed destabilization of nitrosylmyochromogen in a two-step reaction: nitric oxide dissociation from myoglobin, and the oxidation of the resulting nitric oxide and heme groups *(1, 27, 36)*. In the absence of oxygen, the second reaction does not occur, maintaining a more stable color and emphasizing the importance of using oxygen-impermeable packaging for cured meat *(36)*. The mechanisms of color fading also emphasize the importance of oxygen scavengers like ascorbate. Møller et al. investigated the impact on color of numerous factors, including residual oxygen, oxygen transmission ratio of packaging film, product to headspace volume ratio, illuminance level, and nitrite level during curing, and found all factors contributed to discoloration of cured ham *(94)*.

Other color defects can be caused by microbiological or chemical factors and have the potential to occur in cured meat systems. The most common biological contributor to

discoloration is the presence or growth of microbiological contaminants. Bacterial contamination with spoilage organisms, such as some species of *Lactobacillus*, can produce hydrogen peroxide as a metabolic byproduct. The hydrogen peroxide then can react with cured pigment, displacing iron from the heme and destroying the porphyrin ring, giving the product a green color *(48)*. Chemical contamination, in the form of overcure or undercure, can also result in unacceptable colors, such as brown or green. Undercure is essentially due to insufficient levels of nitric oxide for color development, and can often be attributed to the depletion of reducing agents that assist in the conversion of nitrite to nitric oxide; the use of ascorbic acid as an exogenous reductant has minimized the occurrence of this defect. At the other extreme, overcure can occur at concentrations of nitrite at or above 300 ppm, leading to the modification of metmyoglobin to an irreversible green pigment, nitrimetmyoglobin *(112)*.

In some cases, the mere presence of any nitrite at all can be considered a defect, such as in uncured cooked products as in chicken or turkey rolls or roast beef. In such instances, trace levels of nitrite contamination in the process can impart a pink color during thermal processing and, to a consumer, imply that the product is not fully cooked. Heaton et al. stated that panelists could detect pinking with nitrite levels as low as 2, 1, and 4 ppm in turkey, chicken, and pork rolls, respectively *(52)*. In another study of oven roasted turkey breasts, 1 ppm of added sodium nitrite resulted in noticeable pinking *(2)*.

CURED MEAT FLAVOR

The unique color characteristics imparted on meats cured with nitrite have been outlined, yet this ingredient provides a distinctive flavor profile as well. Though research has shown that the prevention of lipid oxidation contributes to the development of cured flavor, the complete

chemistry of cured meat flavor is not well understood *(6, 132)*. The oxidation of lipids is known to deteriorate meat quality and create rancid flavors. This reaction can occur in cooked uncured meats as early as 48 h after thermal processing when stored at 4°C *(112)*. However, the powerful antioxidant capacity of nitrite prevents oxidation and warmed-over flavor in cured products, but does not fully account for the complexity of cured meat flavor *(87, 132)*. Two powerful antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), were substituted for nitrite in frankfurters and subsequently evaluated by panelists, and results reported by Simon et al. indicated that from a flavor perspective, these antioxidants did not compensate for the lack of nitrite in the frankfurters *(135)*.

The use of salt, which is a pro-oxidant, in cured meats presents additional difficulty with regard to controlling lipid oxidation, though the flavor effect of salt, particularly in combination with nitrite, is an important component of cured meat flavor *(23, 135)*. It is generally accepted that the combination of both ingredients is essential for characteristic and desirable cured meat flavor. Froehlich et al. investigated the impact of 0, 50, 100, and 150 ppm nitrite and 0, 1, and 2% salt on flavor of ham, and found that, though cured flavor intensity increased as both nitrite and salt increased, salt imparted a greater effect on flavor *(39)*. Furthermore, a concentration of at least 50 ppm nitrite was required in formulations to develop notable cured flavor. Dethmers et al. identified 50 ppm nitrite or greater as sufficient to inhibit the development of off-flavors and rancidity in thuringer sausage *(26)*.

Consumer preference of cured flavors has also been reported. Sensory evaluation of pork roasts both with and without nitrite showed that panelists preferred the flavor of the roasts with nitrite *(23)*. Thuringer produced with 50, 100, or 150 ppm nitrite was reported to have higher sensory acceptability and lower scores for rancidity and off-flavors than thuringer devoid of

nitrite *(26)*. However, since thuringer is a fermented product, its lower pH could have masked cured color flavor effects by these varied nitrite concentrations. In frankfurters, panelists detected a flavor difference in products produced with 0 or 78 ppm nitrite *(168)*. Simon et al. found that panelists could distinguish flavor variation in frankfurters manufactured with and without nitrite (39, 78, 156 ppm) and that flavor scores increased as nitrite concentration increased *(135)*. In hams cured with 91 or 182 ppm nitrite, panelists were unable to distinguish flavor differences between the treatments *(115)*. Therefore, concentrations of nitrite used in cured meats today clearly are sufficient to impart cured flavor.

MEAT CURING REACTIONS

Cure accelerators. Throughout the previous discussion of nitrite chemistry, the importance of both endogenous and exogenous reducing agents becomes apparent. USDA approval of direct addition of nitrite to meat products led to an increase in nitrite curing and in turn, another meat curing milestone, the discovery of the reducing power of ascorbic acid and its use as an exogenous reductant in meat. Ascorbic acid was found to be very effective in catalyzing the reduction of nitrite to nitric oxide, providing faster cured color development but also reducing levels of residual nitrite and nitrate in finished products *(103)*. Therefore, ascorbic acid or ascorbate, or the isomers erythorbic acid or erythorbate, became regulated by USDA in 1955, and became widely used, interchangeably, as curing adjuncts *(103, 109)*}.

USDA states that these ingredients are allowed in meat curing systems with maximum concentrations as reported in Table 2. In bacon, USDA requires exactly 550 ppm of sodium ascorbate or sodium erythorbate to be used.

Table 2.2. Maximum ingoing cure accelerator for meat and poultry products (ppm) based on meat weight.

Cure Accelerator	Maximum Limit
Ascorbic acid	469
Erythorbic acid	469
Sodium ascorbate	547
Sodium erythorbate	547
Citric acid or sodium citrate	May replace up to half of one of the above

*Table reproduced from the USDA Processing Inspectors' Calculations Handbook *(163)*.

The primary use of cure accelerators in processed meat formulations is to increase the rate of curing reactions, which in turn decreases the overall time required for processing *(112)*. The catalysis of nitrite reduction to nitric oxide by ascorbate was first characterized by Karrer et al. *(66)*. Using kinetic analysis to understand the activity of ascorbate's catalysis of nitric oxide production, Izumi et al. proposed an interaction between nitrous acid and ascorbic acid formed a reactive intermediate species that could then decompose to form nitric oxide and accelerate loss of free nitrite *(59, 60)*.

In the manufacture of sliced bologna, Lin et al. showed that 940 and 500 ppm erythorbate converted 58.46 and 54.27% of total pigment to cured pigment, respectively, as opposed to 52.97% pigment conversion in the absence of ascorbate, and the increased pigment conversion corresponded to decreased residual nitrite after manufacturing, with values of 9.25 and 11.13 ppm when 500 and 940 ppm sodium erythorbate were included, as compared to 22.27 ppm in the absence of erythorbate *(85)*. Sebranek et al. investigated the use of erythorbate with nitrite and the effects on consumer acceptability *(128)*. Different combinations of nitrite (0, 26, 52, and 156 ppm) and erythorbate (0 and 546 ppm) were used to manufacture frankfurters, and the inclusion of erythorbate increased flavor acceptability in treatments containing 26 ppm nitrite *(128)*. Furthermore, cure accelerators act to stabilize color and flavor during storage, based on the

antioxidant capacity of ascorbate and function as oxygen scavengers during storage to prevent deterioration of color and flavor, as heme catalyzed lipid oxidation could promote both pigment oxidation and randicity *(72)*.

Residual nitrite. Due to the complexities of reactions that occur when nitrite is added to a meat system, the fate of nitrite remains difficult to accurately predict, though a portion of added nitrite remains as free, or residual, nitrite. It is estimated that approximately 50% or less of the original formulated nitrite is recoverable at the end of processing *(20, 105, 125)*. Hustad et al. reported that during the emulsification of frankfurters, a loss of 16% of added nitrite was recorded, with at least another 50% lost during the remainder of manufacturing *(55)*. In frankfurters made with 75, 125, or 250 ppm nitrite, 39, 59, and 146 ppm, respectively, remained after cooking and during shelf life, residual nitrite levels continued to decline *(105)*. After processing hams with 182 ppm nitrite without ascorbate, Brown et al. reported residual nitrite concentrations of 49.7, 32.0, and 12.3 ppm after 0, 8, and 16 days, respectively *(12)*. In surveys of cured meats at retail, less than 10 ppm residual nitrite remained in products *(19, 101)*. In a study published in 1997, bacon, ham, and hot dogs at retail, contained residual nitrite concentrations of 7, 6, and 4 ppm, respectively *(19)*. Over a decade later, similar results were published after a nationwide survey of residual nitrite concentrations in both comminuted and whole muscle cured products at retail, with a range of 0.8 to 7.6 ppm nitrite *(101)*.

Endogenous factors affecting residual nitrite depletion include presence of reducing agents, pH, muscle type, and temperature. As previously noted, cure accelerators are significant contributors to the depletion of residual nitrite in cured meats *(77, 103, 112)*. The inclusion of cure accelerators in formulations increases the rate of nitrite reactions that subsequently result in lower concentrations of recoverable residual nitrite *(41, 77, 80)*. For example, in hams cured

with 182 ppm nitrite, Brown et al. reported lower levels of residual nitrite in treatments containing 227, 455, and 568 ppm ascorbate than in those without ascorbate *(115)*.

Another well documented factor in nitrite depletion is pH. Nitrite reactivity increases as pH decreases, since an increasing proportion of the nitrite present is in the reactive nitrous acid form as pH nears pK_a (3.4). Therefore, decreased pH leads to diminished levels of residual nitrite *(78, 103)*. Fox et al. estimated that a decrease of 0.2 pH units doubled the rate of nitric oxide production and color formation while decreasing residual nitrite levels *(37, 38)*. The pH of meat used as raw materials can vary, but Lee et al. documented that the lower pH of white muscle, as opposed to red muscle, resulted in decreased residual nitrite levels when used for curing *(78)*. Alkaline phosphates are used extensively in cooked cured meats and function to increase the pH of the meat system, and in doing so increase the net charge of meat proteins, efficiently increasing overall water binding capacity. However, use of alkaline phosphates can also increase residual nitrite levels due to an increase in pH *(70, 113)*. Kilic et al. manufactured frankfurters with 0.4% sodium tripolyphosphate, an alkaline phosphate used in formulations for quality and economic reasons, and 156 ppm ingoing nitrite, and demonstrated that treatments with phosphate resulted in both a higher pH and a corresponding greater amount of residual nitrite. For example, 1 day after processing, beef and pork frankfurters with and without phosphate had residual nitrite concentrations of 64 and 51 ppm and pH 6.2 and 6.0, respectively *(70)*.

Along with pH, temperature has also been reported to impact nitrite depletion. In a study of both temperature and pH effects on nitrite depletion in canned ham, nitrite depletion rates were found to double for every 12°C increase in temperature or for every 0.86 unit decrease in pH *(100)*. Hustad et al. confirmed the effect of temperature on nitrite depletion by documenting

more rapid depletion rates at 27°C than 7°C storage *(55)*. The temperature impact on nitrite depletion is not limited only to storage temperature, but includes thermal processing as well. Using a response surface design, Kilic et al. demonstrated that increased heat quantity treatments (calculated F-values) used for thermal processing significantly decreased ($P < 0.05$) residual nitrite levels in finished products *(69)*.

Nitrite pathways and products. The complexity of nitrite chemistry is variable and affected by a number of variables *(17, 126)*. Upon addition to a meat system, nitrite transforms so rapidly that it cannot all be recovered after mixing. Based on temperature, pH, reducing agents, and time, the added nitrite reacts and disappears until only a small portion remains in finished RTE products. Attempts to trace these reactions and products have elucidated some of the potential pathways, but in general, have not been able to recover 100% of the added nitrite. ^{15}N-labeled nitrite has been used in model cured meat systems in attempts to trace nitrite pathways *(41, 125, 172)*. Sebranek et al. determined that, in cured comminuted luncheon meat over time, a consistently low level of residual nitrite was attained after the added nitrite rapidly reacted with components of the meat system upon addition. In that study, researchers were able to recover just 72 to 86% of the originally added nitrite in various forms, but could not account for all the originally added nitrite *(125)*. Recovery improved in a model system comprised of nitrite, myoglobin, and ascorbate used by Fujimaki et al. in which nearly all added nitrite was recovered bound to myoglobin, as residual nitrite, nitrate, or gaseous nitrogen products *(41)*.

The challenges associated with recovery of added nitrite from meat systems are due to the enormous complexity of meat systems and the availability of a vast number of components with which nitrite can react once in the meat system. Depending on many exogenous and endogenous factors, added nitrite can react with proteins and lipids in meat, while a portion of added nitrite

remains as residual nitrite *(103)*. Cassens et al. summarized a series of experiments using ^{15}N to trace nitrite reactions with various components of the meat system and estimated that nitrite bound to myoglobin generally accounted for 5-15% of formulated nitrite; nitrate, 1-10%; remaining free nitrite, 5-20%; gas 1-5%; formation of nitrosothiols, 5-15%; lipid, 1-5%; and protein, 20-30% *(20)*.

A major pathway through which nitrite is lost in meat is reaction with heme compounds, most notably myoglobin, which leads to cured color development, but can also form gaseous products. After mixing luncheon meat and trapping headspace gases, Sebranek et al. recovered a small amount of added labeled nitrite (^{15}N) as nitrogen gas and nitric oxide *(125)*. Though there was a greater quantity of the nitric oxide than nitrogen gas, the summation of the two accounted for approximately 5% of originally added nitrite *(125)*. A major portion of the gases formed react with other constituents in a meat system, but some can remain as dissolved gas within products. Nitric oxide can also bind to a number of non-heme proteins and sulfhydryl groups through thionitroso bonding. Woolford et al. reported that of nitrite added to bacon bellies, 25% was bound to proteins *(172, 173)*. Mirna et al. added nitrite to minced meat in a concentration equimolar with sulfhydryl content, and demonstrated that both nitrite and sulfhydryl portions of the samples decreased by approximately 30% during two weeks storage, suggesting nitrosothiols were formed *(93)*. Yet another reaction product when nitrite is added to meat, even in products without added nitrate in formulations, is nitrate. In products cured only with nitrite, a portion of nitrite has been shown to be converted to nitrate *(41)*. Lee et al. suggested that in the presence of oxygen, nitrite and myoglobin can be simultaneously oxidized to metmyoglobin and nitrate *(80)*. Though this reaction can occur in the absence of ascorbate, Lee et al. was able to quantify greater nitrate formation when ascorbate was included in the system *(80)*.

NITRITE AND HUMAN HEALTH

Toxicity and carcinogenicity of nitrite. Though the quality and food safety impacts of nitrite have been demonstrated, nitrate and nitrite have continued to cause debates regarding human health impacts. Nitrite can be toxic to human at relatively low levels, as a 1 g dose could be sufficient to result in death *(34)*. The majority of nitrite used in the manufacturing of processed cured meats is in the form of a blend with sodium chloride and often a colored dye, to distinguish it from other naturally white powdered non-meat ingredients like salt or sugar, while the reduced concentration of nitrite in the blend helps to limit the risk of overconsumption of nitrite and the risk of toxicity due to methemoglobinemia *(42)*. If toxic levels are ingested, the resulting condition presents itself as cyanosis, a blue color of the skin resulting from lack of oxygen delivery to organs and tissues in the body. With its strong heme-oxidizing capacity, nitrite can oxidize hemoglobin to methemoglobin, where iron is in the ferric state, rendering it unable to bind and transport oxygen. The chemistry of this reaction is similar to the formation of metmyoglobin. Methemoglobinemia is of particular consequence in newborn infants and is often referred to as "blue baby syndrome" *(32)*. Newborns are particularly susceptible because their erythrocytes do not have nitrate reductase activity, which in older infants can degrade nitrohemoglobin to release the hemoglobin to functional normally and bind oxygen *(53)*.

The linking of consumption of cured meats to the production of nitrosamines and human cancer has caused controversy for decades. Nitrosamines are N-nitroso compounds that can be formed by the nitrosation of secondary amines at high temperatures, which can occur in cured meats. Such temperatures are not normally applied to most cured meats, with the exception of frying bacon at high temperature. In general, nitrosamines were identified as carcinogenic in

1970, in a report by Lijinsky and Epstein in *Nature (84).* The authors concluded that nitrite and secondary amines were precursers to nitrosamine formation and by eliminating one or both of them, nitrosamine formation would be inhibited. Since cured meats contain both nitrite and secondary amines, they were consequently launched into a high profile controversy surrounding human health. Since that time, numerous studies and task forces have evaluated the concerns that originally were brought to light due to this report, as well as further investigations of nitrite and secondary amines in cured meats.

Research and industry-wide collaboration determined formulation changes to minimize nitrosamines from nearly all bacon produced in the U.S. Therefore, regulatory changes were made for the manufacturing of bacon, as a key product that provided the conditions for potential nitrosamine formation. In order to prevent high levels of residual nitrite in bacon, the maximum ingoing concentrations were reduced from 200 to 120 ppm, and the use of 550 ppm of sodium ascorbate or sodium erythorbate was required, since cure accelerators were shown to reduce residual nitrite concentrations. Because nitrate, if present, can be a reserve source of additional nitrite, added nitrate was banned for use in bacon formulations.

Comprehensive reports from the National Academy of Sciences, in 1981 and 1982, entitled "The Health Effects of Nitrate, Nitrite, and N-Nitroso Compounds" and "Alternatives to the Current Use of Nitrite in Foods", took into account the body of science to date and concluded that cured meats containing nitrite were not a significant human health risk *(97)*. Potential risk to human health due to nitrite was also investigated as part of the National Toxicology Program and was reported in 2001 *(98)*. Conclusions in the report stated that research, to date, did not support the carcinogenicity of nitrite in rats and mice, except some evidence was presented for cancer in the forestomach of female mice.

Yet another, more recent review of nitrite and carcinogenicity was reported by the International Agency for Research on Cancer (IARC) in 2010, which concluded that "ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to humans*" (58).* However, this conclusion can be easily brought into question if only based upon the discovery that nitrite is produced endogenously. Therefore, any policy made based on the IARC conclusion could be refuted by the fact that the human body generates nitrite itself *(92)*. Although clear links do not exist between nitrite consumption and human health risks, and meat processing practices have minimized nitrosamine formation in cured meats, the controversy surrounding nitrite and human health continues today.

Health benefits of nitrite. In studies of human metabolism of nitrate, it was discovered that excretion of nitrate exceeded consumption of nitrite, thus leading scientists to investigate the production or synthesis of nitrite within the human body *(145)*. Tannenbaum et al. reported that 25% of nitrate ingested is acquired from swallowing saliva containing endogenous nitrate *(144)*. Furthermore, about 20% of that endogenous nitrate, is reduced and consumed as nitrite. The discovery of the production of nitrate and nitrite by the salivary glands suggested that these molecules may play a physiological role in human health as well. Summaries of nitrite, nitric oxide, and benefits to human health are readily available *(13, 92, 136, 138)*. The role of nitric oxide as a signaling molecule has been elucidated in recent years, and it has been reported to be involved in variety of physiological functions, including neurotransmission, smooth muscle relaxation, immune response, and wound healing. Nitric oxide serves in a variety of roles involving treatment of cardiac or pulmonary problems, and for example, nitroglycerin, a commonly used drug used to treat cardiac cases, serves as a source of nitric oxide in the body. With science indicating that nitrite is not only consumed but also produced by the body, overall

human exposure has been further analyzed. The National Academy of Sciences in 1981, reported that 39% of dietary nitrite was from cured meats, 34% from baked goods and cereals, and 16% from vegetables *(97)*. However, more recent reports indicate that the majority of ingested nitrite and nitrate come from vegetables and drinking water, with 5% or less coming from cured meats *(3, 19, 124)*. Clearly, the bottom line remains uncertain regarding the human exposure to nitrate, nitrite, and nitric oxides, the sources of these molecules, and the subsequent positive or negative health effects.

ALTERNATIVE CURING

Rationale. Despite the numerous advantages of using nitrite to cure meats, the concerns regarding potential nitrosamine formation and cancer risks have contributed to a market shift in which consumers have increased demand for no-nitrate/nitrite-added products. Consumer perceptions of the safety risks associated with consuming nitrite and nitrate have led many of them to select no-nitrate/nitrite-added products, with expectations that they are safer or healthier choices than traditional, conventionally produced cured meats. This increase in demand for no-nitrate/nitrite-added products has led to the development of alternative curing methods that do not utilize the direct addition of purified sodium nitrite, yet result in a "cured" type of product for consumers. To date, there is a great deal of confusion surrounding these products, from labeling and ingredients to questions regarding food safety *(124, 127)*.

Alternative curing regulations. Traditional or conventional cured meat products contain defined amounts of curing agents (sodium or potassium salts of nitrite or nitrate, as outlined in 9 CFR 424.21 (c)) that produce characteristics easily recognized by consumers, namely color, flavor, shelf life, and safety. Advances in ingredient technology and processing

have allowed meat processors to meet consumer demand by producing alternative cured products with characteristics similar to conventionally cured products made with directly added sodium nitrite. To differentiate, the USDA requires the term "uncured" must be included on the labels of alternative cured products (9 CFR 319.2) *(160)*. The Code of Federal Regulations states the following regarding uncured products *(160)*:

> *"Any product, such as frankfurters and corned beef, for which there is a standard in this part and to which nitrate or nitrite is permitted or required to be added, may be prepared without nitrate or nitrite and labeled with such standard name when immediately preceded with the term "Uncured" in the same size and style of lettering as the rest of such standard 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. And provided further, that labeling for such product complies with the provision of 317.17(c) of this subchapter.*

Further labeling requirements for uncured meats are outlined in 9 CFR 317.17(c).

> *(1) Products described in paragraph (b) of this section or 319.2 of this subchapter, 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. (2) Products described in paragraph (b) of this section and 319.2 of this subchapter 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 F_0 of 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.*

The resulting labels of these products often have the inadvertent effect of confusing consumers. Products manufactured using these techniques are referred to by a variety of names, including uncured, naturally cured, alternative cured, indirectly cured, or nitrite-free, which can become somewhat baffling for the consumer. Adding to this confusion is the combined labeling of Uncured alongside claims such as Natural or Organic on products. Uncured products are not necessarily natural or organic; however, neither natural nor organic products are allowed to be produced with direct addition of purified sodium nitrite, according to the Code of Federal Regulations *(160).*

It is also important to note that the categories natural or organic are not equivalent, but are distinctly different from each other according to USDA. Organic products are fairly well defined and are regulated according to the Organic Foods Production Act, a provision of the 1990 Farm Bill, which defines standards for producing and marketing organic products. The National List of Allowed and Prohibited Substances states that synthetic nitrite is not allowed, yet celery powder, a natural source of nitrate, is allowed in organic foods *(161).* On the other hand, unlike organic, natural is a much more ambiguous term. The claim "Natural" is defined in the USDA Food Standards and Labeling Policy Book as:

> *The term "natural" may be used on labeling for meat products and poultry products, provided the applicant for such labeling demonstrates that: (1) the product does not contain any artificial 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 (158).*

Yet another popular marketing tool for processed meats is termed "clean label". This category is less clearly defined, but at its simplest, is based on removing ingredients from product

formulations that are artificial or unfamiliar to consumers in an effort to make a clean label, free of complicated chemical nomenclature, which allows consumers to more readily understand information regarding their food choices.

Ingredients used in alternative curing. The ingredients and methods utilized for manufacturing alternative cured meats have been outlined by Sebranek et al. *(124)*. Since no substitute for nitrite has been discovered to date, the means for achieving a product with cured characteristics but without direct addition of nitrite utilizes natural sources of nitrate, such as vegetables, coupled with a bacterial starter culture possessing nitrate-reducing enzymes with the ability to reduce nitrate to nitrite. After an incubation step for the conversion of nitrate to nitrite, nitrite is present in the ingredient via a naturally occurring source of nitrate and the bacterial reduction of that nitrate. The resulting nitrite follows the same reaction pathways, in a meat system, as purified chemical nitrite.

Vegetable sources have been identified as having high nitrate concentrations *(97)*. Lettuce, celery, and beets have been reported to contain 1500-2800 ppm of nitrate. Powders or juices prepared from these vegetables facilitate the use of these nitrate sources in food production. Sebranek et al. reported nitrate concentrations of 171, 2114, 2273, and 3227 ppm in carrot, celery, beet, and spinach juice, respectively *(124)*. To date, celery is the most commonly used source of nitrate due to its minimal quality impact, with relatively little impact on color or flavor when used in a processed meat product.

Since nitrite is the active ingredient required for meat curing reactions, and vegetables are sources of nitrate, a bacterial starter culture is required to yield nitrite. Such a culture should contain organisms with nitrate reductase enzymatic activity, for example *Staphylococcus xylosus* or *Staphylococcus carnosus.* When a vegetable powder ingredient containing nitrate and a

bacterial starter culture are added to make an uncured product, the process requires an incubation period prior to thermal processing to allow for conversion of nitrate to nitrite *(16)*. Sindelar et al. investigated the quality of frankfurter-style cooked sausages made with 0.2% or 0.4% celery juice powder and held for 30 or 120 min at 38°C for incubation time *(137)*. The results reported showed that the holding time for incubation was more critical to cured color development than the amount of celery powder (nitrate) added. However, in large diameter hams with 0.2% or 0.35% celery powder, as the longer thermal process used for cooking the hams allowed for nitrate reduction to nitrite, incubation time did not impact objective color measurements.

The amount of residual nitrite present in alternative cured products can vary dramatically. In the aforementioned study, it was calculated that based on nitrate concentration in the celery powder and 100% conversion of nitrate to nitrite, the maximum ingoing nitrite was 69, 120, and 139 ppm for usage rates of 0.2, 0.35, and 0.4% celery powder *(137)*. These nitrite concentrations are well below those for traditional, conventionally cured products, in which ingoing concentrations range from 156 ppm for comminuted or 200 ppm for whole muscle products. Therefore, it is not surprising to see reports of lower residual nitrite concentrations in alternative cured products versus traditional, conventionally cured meats. For example, hams made with 0.2 or 0.35% celery powder and incubated for 120 minutes resulted in 19.5 and 36.1 ppm residual nitrite after incubation, respectively. Furthermore, as compared to a conventionally cured control, alternative cured hams had less residual nitrite at day 0 (21.0 to 36.0 ppm in alternative cured versus 63.4 ppm in control) *(137)*.

More recently, advances in ingredient technologies have resulted in a shift of nitrate reduction from occurring in meat products to taking place during the manufacturing of the ingredients, thus eliminating the incubation step for processors of cured products. The resulting

ingredient is a natural ingredient containing sodium nitrite, commonly referred to as pre-converted celery powder. Much of the meat processing industry has shifted to using pre-converted celery, or cultured celery juice powder, as opposed to the nitrate source and starter culture to both decrease processing time and increase precision over the concentration of nitrite added to formulations.

There have also been advances in natural and organic ingredient options to serve as reducing agents, such as cherry powder (ascorbic acid), to provide natural forms of cure accelerators. Specifically, cherry powder has been shown to function similarly to ascorbate or erythorbate and decrease residual nitrite in a naturally cured products. Terns et al. reported that emulsified cooked sausages manufactured with celery powder and cherry powder resulted in quality and sensory characteristics similar to a nitrite-cured control, and residual nitrite levels in treatments containing cherry powder decreased more rapidly than treatments without cherry powder *(149)*. Additionally, the percentage of total pigment converted to cured pigment at day 0 was higher in treatments containing cherry powder and celery powder, as opposed to celery powder alone, suggesting an increased rate of cured pigment development. Baseler evaluated pork cured with celery powder, with and without 0.28% cherry powder, along with a nitrite-cured control *(9)*. Results showed that over 12 weeks of storage after thermal processing, residual nitrite depleted from 61 to 32 ppm for the nitrite-cured control, 18 to 10 ppm for the celery powder, and 10 to 3 ppm for the celery powder/cherry powder treatment *(9)*. These data show that there is less residual nitrite in an alternative cured system than in one cured with chemical nitrite, and that effect is exaggerated in the presence of cherry powder, a natural cure accelerator.

CURED MEAT SAFETY

Listeria monocytogenes. *L. monocytogenes* is a Gram-positive, rod-shaped, facultative anaerobe that is known to demonstrate unique "tumbling" motility at 25°C. It is a ubiquitous organism and grows amid a wide range of conditions. Isolates have been located in soil, water, vegetation, farm environments, and food processing environments *(169).* The organism may be present on animals destined for slaughter, and can thus make its way into the processing environment and into raw materials. Though this organism is frequently found on raw foods of both plant and animal origins, *L. monocytogenes* can also be isolated from cooked products due to post-thermal processing contamination.

Factors affecting growth and survival. *L. monocytogenes* is a serious threat to consumers of RTE meat products because of its ability to survive and even multiply under standard conditions that control many other potential pathogens in meats. This organism is tolerant to high salt, can grow within a wide range of water activity and pH, is facultatively anaerobic, and perhaps most importantly, can grow at refrigeration temperatures. While the optimum temperature for growth is 37°C, *L. monocytogenes* can multiply at temperatures ranging from 0 to 45°C, making it a particular concern for food processors who rely on temperature control as a hurdle to combat pathogens *(118).* In broth, generation times for 4, 10, and 37°C were 43, 6.6, and 1.1 h, respectively *(7).* The organism is generally inactivated following commercial cooking processes, at temperatures above 50°C *(118).* Salt, a key non-meat ingredient used in cured meats that can have an impact on microbial growth, does not affect *L. monocytogenes* at the concentrations used in processed meats. The organism is halotolerant, and high concentrations of salt (10-12%) do not prevent growth, and reports have shown growth within a pH range of 4.4 to 9.6 *(118).* The optimum water activity for growth is ≥0.97, though

the minimum is as low as 0.93 depending on the strain. In fermented hard salami, *L. monocytogenes* was reported to survive pH as low as 4.4 and water activities of 0.79 to 0.86 for at least 84 days *(63).* There is known variation in growth based on these various intrinsic factors, yet a clear understanding of product parameters like pH, salt concentration, water activity, and nitrite concentration can provide valuable information to estimate the potential for growth of this pathogen. Since there is no current evidence that *L. monocytogenes* survives commercial thermal processes, the presence of the organism on raw materials presents risk to consumers, but emphasizes the necessity of clear separation between raw and RTE environments in food processing facilities as well as the need to address the potential for post-processing contamination and growth.

Incidence of listeriosis. While *L. monocytogenes* has been estimated to cause approximately 1500 cases of foodborne listeriosis annually, a relatively small percentage of total foodborne illness, 94% of those cases result in hospitalizations and 15.9% in death, making it one of the most dangerous foodborne pathogens *(120).* Similar to many foodborne pathogens, populations at greater risk are the young, elderly, and immunocompromised, but pregnant women are especially susceptible to listeriosis, which in that case can result in spontaneous abortion, preterm labor, or stillbirths. It has been recognized as a threat to public health and is associated with RTE foods. A comprehensive risk analysis was conducted by a collaborative effort between the U.S. Food and Drug Administration, USDA, and the Centers for Disease Control (CDC). The results of this analysis found that of 23 categories of RTE foods, RTE meats, including deli meats and frankfurters, posed the greatest risk to consumers *(118).* In the same report, it was estimated that 90% of human listeriosis cases in the U.S. were caused by consumption of deli meats that had been contaminated with *L. monocytogenes (35).* However, it

is important to note that this data was collected and analyzed before the approval and widespread implementation of lactate and diacetate as effective antilisterial agents in such RTE products. Large, multi-state outbreaks of listeriosis have been linked to frankfurters and deli turkey, as well as other processed RTE meat products, drawing more attention to this product category, with respect to regulations and prevention of human illness *(33)*.

Regulations to control *L. monocytogenes.* Because of its ability to grow at refrigeration temperatures, the high mortality rates associated with listeriosis, and outbreaks in which RTE meats have been implicated as vehicles of transmission, a zero-tolerance policy for *L. monocytogenes* in RTE meats was established by USDA *(120, 162)*. Contamination risk can be minimized using the Hazard Analysis Critical Control Point (HACCP) system and is also managed in the food processing environment through prerequisite programs. Rules set forth by FSIS for control of *L. monocytogenes* offer processors three alternatives for minimizing contamination for post-lethality exposed RTE products (9 CFR 430) *(159)*.

> *Alternative 1: Application of a post-lethality treatment (which may be an antimicrobial agent) that reduces or eliminates microorganism on the product and an antimicrobial agent or process that suppresses or limits the growth of L. monocytogenes.*
>
> *Alternative 2: Application of either a post-lethality treatment (which may be an antimicrobial agent) that reduces or eliminates microorganisms on the product or an antimicrobial agent or process that suppresses or limits growth of L. monocytogenes.*
>
> *Alternative 3: Use of sanitary measures only*.

In spite of the vast amount of research and regulatory efforts to control or eliminate *L. monocytogenes* in RTE meats, it remains a major concern for meat processors.

Clostridium perfringens. *C. perfringens* is a Gram-positive, non-motile, anaerobic organism with that forms heat stable spores *(56)*. This organism is one of the most widely distributed bacteria, as it has been isolated from soil, water, intestines, food, and air. It is often a component of the normal intestinal microflora of healthy animals and humans, which can lead to contamination of meat products due to fecal cross contamination during processing. In a commercial abattoir in England, of 100 carcasses each of beef, pork, and lamb, *C. perfringens* was present on 29, 66, and 85 of the carcasses, respectively *(139)*. Because this organism can synthesize only a portion of its essential amino acids, meat is a very suitable, protein rich medium for *C. perfringens* growth *(95)*. In the US, samples of raw, cured whole muscle or ground/emulsified meats were tested for presence of *C. perfringens,* and researchers found 1.6 and 48.7% positives of whole muscle and ground/emulsified samples, respectively *(146)*. Importantly, since any vegetative cells present are normally destroyed by typical cooking procedures and would thus not be a food safety risk in RTE products, samples were also analyzed for spores. None of the whole muscle samples were positive for spores, and 5.3% of the ground/emulsified samples contained spores *(146)*.

Factors affecting growth and survival. *C. perfringens* has been noted for having an remarkably short generation time of less than 10 minutes in meat at temperatures of 43 to 47°C, making it a particular concern in large diameter meat products that may fall within that temperature range during a large part of the cooling process *(123)*. While the optimal temperature range for growth is 43 to 47°C, growth has been noted at temperatures ranging from 12 to 50°C. The heat applied during commercial thermal processing of RTE meat products kills vegetative cells but is sub lethal to spores, and actually serves as a common method of activation for any present spores to begin to germinate. Meanwhile, the optimal temperature for

sporulation is near 37°C, human body temperature, and this contributes to enterotoxin production and resulting illness. Like many organisms, a near neutral pH, between pH 6 and 7, is optimal for growth, while little to no growth is observed at pH values ≤5 and ≥8.3 *(73)*.

Incidence of disease. Five groups (A-E) of *C. perfringens* are classified based on the type of exotoxins produced, with Type A being of greatest concern from a human health perspective due to its production of an enterotoxin. The enterotoxin produced in *C. perfringens* Type A is ultimately responsible for the majority of *C. perfringens* foodborne disease seen in humans, with symptoms that include abdominal pain, nausea, and diarrhea that generally subside after one to two days *(56)*. To lead to illness, a person must consume 10^8 to 10^9 vegetative cells, which would require the appropriate growth environment as well as time to reach those high numbers *(40)*. After an 8-24 hour incubation period, ingested vegetative cells begin to sporulate in response to the harsh gastrointestinal environment, enterotoxin is released in large quantities in the intestine of the host as vegetative cells lyse during sporulation. The enterotoxin binds to receptors in the intestinal wall, disrupts normal cell wall permeability, and results in fluid loss *(74)*. The illness is generally relatively mild and self-limiting in otherwise healthy individuals, though it can be more serious in elderly or debilitated individuals.

Foodborne illness caused by *C. perfringens* results in very few hospitalizations or deaths, but it is estimated to cause nearly 1 million cases of illness in the United States each year *(120)*. While the primary vehicles for *C. perfringens* outbreaks in the US are meat and poultry, a few outbreaks have been identified with cured processed meats, notably two associated with cooked corned beef served at St. Patrick's Day meals in Ohio and Virginia in 1993 *(22)*. The outbreaks that have occurred and linked to such products often are related not to commercial processing, but more often to improper handling, reheating and cooling, at home, retail, or foodservice

establishments. Average outbreaks include 50 to 100 cases, and typically originate in institutional settings, where large quantities of foods are prepared and held before serving. Roast beef, turkey, and chicken, as well as gravies made from these meat products, are often used in food service and institutions for convenience, and are often cooked for long periods of time in large quantities at low temperatures, which usually is adequate to stimulate spore germination of *C. perfringens (40).* To help prevent outgrowth of this organism, suggested holding temperatures for foods are either ≥60°C or ≤4° *(130).*

Regulations to control *C. perfringens*. Commercial thermal processes used to cook RTE meat products effectively destroy vegetative cells of *C. perfringens,* as well as competing organisms, thus leaving a prime growth environment for any *C. perfringens* cells to germinate and multiply during cooling. The risk of outgrowth based on temperature was clearly demonstrated in uncured turkey breasts inoculated with *C. perfringens* spores, cooked to 72°C, and cooled over 3 chilling times, where researchers demonstrated the increased outgrowth when internal product temperature remained in the range of growth for the organism for longer periods of time *(142).*

To aid in mitigating this risk, USDA, FSIS established a Compliance Guideline for Cooling Heat-Treated Meat and Poultry Products (Appendix B) that presents three options for cooling *(157).*

> *Option 1: During cooling, the product's maximum internal temperature should not remain between 130°F and 80°F for more than 1.5 hours nor between 80°F and 40°F for more than 5 hours.*

Option 2: Chilling should begin within 90 minutes after the cooking cycle is completed. All product should be chilled from 120°F to 55°F in no more than 6 hours. Chilling should then continue until the product reaches 40°F.

Option 3: Products cured with a minimum of 100 ppm ingoing sodium nitrite may be cooled so that the maximum internal temperature is reduced from 130 to 80°F in 5 hours and from 80 to 45°F in 10 hours (15 hours total cooling time).

These performance standards were designed to limit outgrowth of *C. perfringens* to a maximum 1 log increase during cooling. This level of growth was determined to be acceptable after the agency (FSIS) evaluated microbial baselines that found 4 log CFU/g of *C. perfringens* in some raw meat and poultry products, leading them to conclude that this level could occur in raw materials used for producing cooked cured meats. Additionally, the Centers for Disease Control estimates that viable counts equal to or greater than 5 log CFU/g of *C. perfringens* in food is likely to result in disease or an outbreak *(73)*. By combining those two pieces of information, possible contamination of 4 log CFU/g and increased potential for an outbreak at levels of 5 log CFU/g, FSIS limited growth of the organism to 1 log during stabilization *(146)*. If these performance standards are not met, a processor has the option to validate a specific cooling process or use one of multiple microbial models now available to estimate the risk of outgrowth during cooling based on temperatures and product parameters.

ANTIMICROBIAL IMPACT OF CURING INGREDIENTS

Antimicrobial impact of nitrite. For many years, the antimicrobial impact of nitrite remained poorly understood, as nitrite was thought to only impact the color and flavor of cured meats. Now, however, nitrite in processed meat products has been well documented as an

effective agent against aerobic and anaerobic, Gram-positive and Gram-negative bacteria, and can be included as one hurdle in a multiple-hurdle approach to food safety *(81, 154)*. Nitrite has been shown to inhibit many organisms, including *Clostridium botulinum, Staphylococcus aureus, Bacillus spp., Clostridium sporogenes, C. perfringens, Streptococcus faecalis, Pseudomonas spp., Enterobacter spp., Micrococcus spp.,* and *Salmonella spp. (21, 29-31, 75, 141, 143).* However, the biochemical reactions responsible for inhibitory effects of nitrite on various pathogens are not yet completely understood.

It is generally accepted that the specific mechanism of inhibition by nitrite varies based on the microorganism *(116, 154).* In 1939, Ingram hypothesized that nitrite interfered with cytochromes, used as electron transporters in respiration of *Bacillus cereus (57).* Subsequently, Tarr et al. evaluated nitrite's impact on *Achromobacter* in broth and found that aerobic respiration was not consistently inhibited by nitrite, suggesting there must be another target within the microorganism affected by nitrite *(147).* Further support for this idea was presented by Castellani et al. in a study investigating relative susceptibility to nitrite across varying microorganisms *(21).* As compared to aerobic environments, authors found nitrite to produce a greater inhibitory effect to microorganisms under anaerobic conditions, suggesting a more complex mechanism than in previous explanations related to respiratory pathways. Furthermore, Castellani et al. proposed that the mechanism must be related to the undissociated nitrous acid form, as approximately a ten-fold increase in bacteriostatic activity for each unit decrease in pH was observed *(21).* This relates to the pK_a of nitrite (3.4) meaning that more nitrous acid is present at more acidic pH than at neutral pH. The importance of nitrous acid as an antibacterial molecule was reiterated by Shank et al. in 1962, after investigating relative effects of nitrite, nitrate, nitric oxide, and nitrous acid on the inhibition of Gram-negative organisms. Shank et al.

hypothesized that the nitrous acid reacted with components of the growth medium, rendering them unavailable for microbial metabolism and growth *(133)*.

In anaerobes, substantial evidence points to pyruvate metabolism as a target affected by nitrite and nitric oxide. Tompkin et al. hypothesized that nitric oxide reacted with iron-sulfur proteins in bacterial cells and interfered with metabolism *(156)*. In clostridia and other obligate anaerobes, iron-sulfur proteins such as ferredoxin are essential to electron transport and energy production, and scientists then believed that nitric oxide could react similarly with the iron in these proteins as with the heme iron in myoglobin. To further understand this potential interaction, Woods et al. added nitrite to *C. sporogenes* in media and observed a decrease in cellular ATP production and an increase in pyruvate excretion, suggesting nitrite interfered with pyruvate metabolism *(171)*. Authors suggested pyruvate-ferredoxin oxidoreductase as a likely target of nitric oxide interaction and metabolism interference *(170, 171)*. In further support, McMindes et al. showed that nitric oxide, but not nitrite, formed iron-sulfur-nitrosyl complexes and inhibited pyruvate-ferredoxin oxidoreductase in *C. perfringens (91).*

While the exact mode of action remains unclear, research has shown that nitrite's effects can be increased when combined with other hurdles *(14, 140)*. Interactions of nitrite with other meat ingredients (e.g. sodium chloride or ascorbate) or specific growth conditions (e.g. oxygen, pH, or temperature) influence the antimicrobial impact of nitrite *(21, 29, 89)*. pH, for example, was one of the first factors recognized to have a significant influence on bacteriostatic impacts of nitrite, when reports that nitrite was a more effective preservative of fish at acidic pH *(147, 148)*. This effect was later confirmed in *C. sporogenes* and *C. botulinum (5, 107)*. Roberts et al. investigated the impacts of salt, along with nitrite and nitrate, on the germination and outgrowth of *Bacillus* and clostridia spores, and found that salt and nitrite provided greater interference with

germination than nitrate, and that the nitrite effect was pH dependent, again pointing to nitrous acid as a key player in the antibacterial impact of nitrite *(117)*. Anaerobic spore outgrowth was tested in a media system at pH 6 and 7, and Duncan et al. concluded that it was nitrite and decreased pH that showed the greatest inhibition *(29)*. Furthermore, in this study, nitrite levels as high as 40,000 µg/g (ppm) allowed for spore germination, but prevented outgrowth. Similar results were reported by Pivnick et al. in an evaluation of germination and outgrowth of *C. botulinum* spores, in which the primary effect of nitrite was observed to be the inhibition of outgrowth of spores or germinated cells remaining after processing *(111)*. This pH effect has also been reported to have an impact on control of *C. perfringens*, where less nitrite was required to inhibit outgrowth of spores at pH 6 than at pH 7 *(75)*.

In the late 1960s and early 1970s, the effect of heating nitrite-containing media and a potential mechanism known as the Perigo effect was first discovered *(107)*. The Perigo effect was suggested to provide microbial inhibition due to the formation of some inhibitor during the heating of nitrite during thermal processing, as a portion of the original added nitrite reacted and was not recoverable as residual nitrite after heating *(107)*. Authors reported that this Perigo factor was more potent than nitrite itself. Reduced nitrite concentrations were required to inhibit *C. sporogenes* after heating the nitrite to 90-130°C in media when compared to adding nitrite after heating. A follow up study evaluated the Perigo effect against other clostridia and reported similar effects of heating the nitrite in media, and also found *C. perfringens* to be most resistant of the species tested *(106)*. For example, at pH 6, an inhibitory concentration of greater than 240 ppm was observed for *C. perfringens* in unheated media, but after heating nitrite in media at 121°C for 20 min, that concentration decreased to 5 to 15 ppm *(106)*.

A similar Perigo-type factor was described by Pivnick et al. in experiments with cured luncheon meat formulated with varied ingoing levels of nitrite and held after processing until all treatments had residual nitrite levels below 2 ppm, at which point they were inoculated with botulinal spores *(110)*. The results of their study indicated that, although residual nitrite concentrations were similar at the point of inoculation, greater amounts of ingoing nitrite led to greater inhibition of growth and toxin formation in samples. A number of studies continued to investigate the Perigo effect and clostridia inhibition in cured meats, with somewhat conflicting results *(4, 5, 79, 93)*. However, the Perigo factor was concluded to be of little importance in cured meats, since the required nitrite concentrations to observe the inhibitory effect were greater than 200 ppm, and thus outside the range of regulated nitrite concentrations for cured meats *(141)*. Furthermore, across much of the literature regarding the inhibitory effect known as the Perigo factor, the transition from bacteriological media to cure meat systems showed that the inhibitory effect was more potent in media than in meat systems. Perhaps the best summary of this research area was given by Tompkin, in which he referred to the formation of the Perigo effect in commercially produced meats as "highly debatable" *(154)*.

While minimum concentrations of nitrite required for bacterial inhibition have been somewhat controversial, the idea that more nitrite provides a greater effect has been generally accepted *(24, 55, 140)*. Curing ingredients, both nitrite and nitrate, were considered directly related to the growth of *C. botulinum* in frankfurters *(55)*. Botulism toxin production was completely inhibited in frankfurters formulated with 50 ppm ingoing nitrite, and authors concluded that nitrate had little effect on toxin production *(55)*. Toxin was detected in liver sausage formulated with nitrite concentrations of 50 to 100 ppm and salt levels of 3.8-4.2% stored at 27°C, but not in sausages formulated with 150 ppm ingoing nitrite *(51)*. Concentrations

of ingoing nitrite below 100 ppm likely have less antimicrobial impact in processed meat products. It is essential to keep in mind that a number of interrelated factors affect the antimicrobial impact of nitrite in meats. Gibson et al. determined that pH, temperature, salt, and nitrite were all influential on growth of *C. perfringens* and the effects of nitrite were increased when combined with decreased temperature or increased salt concentration *(43)*.

Generally, nitrite is not thought to be a very effective inhibitor of *L. monocytogenes*. In broth, nitrite was shown to have little effect on this organism, as little inhibition was observed when 200 ppm nitrite was added to Trypticase soy broth at pH 5.5, 6.0, or 6.5 *(131)*. Shahamat et al. suggested that nitrite was only inhibitory to *L. monocytogenes* at salt concentrations greater than or equal to 3% and nitrite concentrations greater than 200 ppm *(131)*. Buchanan et al. reported that decreasing storage temperatures (37, 28, 19, or 5°C) exhibited a greater effect on *L. monocytogenes* than increasing nitrite concentration (0, 50, 100, or 200 ppm) *(15)*. At storage temperatures of 4°C or 25°C, 30 ppm sodium nitrite was not able to inhibit growth through 28 d in turkey slurries comprised of 25% meat in sterile deionized water *(121)*. Meanwhile, levels as high as 800 ppm sodium nitrite were needed to inhibit growth in beef slurries stored for 24 h at 20°C *(166)*. However, Duffy et al. demonstrated that at 0 and 5°C, *L. monocytogenes* growth rates were decreased and lag times were increased when ingoing nitrite was increased from 0 to 315 ppm *(28)*.

The combined use of multiple hurdles, such as sodium nitrite, pH, and sodium chloride can effectively inhibit *Listeria* growth *(99)*. The relationship between acidic pH and improved bacteriostatic activity of nitrite has been accepted to impact control of many microorganisms. McClure et al. investigated the effects of temperature, pH, sodium chloride, and sodium nitrite on growth of *L. monocytogenes* and reported that at 30°C, even a high concentration (400 ppm)

was unable to delay growth at a pH of 6.0 or higher, but lower levels of nitrite were increasingly more effective at lower pH values *(89)*. Results of *L. monocytogenes* growth on various processed meat products reported by Glass et al. reiterated the limited value of nitrite as a significant inhibitor of growth on meat products *(44)*. However, the increased growth observed in that study in samples of chicken and turkey was likely due to the lack of nitrite in those products as compared to ham and bologna, which would have contained nitrite.

More recently, Myers et al. demonstrated that sliced ham formulated with no nitrite, 100 or 200 ppm sodium nitrite, and 50 or 100 ppm nitrite from celery powder, stored at 4.4°C, *L. monocytogenes* populations in all treatments increased in from approximately 3.4 log to greater than 7.5 log over 21 d *(96)*. Xi et al. tested a cured meat model system with 0, 50, 100, 150, and 200 ppm sodium nitrite at 10°C for 10 d, and showed that after 10 d, treatments with 150 and 200 ppm nitrite had *L. monocytogenes* populations that were 3.13 and 3.25 log less than the control, respectively, suggesting a dose dependent effect based on ingoing nitrite *(174)*. Nitrite provides little inhibition of *L. monocytogenes*.

Antimicrobial impact of cure accelerators. Other commonly used ingredients in cured meats include cure accelerators such as sodium ascorbate and sodium erythorbate, which have been evaluated for antimicrobial impact, with conflicting results reported. When botulinum toxin production was measured in inoculated frankfurters formulated with 0 to 150 ppm sodium nitrite and 0, 105, or 655 ppm sodium ascorbate stored at 28°C, the minimum level of ingoing sodium nitrite to inhibit toxin formation was 100 ppm and ascorbate concentration neither increased nor decreased the effectiveness of nitrite *(11)*. Pork was cured with varying concentrations of nitrite (0, 50, 100, 150, or 200 ppm) and erythorbate (0, 183, 366, or 550 ppm) and inoculated with *C. perfringens* spores, and recovery of spores was affected by the

concentration of nitrite, but not by erythorbate *(119)*. More recent work actually reported greater *C. perfringens* populations after cooling of products formulated with 557 ppm erythorbate and 50 or 100 ppm sodium nitrite as compared to products with sodium nitrite alone *(114)*.

Meanwhile, others have reported an enhancing effect when ascorbate or erythorbate were used in conjunction with nitrite *(21, 64, 151, 153)*. Tompkin reported significantly greater inhibition of *C. botulinum* outgrowth when ascorbate was used in combination with either 50 or 156 ppm nitrite in a comminuted perishable cured meat *(153)*. However, ascorbate alone did not affect *C. botulinum* outgrowth, and 50 ppm nitrite had little effect alone, yet the combination of nitrite and ascorbate was inhibitory. By investigating other compounds that were substituted for the various roles of ascorbate (butylated hydroxyanisole and tertiary butylhydroquinone as antioxidants, sodium sulfide and cysteine as reducing agents, and ethylenediaminetetraacetic acid (EDTA) as a chelating agent without reducing capacity), it was concluded that the additional antimicrobial effects were not due to the reducing capacity or antioxidant effect of ascorbate, but in fact a chelating effect *(151)*. Because the EDTA was not inhibitory in the absence of nitrite, the authors concluded that the inhibition provided by the combined use of EDTA or ascorbate with nitrite was due to nitrite-induced bacterial injury, and then impaired recovery and repair due to the sequestration of one or more cations *(151)*.

Much of the research regarding antimicrobial impacts of cure accelerators has been with clostridia, though Duffy et al. did study the effects of pH, water activity, nitrite, and ascorbate on growth of *L. monocytogenes* in cooked beef, pork, chicken, and turkey at 5°C *(28)*. With similar nitrite and pH levels, *L. monocytogenes* lag time was 9.9 d without ascorbate and increased to a range of 25.4 to 35 d with the addition of 420 ppm sodium ascorbate in cured meats *(28)*. This work suggested that the use of ascorbate enhanced the antimicrobial impact of nitrite on *L.*

monocytogenes. Furthermore, the authors noticed that growth rates tended to be faster when lag was longer and slower when lag was shorter, and attributed that to the continued depletion of residual nitrite during lag, leaving less nitrite in the samples once logarithmic growth commenced.

Antimicrobial impact of residual nitrite. Such observations regarding the complex interactions of ingoing nitrite, cure accelerators, and residual nitrite and the resulting antimicrobial impact, as made by Duffy et al., raised the questions of what impact, if any, residual nitrite has relative to the antimicrobial properties of a meat system to which sodium nitrite is added *(28).* Christiansen et al. investigated botulism toxin formation in canned ham with varied levels of ingoing nitrite (0, 50, 100, 150, or 200 ppm) *(24).* Toxin production was dependent upon the amount of ingoing nitrite, and though residual nitrite was measured after 3 and 7 days, residual levels were directly related to the amount of ingoing nitrite. Conclusions drawn in that study pointed to ingoing nitrite, not residual, as the determining factor for botulism toxin production. Further, Grau et al. reported that higher levels of residual nitrite corresponded to decreased *L. monocytogenes* growth *(49).*

Data collected at Swift and Company and presented by R. Bruce Tompkin to the American Meat Science Association led to the conclusion that the longer an inoculated cooked, cured product is stored before temperature abuse, the less *C. botulinum* inhibition is provided by nitrite *(150).* Tompkin referred to this particular scenario as a "race between spore germination, cell death, and depletion of residual nitrite." In one study, the amount of residual nitrite was manipulated and decreased by adding hemoglobin to the meat system. When 1% bovine hemoglobin powder was added to meat systems containing 50 and 156 ppm nitrite, residual nitrite levels after cooking were 24 and 28 ppm lower, respectively, than treatments without

hemoglobin. Further, the treatments with less residual nitrite exhibited decreased *C. botulinum* inhibition, indicating that not only the ingoing nitrite concentration was important to predict food safety, but also the residual nitrite remaining at the point of temperature abuse *(152, 155).* In contrast, a study was conducted by Pivnick et al., using varying levels of ingoing nitrite to manufacture canned cured luncheon meat *(110). C. botulinum* spores were inoculated into the samples, but only after holding treatments so that all residual nitrite levels had depleted to less than 2 ppm. Reported results demonstrated that even with similar residual nitrite levels at the point of inoculation, the treatments with greater ingoing nitrite concentrations provided greater inhibition of spore outgrowth.

Safety of alternative cured products. What is critical to recognize is that while using slightly different ingredients, curing meat with either a direct delivery of nitrite or an indirect, natural source of nitrite results in products that have the same quality attributes from a consumer's perspective, yet one important difference is that alternative cured products are known to have less ingoing and less residual nitrite *(124).* Furthermore, lower levels of nitrite indicate, at least to some degree, that alternative cured products could provide less inherent protection against the outgrowth of bacterial pathogens. This is of particular concern to food processors because consumers may notice the similar quality of alternative cured meats and conventional products, and consequently expect them to have the shelf life and level of food safety that are predictable in traditional, conventionally cured products cured with direct addition of chemical sodium nitrite.

Decreased inhibition of pathogens, including *L. monocytogenes, C. perfringens,* and *C. botulinum,* has been documented in hams, frankfurters, and bacon when alternative cured products were compared to purified sodium nitrite cured controls *(62, 122, 167).* Wanless

reported that frankfurters and hams made with pre-converted celery powder as a nitrite source exhibited shorter times to *C. botulinum* toxin production than conventionally cured controls *(167)*. For inoculated commercial frankfurters, hams, and bacon, those made with nitrite from natural sources supported greater growth of *C. perfringens* than purified nitrite-added controls during storage at 20°C *(62)*. However, in the same study, authors recognized a considerable amount of variation in growth patterns in the commercially prepared frankfurters, hams, and bacons tested, regardless of nitrite source, indicating that there remains a great deal to understand about the microbiological safety of these products.

Schrader reported similar growth patterns for *L. monocytogenes*. Treatments containing celery powder as a source of nitrite were not as effective to control *L. monocytogenes* growth as conventionally cured controls when stored for 35 d at 10°C *(122)*. Myers et al. evaluated sliced ham made with 100 or 200 ppm sodium nitrite, and 50 or 100 ppm nitrite from celery powder against uncured control, and found that there was not a difference in *L. monocytogenes* growth among treatments through 12 d storage *(96)*. However, at days 12 and 14, the uncured control allowed more growth than other formulations, and 200 ppm sodium nitrite showed less growth ($P < 0.05$) than uncured, 50 ppm, and 100 ppm nitrite from celery powder treatments.

Golden et al. presented support for the hypothesis that the concentration, but not the source, is the determining factor regarding nitrite effects on *L. monocytogenes*, using a sliced deli-style turkey product cured with 80 or 120 ppm nitrite from either purified nitrite or from celery powder *(47)*. This information was critical in the discussion of antimicrobial impacts of nitrite when looking at alternative sources of the ingredient. This verification that nitrite acts similarly in a product regardless of its source is essential in order to utilize previously published knowledge of nitrite as an antimicrobial in determining the safety of alternative cured products,

with lower nitrite concentrations. After 8 weeks of storage, no difference ($P < 0.05$) in *L. monocytogenes* growth was reported in turkey breast treatments with 80 ppm nitrite from either purified or celery powder sources, or in treatments with 120 ppm nitrite from either source. The authors also reported that neither of these nitrite concentrations was sufficient to completely inhibit growth, since all treatments supported greater than 2 log increases after 8 weeks at 4°C.

ADJUNCT ANTIMICROBIALS

Conventional antimicrobials. To enhance the safety of RTE meats, many antimicrobial agents have been identified and approved for use in formulations. Many of the adjunct antimicrobial compounds used today are forms of organic acids or their respective salts. Acetic, lactic, propionic, sorbic, and benzoic acids are among the most active, and antimicrobial activity of these acids is pH-dependent. The main mode of action is through the undissociated form of the acid, so pK_a of the acid relative to the product pH also becomes an important factor in effectiveness. In general, the undissociated form of the acid is able to cross the microbial membrane, and once encountering the near-neutral pH in the cytoplasm, dissociation occurs *(25)*. The released protons acidify the cytoplasm, which can lead to changes in conformation of many essential components for cell metabolism, growth, and survival, such as enzymes, structural proteins, phospholipids, and nucleic acids. Organic acid salts, in particular sodium lactate and sodium diacetate, have been found to be effective in controlling growth of *L. monocytogenes*, particularly in the presence of nitrite, and have thus become commonly used in conventional RTE meat products *(121)*. Sodium and potassium lactate are allowed to be used up to 4.8% (21 CFR 184.1768 and 21 CFR 184.1639).

Compelling evidence supports the efficacy of the combined use of lactate and diacetate in the inhibition of *L. monocytogenes* growth during refrigerated storage of RTE meat products, and many meat processors incorporate these ingredients into product formulations to enhance the safety of such products. Glass et al. demonstrated that >3% sodium lactate or the combination of 1% lactate with 0.1% diacetate could inhibit *L. monocytogenes* growth in bratwursts for 60 days at 4.5°C *(45)*. Storage temperature also affects the efficacy of these antimicrobials. For example, at 7 and 3°C, 3.4% lactate with 0.1% diacetate was able to delay growth for 4 and 12 weeks, respectively *(45)*. In an uncured comminuted beef model system (pH 6.3, 79% moisture) stored at 10°C, 2.5% sodium lactate and 0.2% sodium diacetate was bacteriostatic to *L. monocytogenes* for 20 d, whereas decreasing the storage temperature to 5°C decreased the antimicrobial concentrations required for a similar antilisterial effect to 1.8% sodium lactate and 0.1% sodium diacetate *(88)*. Additionally, other organic acid salts have been shown to inhibit *L. monocytogenes.* For example, in ham stored 12 weeks at 4°C, 0.1% benzoate, 0.2% propionate, 0.3% sorbate provided similar *L. monocytogenes* growth inhibition as 1.6% sodium lactate with 0.1% diacetate *(46)*. The use of nitrite also can enhance these organic acid salts. Glass et al. demonstrated less *L. monocytogenes* growth inhibition at 4°C in uncured turkey as compared to nitrite-cured ham *(46)*. In frankfurters made with 156 ppm ingoing sodium nitrite, Barmpalia et al. demonstrated that 1.8% sodium lactate combined with 0.25% sodium diacetate inhibited *L. monocytogenes* for 40 d at 10°C *(8)*.

Since effective usage rates of lactate and diacetate vary based on other products parameters, such as moisture, pH, salt, and nitrite, a variety of models have been developed to help processors determine appropriate levels of antimicrobial required to achieve a certain margin of safety depending on their product and desired shelf life. Seman et al. used response

surface modeling to model the effects of salt and moisture content along with usage of lactate and diacetate to optimize formulations and inhibit *L. monocytogenes* growth in RTE products *(129)*. Perhaps one of the most commonly used models is the Corbion® Listeria Control Model 2012, provided by Corbion Purac North America (Lenexa, KS; http://www.corbion.com/food/meat-and-poultry/solutions/listeria-control-model-2012), which allows processors to incorporate their product parameters to accurately formulate antimicrobials to achieve a level of safety during shelf life.

Besides its impact on *L. monocytogenes*, lactate has been reported to affect other pathogens in RTE products as well. Lactate has been shown to delay *C. botulinum* toxin formation, and lactate, diacetate, acetate, as well as citrate have also been proven to inhibit *C. perfringens* outgrowth during cooling of products *(65, 67)*. Ground, cook-in-bag turkey was made with 0, 2, 2.5, 3, or 3.5% sodium lactate and stored at 27°C; data showed that while toxin was detected in 3 days in the control treatment without lactate, toxin formation was delayed as long as 5 days at the highest concentration of lactate *(86)*. Organic acid salts (1% sodium lactate, 1% sodium acetate, 1% sodium citrate, and 1% sodium citrate with sodium diacetate) provided significant inhibition of *C. perfringens* outgrowth during 15 h of exponential cooling of ground turkey *(65)*. Based on comparisons between studies by the same researchers, Juneja addressed the greater spore germination and outgrowth of *C. perfringens* observed in turkey versus beef or pork *(65)*. Kennedy et al. confirmed that 2% potassium lactate in ground turkey breast was sufficient to limit *C. perfringens* outgrowth to less than 1 log during 10 or 12 h linear cooling *(67)*.

Natural, organic, or clean label alternative antimicrobials. Since the purified forms of organic acids are considered preservatives, they are not allowed in natural or organic

formulations, nor would they fit on a "clean label", with chemical names such as sodium lactate or sodium diacetate. Fortunately, many weak organic acids are naturally occurring or can be generated using naturally occurring ingredients and processes. For example, acetic acid is the primary component of vinegar, lactic acid is naturally produced by bacterial fermentation of sugars, and citric acid can be found in high concentrations in many fruits.

With the growth in natural, organic, or "clean label" RTE meat products, there has been a corresponding increase in the number of investigations of natural sources of antimicrobials. Lemon juice and vinegar, natural sources of citric and acetic acids, respectively, have been shown to inhibit *C. perfringens* outgrowth in uncured turkey and roast beef during chilling *(83, 165).* Li et al. showed that a 2.0% lemon juice and vinegar blend limited outgrowth of *C. perfringens* spores to less than 1 log during cooling of roast beef, even when cooling took as long as 21 hours from 54.4 to 4.4°C *(83).* In turkey breast, 3.5% of the lemon and vinegar blend was required to limit outgrowth to less than 1 log during 21 h of chilling, and 2.5% buffered vinegar also provided similar inhibition *(165).*

Though some work has dealt with natural or clean label antimicrobials in products without nitrite, minimal research has investigated the use of natural antimicrobials in in alternative cured products, with nitrite from natural sources like celery powder. Jackson et al. investigated the use of natural antimicrobial ingredients in conventional and alternative cured frankfurters and ham *(61).* Cooked product was surface inoculated with *C. perfringens* spores, heat shocked, cooled according to the uncured regulations of Appendix B, and sampled over time while stored at 20°C. Results showed that alternative cured formulations allowed for greater increases in *C. perfringens* than conventionally cured controls, but ingoing nitrite levels were not controlled. While additional antimicrobials showed some inhibition when combined with nitrite,

results were variable, and the combination of antimicrobials and natural sources of nitrite were not as effective as conventionally cured sodium nitrite formulations made with 156 ppm ingoing nitrite. Blends of cultured sugar and vinegar as well as cherry, lemon, and vinegar were shown to effectively inhibit *L. monocytogenes* growth in alternative cured frankfurters *(122)*. Similar results were observed in an investigation of *C. botulinum* toxin production using the same antimicrobials in a model system *(167)*.

McDonnell et al. screened a number of ingredients for antimicrobial properties in uncured and alternative cured model systems *(90)*. In uncured turkey systems, 1.5% vinegar/lemon/cherry powder blend, 2.0% buffered vinegar, 2.5% vinegar/lemon juice blend, 0.05% tea tree oil, or 3.0% cultured sugar/vinegar inhibited *L. monocytogenes* outgrowth at 4°C for 4 weeks. In a system containing nitrite from purified or natural sources, the antimicrobial impact of nitrite was enhanced by the use of 0.03% grapeseed extract, 1.0% liquid smoke extract, or 0.5% cherry powder during the same storage period. The most successful antimicrobial ingredients from the screening, 1.5% vinegar/lemon/cherry blend, 3.0% cultured sugar/vinegar, and 2.0% buffered vinegar, were subsequently validated in commercial deli-style turkey breast, roast beef, and ham, confirming that natural antimicrobial alternatives are available and can be effective in preventing *L. monocytogenes* growth during refrigerated storage. Golden et al. showed that turkey breast products supplemented with 1.0% cultured sugar/vinegar blend and 80 ppm nitrite from celery powder had no *L. monocytogenes* growth through 12 weeks at 4°C *(47)*. Lavieri et al. recently reported that 1.0% dried vinegar or 2.5% lemon/vinegar blend in alternative cured frankfurters with 50 ppm ingoing nitrite from celery powder prevented *L. monocytogenes* growth through 98 days at 4°C, while a control with nitrite but no antimicrobials allowed for populations to reach approximately 8 log *(76)*. While the use of antimicrobials has

been extensively studied, both in uncured products and those cured with direct addition of sodium nitrite, the same level of confidence and in-depth understanding remains to be determined when referring to natural or clean-label antimicrobials and alternatively cured meat products.

SUMMARY OF LITERATURE

Sodium nitrite, the compound responsible for the characteristic color and flavor of cured meats, has long been associated with increasing the safety of RTE meat products through its antimicrobial effects on pathogens including *C. botulinum, C. perfringens,* and *L. monocytogenes.* While the intricacies of the *C. botulinum* inhibition by nitrite in cured meats have received a great deal of attention, a more complex and less understood relationship exists between nitrite and both *C. perfringens* and *L. monocytogenes.* Less is known about the concentrations of ingoing nitrite or residual nitrite that impact these two pathogens, making it increasingly difficult to maximize the safety impact of nitrite. As consumers insist on minimizing or eliminating other ingredients that contribute to food safety in an effort to reduce consumption of preservatives, maximizing the antimicrobial effects of nitrite in RTE products remains of critical importance.

As the consumer's focus continues to shift toward what are perceived as safer, healthier, and more wholesome choices, alternative cured RTE meats have become more widely available. With that, technological advances in ingredients and processing have brought forth alternative sources of such ingredients as ascorbate and organic acid based antimicrobials to be used in formulations of alternative cured products. A clearer picture of the resulting safety impact of the combination of these ingredients is necessary in order to provide consumers with alternative

cured products with equivalent microbiological food safety characteristics to the high degree of safety that has become synonymous with traditionally cured meats.

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CHAPTER 3: INVESTIGATING THE IMPACT OF INGOING SODIUM NITRITE AND SODIUM ASCORBATE CONCENTRATIONS ON GROWTH OF *LISTERIA MONOCYTOGENES* IN A COOKED, CURED MEAT MODEL SYSTEM

ABSTRACT

Nitrite is well known to possess antimicrobial properties with regards to *Listeria monocytogenes,* but it does not completely inhibit growth. However, the level or mode of inhibition is not easily predicted using our current knowledge of the inhibitory effects from nitrite. The objective of this study was to determine the impact of ingoing sodium nitrite, ascorbate, and residual nitrite on *L. monocytogenes* growth. Ready-to-eat sausages were manufactured using 80/20 pork, 2.0% sodium chloride, one of four levels of sodium nitrite (0, 100, 200, or 500 ppm), and one of three levels of sodium ascorbate (0, 500, or 1000 ppm). Cooked, sliced meat was inoculated with 3 log CFU/g of a 5-strain mixture of *L. monocytogenes,* vacuum packaged, and stored at 7°C for 40 d. *L. monocytogenes* was enumerated at 0, 7, 14, 21, 28, and 40 d by rinsing samples with Butterfield's phosphate buffer and plating appropriate dilutions on modified oxford agar. Residual nitrite analysis was performed on d 0, 7, 14, 21, 28, and 40 using a modified AOAC nitrite determination method. Results showed that uncured treatments (no nitrite) allowed for greater population increases of *L. monocytogenes* than those containing nitrite. Ascorbate did not consistently affect microbial growth in uncured or cured treatments. Though growth decreased as ingoing nitrite increased, only treatments with 500 ppm ingoing nitrite significantly inhibited growth relative to uncured treatments throughout the experiment. Calculated growth rates showed that addition of ≥100 ppm ingoing nitrite reduced growth rates to less than half of the uncured growth rates. Residual nitrite concentration depleted over time, and tended to deplete more rapidly in the presence of ascorbate, but neither ascorbate nor residual nitrite were significant factors determining *L. monocytogenes* growth. Results confirm that increased levels of ingoing sodium nitrite provide greater inhibition of *L. monocytogenes* growth in RTE meats.

Listeria monoycytogenes is a dangerous pathogen, particularly for pregnant women and immunocompromised individuals. Although listeriosis accounts for approximately 1500 annual cases, a relatively small portion of the total foodborne illness recorded in the U.S. each year, but a high percentage of cases lead to hospitalization (94%) and even death (15%) *(37)*. This Gram-positive, facultative anaerobe is ubiquitous in the environment and is able to survive and grow in a temperature range of 0 to 50°C and from pH range of 4.6 to 9.2 *(24)*. Commercial thermal processing procedures used for ready-to-eat (RTE) meats are generally sufficient to inactivate *L. monocytogenes* that may be present in raw materials, but post-processing contamination can occur at several stages before human consumption, such as during slicing or packaging at a manufacturing facility or retail deli *(30)*. If such contamination occurs, this pathogen's ability to multiply during refrigerated storage makes it a major food safety concern for processors of RTE meat and poultry products *(18)*. Therefore, USDA FSIS maintains a zero tolerance policy for the presence of *L. monocytogenes* in products manufactured in meat processing facilities *(51)*. A number of control measures to reduce the risk of contamination have been put in place during the manufacturing of RTE meats to prevent post-processing contamination, including improved sanitation and environmental monitoring procedures *(44)*. Additionally, a number of ingredients for use in product formulations have been identified and implemented in many products as growth inhibitors of *L. monocytogenes* to slow or prevent growth if products become contaminated *(20, 42, 52)*.

Sodium nitrite (nitrite) is responsible for the characteristic color and flavor of cured meats, and has also been identified to play an important antimicrobial role in RTE meats *(47)*. The maximum allowable nitrite concentrations permitted in ground/comminuted and massaged/pumped products are 156 and 200 ppm, respectively, according to regulations set by

the USDA FSIS *(50)*. Recently, in response to consumer efforts to limit preservatives in foods, an alternative curing process was developed to cure meats without the direct addition of the preservative sodium nitrite *(39)*. Since no substitute for nitrite has been found to yield both the typical quality characteristics and provide the level of microbiological safety synonymous with cured meats, natural sources of nitrite (e.g. celery powder) were identified and isolated as replacements for purified nitrite *(39)*. Though these alternative ingredients provide sufficient nitrite in meat formulations to create cured color and flavor, current ingredient usage normally equates to ingoing nitrite concentrations less than 100 ppm, sometimes as low at 40 to 60 ppm, which has led to concerns regarding the inherent safety of alternative cured meats with diminished nitrite concentrations *(39)*.

Even though ingoing concentrations of nitrite are defined by formulation, once added to a meat system, nitrite is extremely reactive and can follow a number of chemical pathways to yield many diverse products *(7, 10)*. However, a portion of the originally added nitrite remains recoverable as free, or residual nitrite, at a level which continues to react and decline during storage *(9, 10, 40)*. Sodium ascorbate (ascorbate) is one of a select group of compounds commonly referred to as cure accelerators, which can be added to cured meat formulations up to 547 ppm to speed up nitrite-related reactions that yield nitric oxide for cured color development *(4, 13, 50)*. In doing so, ascorbate reduces the concentration of residual nitrite remaining in the product after processing, and can also increase the rate of residual nitrite depletion during storage *(15, 16, 25, 36)*. The reactivity of nitrite is complex and difficult to predict or control, since its chemistry is influenced by many factors, including pH, temperature, and the presence of cure accelerators, such as ascorbate, to name a few *(10, 40)*.

Although nitrite has been shown to contribute to inhibition of *L. monocytogenes* growth, it has long been considered a primary control measure to prevent growth and toxin formation by *Clostridium botulinum (8, 47).* Nitrite inhibits botulinal toxin production in a dose-dependent manner based on ingoing concentration, and both ascorbate and residual nitrite have been demonstrated to contribute to preventing botulinal toxin formation in meats *(23, 43, 48)*. Furthermore, ascorbate was recently shown to enhance nitrite-related inhibition of *Clostridium perfringens* during chilling of deli-style turkey breast *(11, 28, 45, 48, 49).* Relative to its impact on *C. botulinum* control, nitrite plays a smaller role in *L. monocytogenes* control in RTE meats, and less is understood about this relationship than the more well established effects of nitrite on clostridia *(47)*.

Nitrite has been reported to increase lag time of *L. monocytogenes*. At 0 and 5°C, the inclusion of 315 ppm ingoing nitrite (compared to uncured, no nitrite controls) was shown to double the lag time observed in uncured (no nitrite) vacuum packaged, cooked sliced meats inoculated with *L. monocytogenes (12).* Furthermore, Xi et al. showed the dose-dependent relationship between ingoing nitrite concentration and *L. monocytogenes* growth in cooked, cured pork sausage, stored for 10 d at 10°C *(53)*. Authors reported population increases of approximately 2 log over the 10 d storage period for treatments containing 150 and 200 ppm nitrite, yet these final populations were 3.13 and 3.25 log less than uncured (no nitrite) controls, respectively, and less than treatments containing 50 or 100 ppm nitrite *(53)*.

Although reports suggest ascorbate and residual nitrite concentrations impact cured meat safety relative to *C. botulinum,* limited knowledge exists to elucidate their effects on *L. monocytogenes* growth. Duffy et al. reported increased *L. monocytogenes* lag times when 315 ppm nitrite was supplemented with 420 ppm ascorbate in RTE meats as compared to nitrite alone

(12). Antimicrobial impact of residual nitrite was tested by storing cooked cured pork (156 ppm nitrite), inoculated with *C. botulinum* spores, at 10°C before temperature abuse (27°C), allowing residual nitrite concentrations to decrease. Authors reported that residual nitrite declined to levels as low as 5 ppm, and correspondingly, the inhibition of outgrowth diminished as residual nitrite declined, concluding that the concentration of residual nitrite remaining at the point of temperature abuse was critical for the control of *C. botulinum* outgrowth *(46)*. To date, it is unknown whether the same is true for *L. monocytogenes.* Since the potential for *L. monocytogenes* contamination of RTE products exists post-thermal processing, and a significant portion of originally added nitrite is lost during processing, particularly thermal processing, the organism is theoretically never exposed to nitrite concentrations as high as the originally formulated levels *(10)*. However, whether the bacteriostatic effects of nitrite on growth of *L. monocytogenes* are dependent solely upon ingoing nitrite concentration or are also affected by residual nitrite levels, or other products from the conversion of nitrite, remains to be determined.

While the use of nitrite is insufficient as a sole antilisterial agent in meats, it can be significantly enhanced when combined with other factors, such as pH, sodium chloride, and temperature *(6, 12, 31)*. Several studies reported that combinations of pH, temperature, sodium chloride, sodium nitrite concentrations, and vacuum packaging affect *L. monocytogenes* growth *(5, 31)*. Decreased pH can enhance the antimicrobial effect of nitrite. For example, at pH 6.0 or greater, nitrite concentrations as high as 400 ppm were unable to delay growth, whereas concentrations as little as 50 ppm exhibited inhibitory effects at pH less than 6.0 *(31)*. Many adjunct antimicrobial ingredients shown to control *L. monocytogenes* growth during refrigerated storage are more effective in cured products than in uncured, demonstrating synergy between nitrite and added antimicrobials *(17, 22, 26, 35, 38, 54)*. For example, in RTE turkey, ingoing

nitrite concentrations as low as 30 ppm were sufficient to increase lactate-diacetate inhibition of *L. monocytogenes* during refrigerated storage *(19)*. Though the availability of supplemental antimicrobials for use in RTE processed meats is increasingly widespread, a more thorough understanding of the impact of nitrite would help to ensure that nitrite is formulated at levels that maximize its antimicrobial impact.

Due to the complex nature of nitrite within a meat system and the lack of a defined mechanism by which nitrite hinders growth of *L. monocytogenes,* factors such as ingoing nitrite concentration, residual nitrite concentration, and added ascorbate could play roles in the inhibitory effect observed in cured meats. Therefore, the objective of this study was to investigate ingoing sodium nitrite and sodium ascorbate concentrations for impact on *L. monocytogenes* growth in a cooked, cured pork sausage. Additionally, by varying the concentrations of nitrite and ascorbate, it was expected that a range of residual nitrite concentrations would be present at the point of inoculation and during refrigerated storage, providing insight into nitrite depletion and residual nitrite, and the potential relationship between these factors and *L. monocytogenes* growth.

MATERIALS AND METHODS

Preparation of cured meat model system. Cooked, cured pork sausages were manufactured with varying concentrations of ingoing sodium nitrite and sodium ascorbate. Formulations included 2.0% sodium chloride, and treatments were defined by ingoing sodium nitrite and sodium ascorbate according to a 4 x 3 factorial. Ingoing nitrite concentrations were 0, 100, 200, or 500 ppm (Prague powder: 6.25% sodium nitrite, 93.75% sodium chloride) and sodium ascorbate levels were 0, 500, or 1000 ppm (purified sodium ascorbate). The

concentrations were chosen to be inclusive of today's commonly used concentrations of these ingredients in commercial cured products, but nitrite and ascorbate were also included at 500 ppm and 1000 ppm, respectively, more than twice the amounts that would legally be allowed in products for the purpose of exaggerating any differences that may be more difficult to resolve at lower levels.

Fresh pork Boston butts were obtained from a commercial supplier and stored vacuum packaged at 2.2 to 4°C until use (within 4 d). The Boston butts were deboned and trimmed to approximately 80% lean (20% fat) and then ground through a 19.05 mm plate and again through a 9.53 mm plate attached to a grinder (Model 4732, Hobart Corp., Troy, OH). The ground pork was weighed and transferred to a mixer (Model AS200, Hobart Corp.), where it was mixed with sodium chloride, sodium nitrite and sodium ascorbate according to treatment. Sodium nitrite was mixed for 1 min, followed by mixing salt for 1 min, and finally, sodium ascorbate for 1 min, so that all treatments were mixed for a total of 3 min.

Treatments were then stuffed into moisture impermeable plastic casings (9.5 cm diameter) using a table top piston stuffer and held overnight at 4°C to allow nitrite-related reactions to occur before thermal processing. A step-up steam cooking (100% humidity) schedule was used to thermally process the treatments in a single truck smokehouse (Model 450 MiniSmoker, Alkar Engineering Corp., Lodi, WI) to an internal temperature of 71.1°C. Thermal processing steps included 54.4°C for 30 min, 62.8°C for 30 min, 71.1°C for 30 min, and 79.4°F until product reached an internal temperature of 71.1°C. After the completion of thermal processing, treatments were transferred to a cooler (2°C) and cooled overnight (16-20 h). Thermal processing and cooling data were recorded using a temperature recording logging device (TempTale®4, Sensitech, Beverly, MA) to ensure target heating and cooling parameters

were met. After cooling, products were aseptically removed from the casing, sliced using a table top slicer (Model 919E, Berkel Incorporated, Troy, OH) sanitized with 70% ethanol to yield approximately 17 g per slice, vacuum packaged in gas-impermeable pouches (3 mil high barrier pouches; oxygen transmission rate, 50-70 cm^3/m^2, 24 h at 25°C and 60% relative humidity; water transmission rate, 6-7.5 g/m^2, 24 h at 25°C and 90% relative humidity; UltraSource, Kansas City, MO), and transported to the University of Wisconsin-Madison Food Research Institute (Madison, WI) for inoculation.

Preparation of inoculum. *Listeria monocytogenes* strains LM 101 (hard salami isolate, serotype 4b), LM 108 (hard salami isolate, serotype 1/2b), LM 310 (goat's milk cheese isolate, serotype 4b), FSL-C1-109 (human isolate, serotype 4b), and V7 (raw milk isolate, serotype 1/2a) were grown individually in 10 ml of Trypticase™ soy broth (BBL, BD, Sparks, MD) at 37°C for 18 to 20 h. Cells were harvested by centrifugation (2,500 x *g*, 20 min) and suspended in 4.5 ml 0.1% buffered peptone water (pH 7.2). Equivalent populations of each isolate were combined to provide a five-strain mixture of *L. monocytogenes* to yield target level of 3 log CFU/g meat (4 log CFU/50 g package). Purity of each strain was verified by streaking on modified Oxford agar (MOX; *Listeria* selective agar base, Difco, BD, Sparks, MD) and Trypticase™ soy agar (TSA, BBL, BD, Sparks, MD), and populations of each strain and the mixtures were verified by plating on MOX.

Inoculation and testing. Each sample consisted of 3 slices, which were surface-inoculated between slices with 250 µL of the *L. monocytogenes* mixture to yield a target level of 3 log CFU/g (3 log CFU/ml rinse material when using 50 ml rinse for enumeration). Each package was vacuum packaged (Multivac AGW, Sepp Haggemuller KG, Wolfertschewenden, Germany) in gas-impermeable pouches (3 mil high barrier pouches; oxygen transmission rate,

50-70 cm^3/m^2, 24 h at 25°C and 60% relative humidity; water transmission rate, 6-7.5 g/m^2, 24 h at 25°C and 90% relative humidity; UltraSource, Kansas City, MO) and stored at 7°C.

Triplicate inoculated samples were assayed for *L. monocytogenes* populations at d 0, 7, 14, 21, 28, and 40. Additionally, duplicate uninoculated samples were assayed for lactic acid bacteria and pH at each sampling interval. To enumerate bacterial populations, 50 ml Butterfield's phosphate buffer was added to each 50 g package and massaged externally by hand for approximately 2 min to release attached cells *(21)*. The rinse and appropriate ten-fold serial dilutions were surface plated on MOX (35°C; 48 h). Populations of lactic acid bacteria were enumerated by plating rinse material on All Purpose Tween agar (APT; Difco, BD, Sparks, MD) supplemented with 0.004% bromcresol purple (25°C; 48 h). Changes in pH of each uninoculated sample were measured by removing a 10 g representative portion of each uninoculated sample before rinsing in buffer. The 10 g portion was homogenized with 90 mL of deionized water with a lab blender (Stomacher 400, A.J. Seward, London, UK) and the pH was measured on the slurry (Accumet Basic pH meter and Orion 8104 combination electrode, Thermo Fisher Scientific, Waltham, MA).

Residual nitrite analysis. Residual nitrite analysis was performed on duplicate uninoculated samples on d 0, 7, 14, 21, 28, and 40, using a modification of the colorimetric method for measuring nitrites in cured meat (AOAC 973.31) *(1)*. Vacuum packaged, uninoculated samples were removed from 7°C storage and immediately frozen (-20°C) until analysis. The analysis method was modified to prevent interference based on the varied levels of ascorbate between treatments that could react with residual nitrite during the 2 h steaming extraction step *(14)*. By the addition of 4 ml of 0.1M sodium borate to the approximately 300 mL of sample, a pH near 8.0 was maintained to prevent the accelerated nitrite reactivity that

occurs at acidic pH. Additionally, 4 ml of 30% (w/v) zinc sulfate was added at the end of cooling, before the filtration step to precipitate protein remaining in the sample. Four observations per treatment at each time point were collected for residual nitrite analysis.

Proximate analysis. Duplicate uninoculated samples for each treatment and replication were analyzed for moisture (5 h, 100°C vacuum oven method *(2)*), pH (measured in a slurry prepared by homogenizing 10 g with 90 ml deionized water), NaCl (measured as % Cl^- with Quantab Titrators for Chloride, High Range Titrators –300 to 6000 ppm Cl, Hach Company, Loveland, CO) and water activity (Decagon AquaLab 4TE water activity meter, Pullman, WA) *(2, 32, 41)*.

Data analysis. This study was designed using a 4 x 3 factorial, with sodium nitrite and sodium ascorbate as the two factors. Nitrite was tested at 4 levels (0, 100, 200, and 500 ppm), while ascorbate was tested at 3 levels (0, 500, and 1000 ppm). Two independent replications were performed, and data were compared with analysis of variance using the linear models procedure in JMP (JMP Pro 11, SAS Institute, Cary, NC). The model included main effects (nitrite and ascorbate) and any significant interactions. All least significant differences were found using the Tukey-Kramer pairwise comparison method with significance levels determined at $P < 0.05$.

Growth of *L. monocytogenes* was measured over time and used to generate simple growth rates according to the equation:

L. monocytogenes count = Initial count + Span$(1 - e^{-kt})$

where Initial count is the number of inoculated organisms (log CFU/g), Span is the span in counts from the initial population to maximum concentration (log CFU/g), k is the growth rate constant (per d), and t is time (d) *(42)*. In this study, the initial *L. monocytogenes* count was 3.30

log CFU/g, while the maximum observed count was 8.50 log CFU/g, making the span 5.20 log CFU/g. These values were used to calculate growth rate constants for all treatments in this study. This treatment of the *L. monocytogenes* growth data allowed for a simplified approach to analyze the impact of nitrite and ascorbate on *L. monocytogenes*.

In a meat system, residual nitrite has been shown to deplete according to first-order rate kinetics *(3, 15)*. Therefore, the residual nitrite measured over time in this study was used to calculate a depletion rate according to the equation:

$$\text{Residual concentration} = \text{Ingoing concentration} \cdot e^{-dt}$$

where Ingoing concentration is the formulated level of sodium nitrite, *d* is the depletion rate constant (per d), and *t* is time (d). Additionally, an arbitrary data point, 10 ppm at 90 d, was included in the analysis of each treatment, which allowed for more accurate curve fitting using nonlinear regression. This arbitrary data point represents the continued nitrite depletion that would take place during shelf life, beyond the scope of the sampling period in this experiment, and recent surveys of residual nitrite in retail cured meats revealed that the majority of products contain approximately 10 ppm residual nitrite *(9, 34)*. *L. monocytogenes* growth rates and residual nitrite depletion rates were calculated for each treatment and replication and analyzed linear modeling procedures in JMP, as previously outlined.

RESULTS AND DISCUSSION

Proximate analysis showed that treatments of cooked, cured pork sausage had an average 60.3 ±2.1% moisture, 2.12 ±0.20% NaCl, pH 6.5 ±0.1, and a_w 0.975 ±0.007 with no significant differences among treatments ($P > 0.05$). Though sporadic increases of lactic acid bacteria were observed, levels were typically below the level of detection (<1 log CFU/g), and did not appear

to confound the study results, or affect *L. monocytogenes* growth (data not shown). No changes in pH were observed throughout this study ($P > 0.05$, data not shown).

Changes in populations of *L. monocytogenes* and residual nitrite concentrations are reported in Figure 3.1. There was no significant difference in initial pathogen populations ($P > 0.05$, average inoculum level of 3.3 log CFU/g). Results support that there is a dose dependent, inverse relationship between ingoing nitrite concentration and *L. monocytogenes* growth ($P < 0.05$), but ascorbate did not affect growth ($P > 0.05$) in this study. Uncured treatments, regardless of ascorbate level, grew to >8 log by d 14 and populations remained stable throughout the remainder of the study. At 7 d, populations of *L. monocytogenes* were significantly greater ($P < 0.05$) in uncured treatments, regardless of ascorbate, than treatments formulated with 500 ppm ingoing nitrite (Table 3.1). Treatments with ≤ 200 ppm ingoing nitrite did not consistently limit growth of *L. monocytogenes* relative to the uncured treatments ($P > 0.05$). Further, on d 14, populations in the uncured treatments were greater than all cured treatments, and treatments with 500 ppm ingoing nitrite supported significantly less growth ($P < 0.05$) than the uncured treatments throughout the study. Meanwhile, treatments with 100 ppm ingoing nitrite with and without ascorbate and treatments with 200 ppm nitrite and no ascorbate appeared to reach maximal populations by d 21, which remained stable through d 40 ($P > 0.05$). Treatments with 200 ppm ingoing nitrite + ascorbate and 500 ppm ingoing nitrite with and without ascorbate continued to increase from d 21 to d 40.

Residual nitrite recovered at d 0 ranged from approximately 50 to 75% of ingoing nitrite, which corresponds with previous reports that 50% of nitrite is lost during processing, including mixing and thermal processing *(9, 40)*. By d 40, approximately 20 to 40% of the originally formulated nitrite was recovered as residual nitrite, showing that concentrations depleted over

time. At d 0, the addition of ascorbate appeared to have accelerated nitrite-related reactions during processing and significantly reduced ($P < 0.05$) post-processing residual nitrite in treatments containing ≥200 ppm ingoing nitrite, but did not produce a similar effect at 100 ppm ingoing nitrite levels (Table 3.2). It is possible that naturally occurring reductants in the meat were sufficient to react with this lower concentration of nitrite (100 ppm) and the addition of ascorbate as an exogenous reductant did not accelerate nitrite-related reactions. Further, with 500 ppm ingoing nitrite, increasing the ascorbate concentration from 500 to 1000 ppm decreased ($P < 0.05$) residual nitrite at d 0, and that difference was maintained throughout the study, as 500 ppm nitrite + 500 ppm ascorbate had greater ($P < 0.05$) residual nitrite levels than 500 ppm nitrite + 1000 ppm ascorbate on all d. Throughout storage time, ascorbate had a negligible effect ($P > 0.05$) on residual nitrite concentrations when 100 ppm nitrite was originally added to treatments. At 200 ppm ingoing nitrite levels, the presence of ascorbate in formulations reduced ($P < 0.05$) residual nitrite concentrations, but the higher concentration of ascorbate did not potentiate that effect. Residual nitrite levels tended to stabilize in most treatments during this 40 d storage period, and results show that a more rapid rate of depletion occurred in the first two weeks of storage, and the rate of conversion of residual nitrite decreased with time.

As expected, varying levels of residual nitrite were present at the point of inoculation, based on usage of ascorbate at each ingoing nitrite concentration. However, since each residual level was covariate with ingoing nitrite and ascorbate levels, the effect of residual nitrite on inhibition of *L. monocytogenes* growth in RTE meats could not be isolated and analyzed as a factor. A different experimental design would be needed to address the hypothesis that residual nitrite is a factor in the inhibition of *L. monocytogenes* growth in RTE meats. Subsequent to the completion of this experiment, knowledge generated from these results was used to develop a

follow-up study to better isolate residual nitrite as a factor in mapping the overall effects of nitrite, ascorbate, and residual nitrite on *L. monocytogenes*, presented as Chapter 4 of this dissertation *(27).*

Growth rates were generated for each combination of nitrite and ascorbate are shown in Table 3. Uncured treatments (0 nitrite) had growth rates ranging from 0.142 to 0.167 log/d, whereas growth rates for treatments with a minimum 100 ppm ingoing nitrite were ≤ 0.061 log/d, less than half of the rates for the uncured treatments. Similar to the results of the *L. monocytogenes* populations over time, the generated growth rates were significantly greater ($P < 0.05$) in the uncured treatments than the cured formulations containing ≥100 ppm ingoing nitrite. However, increasing ingoing nitrite concentration up to 500 ppm did not decrease overall growth rates ($P > 0.05$). Though not statistically different, rates tended to decrease slightly with increasing nitrite concentration. Ascorbate did not significantly impact growth rates ($P > 0.05$), but at each ingoing nitrite level, *L. monocytogenes* growth rates were slightly less in treatments containing nitrite and ascorbate than those with nitrite alone. Nitrite depletion rates (ppm/d) were not different among treatments ($P > 0.05$). However, depletion rates tended to increase, though not significantly, as ascorbate concentration increased.

The results of this study confirmed that while nitrite is not bactericidal to *L. monocytogenes*, it does provide a bacteriostatic effect which is greater with increased ingoing nitrite concentration. Estimating lag time was not part of the design of this experiment, and would have been difficult to estimate with much precision since the majority of the treatments (9 of 12) displayed growth between the first and second sampling points (0 and 7 d). This experiment was designed to evaluate the interaction of ascorbate with nitrite during refrigerated storage of RTE meats and overall effects on listerial inhibition. The experiment was not

specifically designed to discriminate at what point lag phase for *L. monocytogenes* ended and exponential growth began. However, the two parameters, lag time and growth rate, are interrelated. For example, Duffy et al. observed that, in sliced cured meats, calculated growth rates tended to be less when lag time was shorter, while rates were generally greater when lag time was longer *(12)*. To that end, the equation used to calculate growth rates in this study does not account for any lag that may have occurred, and thus, the decreased growth rates estimated as ingoing nitrite concentration increased could have been affected by longer lag times. In future studies, more frequent sampling intervals may help to differentiate lag time and growth rate to determine nitrite impacts on each parameter, individually.

Previous studies have reported that lag periods were extended as nitrite concentration increased, supporting a general dose dependent effect of nitrite against *L. monocytogenes,* which is further substantiated by the results of this study *(12, 18)*. In this study, there was little difference observed in growth rates between 100 and 200 ppm ingoing nitrite, but slightly more inhibition at 500 ppm ingoing nitrite. From an application perspective, this suggests that there is a greater effect from including 100 ppm ingoing nitrite than from increasing from 100 to 200 ppm ingoing nitrite. Since the 500 ppm ingoing concentration is beyond regulatory limits, the degree of growth inhibition is not practical for commercial formulations, but does still support the dose dependence of nitrite as an antimicrobial. Buchanan et al. reported that increasing nitrite concentration up to 200 ppm (0, 50, 100, or 200 ppm) provided less inhibition of *L. monocytogenes* than decreasing storage temperature (37, 28, 19, or 5°C) *(6)*. In contrast, Myers et al. reported that 200 ppm ingoing nitrite allowed for less *L. monocytogenes* growth in sliced ham versus lesser concentrations of nitrite, and a recent study by Golden et al. reinforced that nitrite concentration (80 and 120 ppm) contributed to the inhibition of *L. monocygtogenes* in

sliced deli-style turkey *(22, 33)*. Collectively, it appears that concentrations of nitrite normally found in cured meats contribute an indeterminate level of *L. monocytogenes* inhibition, though levels <100 ppm ingoing nitrite tend to have little inhibitory effect.

Ascorbate did not significantly enhance the antilisterial effect of nitrite in this study. Duffy et al. reported that 420 ppm ascorbate did not inhibit growth in uncured products, but when added to nitrite, the combination of the two ingredients resulted in significantly reduced *L. monocytogenes* growth *(12)*. Though this conclusion was reported, specific methods and data used to reach the conclusion were note reported, making it difficult to further investigate reasons for the difference between those results and the results of this study. In this study, ascorbate was not shown to directly affect the antimicrobial impact of nitrite against this pathogen, but as a cure accelerator and reducing agent, altered the environment within the meat system by changing the residual nitrite concentration over time. Though statistical analysis does not support an antimicrobial effect of adding ascorbate to cured meat formulations, trends in the data suggest that ascorbate could slightly enhance the bacteriostatic impact of nitrite on *L. monocytogenes.* Meanwhile, the experimental design used in this study was unable to provide a clear conclusion regarding the impact of residual nitrite on pathogen growth, but outcomes and knowledge gained from this study were utilized to design a follow up experiment better suited to evaluated residual nitrite as a factor in *L. monocytogenes* growth *(28)*.

Similar to previous reports, this study confirms that concentrations of ingoing nitrite used in cured meats today are not sufficient to prevent growth of *L. monocytogenes*, yet nitrite is an effective hurdle in a multiple hurdle approach to food safety *(29)*. Results indicate that including at least 100 ppm nitrite significantly slows the rate at which *L. monocytogenes* multiplies within a product. While the majority of meats cured with purified sodium nitrite contain >100 ppm

ingoing nitrite, the knowledge gained from this study is also useful for manufacturers of products formulated with alternative sources of nitrite, such as cultured celery juice powder, and emphasize the importance of targeting ≥100 ppm ingoing nitrite to achieve a significant impact on growth of *L. monocytogenes* in RTE products. However, results also emphasize the need to elucidate adjunct ingredients to supplement the limited antimicrobial impact of nitrite on this pathogen. Overall, results of this study coincide with literature to conclude that while nitrite does not prevent *L. monocytogenes* growth, it can slow growth, and does so to a degree related to the ingoing nitrite concentration.

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TABLE 3.1. *Populations of* L. monocytogenes *in cooked, cured pork sausage at 7°C.*

Nitrite (ppm)[a]	Ascorbate (ppm)[a]	*L. monocytogenes* CFU/g[b] d 0	d 7	d 14	d 21	d 28	d 40
	0	3.3 AJ	5.4 ABI	8.0 AH	8.3 ABH	8.2 ABH	8.4 AH
0	500	3.4 AJ	6.7 AI	8.4 AH	8.7 AH	8.4 AH	8.2 ABH
	1000	3.5 AJ	6.5 AI	8.5 AH	8.4 ABH	8.4 AH	8.5 AH
	0	3.4 AK	5.0 BCJ	6.3 BI	7.4 BCHI	7.4 ABCHI	7.7 ABCH
100	500	3.3 AJ	4.1 CDEJ	5.9 BI	6.5 CDEFHI	7.1 BCDH	7.3 CDH
	1000	3.3 AJ	4.5 BCDI	6.4 BH	6.7 CDEH	7.1 BCDH	7.3 BCDH
	0	3.3 AJ	4.3 BCDEJ	6.0 BI	6.8 CDEHI	7.0 CDEHI	7.7 ABCH
200	500	3.2 AJ	4.0 CDEJ	5.2 BCDI	6.0 DEFGI	6.9 CDEH	7.2 CDH
	1000	3.3 AJ	3.6 DEJ	5.8 BCI	6.3 DEFGI	6.9 CDEH	7.2 CDH
	0	3.3 AK	3.4 DEK	4.6 CDJE	5.4 GI	6.1 DEFHI	6.8 DH
500	500	3.3 AJ	3.3 DEJ	3.9 EJ	5.5 FGI	5.6 FI	6.7 DH
	1000	3.3 AK	3.2 EK	4.6 DEJ	5.7 EFGI	6.0 EFHI	6.7 DH
SEM[c]		0.1	0.3	0.3	0.2	0.2	0.2

[a]Ingoing sodium nitrite from Prague powder (6.25% sodium nitrite) and purified sodium ascorbate formulated on a meat weight basis.
[b]Means within a column (A-G) or row (H-K) bearing a common letter are not significantly different ($P < 0.05$).
[c]SEM = standard error of the means.

TABLE 3.2. *Residual nitrite concentrations in cooked, cured pork sausage at 7°C.*

Nitrite	Ascorbate	Residual nitrite (ppm)[b]											
(ppm)[a]	(ppm)[a]	d 0		d 7		d 14		d 21		d 28		d 40	
	0	66	FH	56	EI	57	FGHI	50	DEIJ	43	CJ	42	CDJ
100	500	54	FH	47	EHI	50	GH	40	DEIJ	37	CDJ	36	CDJ
	1000	50	FH	44	EH	38	HH	37	EH	18	DI	20	DI
	0	147	DH	129	CI	118	DI	102	CJ	96	BJ	57	CK
200	500	104	EH	80	DI	81	EI	58	DJ	51	CJK	39	CDK
	1000	105	EH	80	DI	71	EFI	57	DJ	50	CJK	41	CDK
	0	303	AH	250	AI	243	AIJ	217	AJ	187	AK	172	AK
500	500	257	BH	236	AH	217	BHI	191	BIJ	171	AJ	156	AJ
	1000	195	CH	180	BHI	165	CI	114	CJ	106	BJ	94	BJ
SEM[c]		6		5		3		4		5		6	

[a]Ingoing sodium nitrite from Prague powder (6.25% sodium nitrite) and sodium ascorbate (purified sodium ascorbate) formulated on a meat weight basis.
[b]Means within a column (A-G) or row (H-K) bearing a common letter are not significantly different ($P < 0.05$).
[c]SEM = standard error of the means.

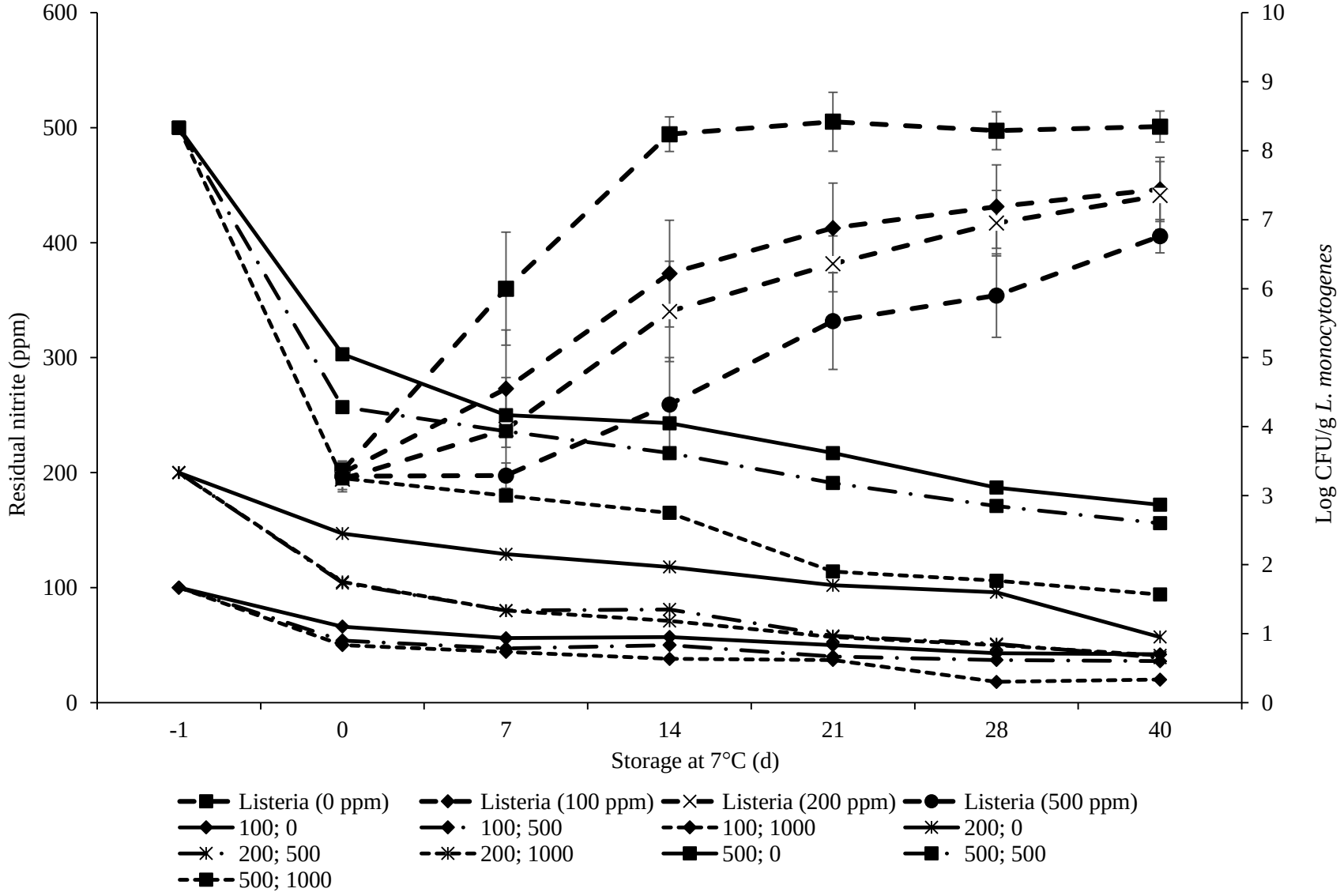
TABLE 3.3. *Growth rate of* L. monocytogenes *(log/d) and residual nitrite depletion rate (ppm/d) in cooked, cured pork sausage at 7°C.*

Nitrite (ppm)[a]	Ascorbate (ppm)[a]	Growth rate (log/d)[b]	Depletion rate (ppm/d)[b]
0	0	0.142 A	ND
	500	0.174 A	ND
	1000	0.167 A	ND
100	0	0.061 B	0.050 A
	500	0.043 B	0.073 A
	1000	0.049 B	0.515 A
200	0	0.047 B	0.040 A
	500	0.035 B	0.098 A
	1000	0.038 B	0.107 A
500	0	0.025 B	0.047 A
	500	0.021 B	0.057 A
	1000	0.025 B	0.601 A
SEM[c]		0.013	0.222

[a]Ingoing sodium nitrite from Prague powder (6.25% sodium nitrite) and sodium ascorbate (purified sodium ascorbate) formulated on a meat weight basis.
[b]Means within a column bearing common letters are not significantly different ($P < 0.05$).
[c]SEM = standard error of the means.

FIGURE 3.1. Residual nitrite concentrations (primary y-axis; treatments defined as "ppm ingoing nitrite; ppm ascorbate") and populations of *L. monocytogenes* (secondary y-axis; treatments defined as "Listeria (ppm ingoing nitrite)") in cooked, cured pork sausage stored at 7°C.

FIGURE 3.1


Residual nitrite (ppm)
Log CFU/g L. monocytogenes
Storage at 7°C (d)
600
500
400
300
200
100
0
10
9
8
7
6
5
4
3
2
1
0
-1
0
7
14
21
28
40
Listeria (0 ppm)
Listeria (100 ppm)
Listeria (200 ppm)
Listeria (500 ppm)
100; 0
100; 500
100; 1000
200; 0
200; 500
200; 1000
500; 0
500; 500
500; 1000

CHAPTER 4: MODELING THE IMPACT OF INGOING SODIUM NITRITE, SODIUM ASCORBATE, AND RESIDUAL NITRITE CONCENTRATIONS ON GROWTH PARAMETERS OF *LISTERIA MONOCYTOGENES* IN COOKED, CURED PORK SAUSAGE

ABSTRACT

Sodium nitrite has been identified as a key antimicrobial ingredient to control pathogens in ready-to-eat meat and poultry products including *Listeria monocytogenes.* This study was designed to more clearly elucidate the relationship between chemical factors (ingoing nitrite, ascorbate, and residual nitrite) and *L. monocytogenes* growth in RTE meats. Treatments of cooked, cured pork sausage (65% moisture, 1.8% salt, pH 6.6) were based on response surface methodology (RSM) with ingoing nitrite and ascorbate concentrations as the two main factors. Concentrations of nitrite and ascorbate, including star points, ranged from 0 to 352 and 0 to 643 ppm, respectively. At one of two time points after manufacturing (d 0 and d 28), half of each treatment was surface inoculated to target 3 log CFU/g of a 5-strain *L. monocytogenes* cocktail, vacuum packaged, and stored at 7°C for up to 4 weeks. Growth of *L. monocytogenes* was measured twice per week, and enumerations were used to estimate lag time and growth rates for each treatment. Residual nitrite concentrations were measured at d 0, 4, 7, 14, 21, and 28, and nitrite depletion rate was estimated using first-order kinetics. The RSM was used to model *L. monocytogenes* lag time and growth rate based on ingoing nitrite, ascorbate, and the residual nitrite remaining at the point of inoculation. Modeling results showed that lag time was impacted by residual nitrite concentration remaining at inoculation as well as the squared term of ingoing nitrite, whereas growth rate was affected by ingoing nitrite concentration but not by the remaining residual nitrite at the point of inoculation. Residual nitrite depletion rate was dependent upon ingoing nitrite concentration and was only slightly affected by ascorbate concentration. This study confirmed that ingoing nitrite concentration influences *L. monocytogenes* growth in RTE products, yet residual nitrite concentration contributes to the antimicrobial impact of nitrite as well.

Listeria monocytogenes is a significant concern to processors of ready-to-eat (RTE) meat and poultry products. As a ubiquitous organism with the capacity to grow at refrigeration temperatures, *L. monocytogenes* can be present in a post-thermal-processing environment, and if product becomes contaminated, subsequent growth can occur in RTE meats during refrigerated storage *(20)*. While listeriosis causes a relatively small proportion of all foodborne illness reported each year, an estimated 1500 cases, 94% of those cases result in hospitalizations and 15% result in death *(32)*. A subset of the population is particularly susceptible to infection with this organism, and one of the most at-risk demographics are pregnant women, in whom listeriosis can result in spontaneous abortion *(31)*. RTE meats which support the growth of *L. monocytogenes* are considered high risk foods for attribution of listeriosis cases *(31, 32)*. Along with environmental and sanitation controls to prevent post-processing contamination, a number of inhibitors, such as organic acids, have been identified for use in formulations and other post-processing intervention technologies, such as high-pressure-processing, have been developed to enhance the safety of these products *(37)*. However, no universal "silver bullet" answer has been discovered for the guaranteed control of *L. monocytogenes* in RTE meats, highlighting the importance of a thorough understanding of the available hurdles to increase overall food safety.

One such formulation-based hurdle is sodium nitrite, an ingredient that has long been used as a preservative and curing agent in formulations which is also responsible for imparting the unique colors and flavors associated with cured meats *(27)*. In addition to significant quality-related contributions, nitrite has also been noted to contribute to the control of pathogens, most notably *Clostridium botulinum (43)*. Due to the severity of *C. botulinum* toxin production and the impact of nitrite on mitigating this risk in RTE products, a significant research effort has existed in this subject area for decades. Therefore, the majority of what is currently understood

about the antimicrobial impact of nitrite relates specifically to *C. botulinum.* Hustad et al. demonstrated the significant effects of ingoing nitrite concentration on botulinal toxin production, and reported that frankfurters formulated with 50 ppm nitrite delayed toxin formation until 56 d of storage at 27°C, as compared to toxin detection in uncured samples at 14 d *(22)*.

The antimicrobial activity of nitrite is dose dependent and is based on the ingoing nitrite concentrations, but other components of cured meat systems, ascorbate and residual nitrite, in particular, have also been suggested to impact pathogen growth. When added to a meat system, nitrite is extremely reactive and can proceed through one of many chemical pathways with the constituents of meat products *(10)*. As such, the concentration of added nitrite that remains as free nitrite decreases drastically upon addition to a meat mixture, and continues to react and deplete throughout processing and storage time *(11, 12)*. Sodium ascorbate, commonly referred to as a cure accelerator, increases the rate of some of these chemical reactions to expedite the development of cured color during processing. As a result of its function to reduce nitrite to nitric oxide, the use of ascorbate also results in a more rapid depletion of residual nitrite during the curing process *(23)*. Although residual nitrite concentrations deplete during processing and storage regardless of the presence of ascorbate, the loss of added nitrite can be more pronounced when ascorbate is included *(6, 28)*.

The bacteriostatic impact of nitrite on *L. monocytogenes* is less pronounced than its inhibitory properties affecting growth and toxin production by *C. botulinum,* which has been reported to occur at relatively low concentrations (50 to 100 ppm) *(21, 22)*. As a factor of this less prominent effect, some studies have concluded that concentrations of nitrite representative of those used in cured meats were insufficient to effectively control *L. monocytogenes* growth

(17, 29, 33). Many studies, however, have evaluated multiple product parameters in combination, including nitrite concentration, salt level, pH, and temperature, and found that nitrite generally enhances bacteriostatic activity in RTE meats *(8, 9, 16, 24)*. Though the relationship between nitrite and *L. monocytogenes* remains ill-defined, nitrite does play a contributing role in *L. monocytogenes* control, particularly in combination with additional hurdles. However, with more detailed understanding of the direct antimicrobial effects of nitrite, its contribution to the safety of RTE meats can be maximized, both alone and in combination with some of the aforementioned hurdles.

The high reactivity of nitrite in a meat system makes it a particularly challenging matrix to study in detail. Beyond the documented effect of ingoing nitrite, ascorbate and residual nitrite have both been suggested to have food safety roles. The antimicrobial impact provided by these two factors, either individually, collectively, or synergistically, are less clearly understood than ingoing nitrite concentration and the related pathogen control response. When used with nitrite, ascorbate has been suggested to enhance the control of both *C. botulinum* and *L. monocytogenes (16, 41, 42)*. Tompkin et al. demonstrated an additional layer of prevention against botulinal toxin formation when ascorbate was added to cured meats and proposed that the effect was likely due to the ability of ascorbate to chelate some essential cation required for recovery from nitrite-induced injury to *C. botulinum (41).* Duffy et al. demonstrated that 420 ppm ascorbate used with up to 315 ppm nitrite significantly improved nitrite's ability to influence *L. monocytogenes* lag time, reporting a difference of 25.4 versus 9.9 d of lag time in cured treatments with and without ascorbate, respectively *(16)*.

Investigations of the antibotulinal impact of residual nitrite were reported by Tompkin et al. and Christiansen et al., concluding that in addition to ingoing nitrite concentration, the level

of residual nitrite at the point of temperature abuse was important for controlling growth and toxin production and determining the safety of cured products *(13, 40, 45)*. Since the time of contamination and critical growth period is uniquely different for *L. monocytogenes* than for *C. botulinum*, the question becomes whether the concentration of remaining residual nitrite at the point of contamination impacts the growth of this organism as well. This study was designed to investigate ingoing and residual nitrite concentrations and to model their impact on growth of *L. monocytogenes* and provide insight to the mechanism by which nitrite contributes to the safety of RTE meats.

MATERIALS AND METHODS

Experimental design. A central composite response surface methodology (RSM) design was used for this two-factor experiment. The 2 factors, sodium nitrite and sodium ascorbate, and the factor levels used in the study are reported in Table 4.1. USDA FSIS regulations limit maximum ingoing sodium nitrite concentrations to 156 ppm for comminuted products and 200 ppm for immersion, massaged, or pumped products, on a meat weight basis, and sodium ascorbate can be used in cured products at up to 547 ppm *(46)*. However, to provide insight into mechanistic effects of nitrite and ascorbate on *L. monocytogenes*, ingoing nitrite concentrations were expanded beyond maximum regulatory limits for this study. Although not a specific research objective for this study, the lower levels of the two factors would be considered representative of ingoing concentrations of the natural sources for both ingredients (natural nitrite from celery powder and natural ascorbate from cherry powder) utilized to produce alternatively cured meats in which would be substituted for synthetic sodium nitrite and sodium ascorbate *(34)*. Since ascorbate accelerates nitrite-related reactions and the depletion of residual

nitrite, the combination of varying levels of ascorbate with nitrite was expected to yield varied residual nitrite concentrations after processing, at the point of inoculation.

The RSM design consisted of 4 factorial treatments, 4 star points, and 2 center points, for a total of 10 treatments (Table 4.2). The radius for star points was calculated as $2^{n/4}$, where *n* is the number of factors in the model, resulting in a radius of 1.414. These treatments and the factor levels were determined using Design of Experiments in JMP (JMP Pro 11, SAS Institute, Cary, NC) and are shown in Table 4.2. The entire design was repeated a second time, resulting in 4 total replications of the center point, and 2 replications of each treatment (combination of ingoing nitrite and ascorbate concentration, coded "a" and "b"). Since residual nitrite depletes over storage time, the initial inoculation point was expected to have greater concentrations of residual nitrite than the delayed inoculation point for each of the formulations. Residual nitrite, measured at each inoculation point (d 0 and d 28), was included as a factor in addition to the two-factor RSM design. Therefore, the model was designed to provide knowledge about the mechanistic relationship between nitrite and *L. monocytogenes,* while remaining within an accurate range of ingredient usage in today's processed meat products.

Sample preparation. Fresh pork Boston butts were obtained from a commercial supplier and stored vacuum packaged at 2.2 to 4°C until use (within 4 d). The Boston butts were deboned and trimmed to approximately 80% lean (20% fat) and then ground through a 19.05 mm plate and again through a 9.53 mm plate using a meat grinder (Model 4732, Hobart Corp., Troy, OH). The ground pork (13.62 kg meat block per treatment) was weighed and transferred to a mixer (Model 100DA, Leland Detroit Manufacturing Company, Detroit, MI) and mixed with 2% salt for 2 min. A brine solution was made by dissolving other non-meat ingredients (0.3% sodium tripolyphosphate, and sodium nitrite (SureCure; 6.25% sodium nitrite, 93.75% sodium chloride)

and sodium ascorbate according to treatment (combination of ingoing nitrite and ascorbate concentrations, defined in Table 4.2) in 5% water (4°C). After salt was incorporated and mixed for 2 min, the brine was added to the mixer and mixed an additional 3 min.

Each treatment was transferred to a rotary-vane vacuum stuffer (VF 608 Plus vacuum filler, Handtmann CNC Technologies Inc., Buffalo Grove, IL) and stuffed into moisture impermeable plastic casings (9.5 cm diameter) to minimize variations in yield and finished product composition. Within 2 h after mixing, treatments were thermally processed using a step-up steam cooking (100% humidity) schedule in a single truck smokehouse (Model 450 MiniSmoker, Alkar Engineering Corp., Lodi, WI) to an internal temperature of 71.1°C. Thermal processing steps included 54.4°C for 30 min, 62.8°C for 30 min, 71.1°C for 30 min, and 79.4°F until product reached an internal temperature of 71.1°C. After the completion of thermal processing, treatments were transferred to a cooler (2°C) and cooled overnight (16-20 h) to an internal temperature <4°C. Thermal processing and cooling temperature data were recorded using a temperature recording logging device (TempTale®4, Sensitech, Beverly, MA) to ensure target heating and cooling parameters were met.

Due to thermal processing space limitations, treatments were randomly assigned to one of two blocks for thermal processing (5 treatments per block). Within each block the treatments were randomized for the order in which they were manufactured. Thermal processing began within two hours of beginning of mixing of the first treatment of each block. The stuffed treatments were not held for an appreciable period of time before thermal processing, as that would negate some of the effect of ascorbate on the speed of nitrite reactions occurring in the treatments, which was a primary factor of exploration in the experimental design. After cooling to an internal temperature of <4°C, approximately half of each treatment was aseptically

removed from the casing, sliced using a commercial table top slicer (Model 919E, Berkel Incorporated, Troy, OH) sanitized with 70% ethanol to yield approximately 17 g per slice, vacuum packaged in bulk, and stored at 3 to 4°C until transportation to the University of Wisconsin-Madison Food Research Institute (Madison, WI) for subsequent inoculation (initial inoculation point=time 0). The time 0 inoculation time point was 2 d post processing. The remainder of each treatment was stored as intact, unsliced chubs in moisture impermeable plastic casings, under the same conditions (7°C) as inoculated time 0 samples. The remaining half of each treatment was sliced after 27 d of storage for inoculation after 28 d of storage at 7°C (delayed inoculation point; 30 d post processing). The use of the delayed inoculation allowed for residual nitrite levels to decline over time as a normal occurrence during shelf life of RTE meats, thus providing a different environment with respect to the residual nitrite concentration for the microorganisms between the initial and delayed inoculation points.

Sample proximate analysis. Triplicate uninoculated samples for each treatment were analyzed for moisture (5 h, 100°C vacuum oven method *(3)*), pH (measured in a slurry prepared by homogenizing 10 g with 90 ml deionized water), NaCl (measured as % Cl^-, $AgNO_3$ potentiometric titration, Mettler DL22 food and beverage analyzer, Columbus, OH) and water activity (a_w, Decagon AquaLab 4TE water activity meter, Pullman, WA) *(3, 38)*.

Objective color analysis. Duplicate uninoculated samples were vacuum-packaged and designated for objective color analysis during storage at 7°C, under the same conditions as inoculated samples. At predetermined time intervals (d 0, 14, 28, 42, and 56 for initial inoculation samples and d 0, 14, 28 for delayed inoculation samples), instrumental color was measured using a chroma meter (Model CR-310, Minolta Camera Co., Ltd., Osaka, Japan; 50 mm aperture, illuminant D_{65}, 0° viewing angle) and a scanning reflectance spectrophotometer

(Model UV-2401 PC, Shimadzu Corp., Kyoto, Japan) with attached multipurpose large sample compartment (Model MPC-2200; Shimadzu Corp., Columbia, MD). The scanning reflectance spectrophotometer was configured for a sampling interval of 1.0 nm, slit width of 5.0 nm, and fast scan speed at a wavelength range of 500 to 700 nm. Both instruments were standardized by placing the same packaging material as used on the samples over a white standardization tile. Values for the standardization tile were CIE L*=97.06, a*=-0.14, b*=1.93. Color was measured using the Commission Internationale de l'Eclairage (CIE) L* (lightness), a* (redness), and b* (yellowness) system *(2)*. At each time point and for both color measurement methods, measurements were taken at two locations on each of the two packaged samples for each treatment, and the samples were placed on the white calibration plate during measurement with the chroma meter. Using the spectrophotometer, cured color pigment was estimated using a ratio of the percentage reflectance at 650 nm divided by the percentage reflectance at 570 nm *(2)*.

Residual nitrite analysis. Residual nitrite was analyzed on duplicate uninoculated meat samples from each treatment at each designated time point (d 0, 4, 7, 11, 14, 21, and 28). A dedicated HPLC instrument was used to quantify residual nitrite according to methods previously reported by Bryan et al. *(7)*. At each predetermined time point, two uninoculated samples per treatment were homogenized using a Waring blender and liquid nitrogen and then stored at -80°C until analysis. On each day of analysis, 5 g of powdered sample was homogenized with 45 ml of pH 7.4 100 µM phosphate buffer, which was then split into 2 slurries and centrifuged at 10,000 x *g*, 4°C for 5 min (Avanti J-E with JA-25.50 rotor, Beckman Coulter, Indianapolis, IN). After centrifugation, 400 µl of supernatant from each slurry and 400 µl of methanol were transferred to a 1.5 ml snap cap centrifuge tube. This mixture was vortexed on high speed for 3 to 5 s and allowed to sit at 4°C for at least 10 min to allow methanol to break

down any remaining protein in the sample. The samples were then centrifuged for 8 min at 13,000 x *g* at 4°C (Eppendorf model 5424 centrifuge, Brinkmann Instruments, Inc., Westburg, NY) and supernatant was transferred to a new 1.5 ml snap cap tube.

This extraction process yielded 4 subsamples per treatment and time point, which were collected using the ENO-20 NOx Analyzer (EiCom USA, San Diego, CA) and Data Processor (EICOM EPC-500) and analyzed in PowerChrom (version 2.3, eDaq Ltd., Denistone East, New South Wales, Australia). Analyzer settings were: reactor pump 0.22 ml/min, carrier pump 0.66 ml/min, and injection 50 µl. NO_x standards were made from $NaNO_2$ powder diluted with MilliQ water into 0, 20, 40, 60, 80, and 100 µM of $NaNO_2$ solution. Standards and samples were analyzed following the same procedure to determine concentration of $NaNO_2$.

Inoculum preparation. *L. monocytogenes* strains LM 101 (hard salami isolate, serotype 4b), LM 108 (hard salami isolate, serotype 1/2b), LM 310 (goat's milk cheese isolate, serotype 4b), FSL-C1-109 (human isolate, serotype 4b), and V7 (raw milk isolate, serotype 1/2a) were grown individually in 10 ml of Trypticase™ soy broth (BBL, BD, Sparks, MD) at 37°C for 18 to 20 h. Cells were harvested by centrifugation (2,500 x *g*, 20 min) and suspended in 4.5 ml 0.1% buffered peptone water (pH 7.2). Equivalent populations of each isolate were combined to provide a five-strain mixture of *L. monocytogenes* to yield a target level of 3 log CFU/g meat (4 log CFU/50 g package). Purity of each strain was verified by streaking on modified Oxford agar (MOX; *Listeria* selective agar base, Difco, BD, Sparks, MD) and Trypticase™ soy agar (TSA, BBL, BD, Sparks, MD), and populations of each strain and the mixtures were verified by plating on MOX.

Inoculation and storage. Each sample consisted of 3 slices, which were surface-inoculated between slices with 250 µl of the *L. monocytogenes* mixture to yield a target level of

3 log CFU/g (equivalent to 3 log CFU/ml rinse material when using 50 ml rinse for enumeration). Each package was vacuum packaged (Multivac AGW, Sepp Haggemuller KG, Wolfertschewenden, Germany) in gas-impermeable pouches (3 mil high barrier pouches; oxygen transmission rate, 50-70 cm^3/m^2, 24 h at 25°C and 60% relative humidity; water transmission rate, 6-7.5 g/m^2, 24 h at 25°C and 90% relative humidity; UltraSource, Kansas City, MO) and stored at 7°C for up to 8 weeks. This study consisted of two inoculation points. As previously stated, approximately half of each treatment was aseptically sliced for inoculation at time 0 (initial inoculation), whereas the remaining half was inoculated after 28 d at 7°C (delayed inoculation).

Microbiological enumeration. At time 0 and predetermined intervals (d 0, 4, 7, 11, 14, 18, 21, 25, and 28) throughout storage, triplicate inoculated samples were assayed for *L. monocytogenes* populations and duplicate uninoculated samples were assayed for lactic acid bacteria and pH. Bacterial populations were enumerated in rinse material after adding 50 ml Butterfields phosphate buffer to each package and massaging externally by hand for approximately 2 minutes to release attached cells. The rinse and appropriate ten-fold serial dilutions were surface plated on MOX (35°C; 48 h). Populations of lactic acid bacteria were enumerated weekly by plating rinse material on All Purpose Tween agar (Difco, BD, Sparks, MD) with 0.004% bromcresol purple (25°C; 48 h). Changes in pH of each uninoculated sample were measured weekly by removing a 10 g representative portion of the sample before rinsing in buffer for microbiological analysis. The 10 g portion was homogenized with 90 ml of deionized water with a lab blender (Stomacher 400, A.J. Seward, London, UK) and the pH of the slurry was measured (Accumet Basic pH meter and Orion 8104 combination electrode, Thermo Fisher Scientific, Waltham, MA).

Data analysis. A general linear model function of JMP was used to evaluate difference ($P < 0.05$) in the physicochemical properties (pH, moisture, salt, and a_w) of samples as well as the objective color measurements. Two microbiological responses were calculated from the collected data: maximum growth rate (μ_{max}; log/d) of *L. monocytogenes* and lag time of *L. monocytogenes.* Growth responses for *L. monocytogenes* were estimated using DMFit, an add-on for use with Excel (version 2007, Microsoft, Seattle, WA), which calculated growth rate (μ_{max}) and lag time for each treatment was determined based on the average log CFU/g from 3 enumerated samples at each sampling point *(4)*. The statistical analysis of the central composite design was carried out using the RSM function in JMP. All main effects (ingoing nitrite, ascorbate, and residual nitrite) were left in the models even at $P > 0.05$, since they were the factors of main interest in the initial design of the experiment. Insignificant ($P > 0.05$) two-way interactions and second-order terms were eliminated, so that the final models reflected the influence of the main factors and included the significant second-order terms.

The residual nitrite depletion rate was calculated using non-linear regression in JMP. The first-order decay kinetics model was fit to residual nitrite concentrations measured at d 0, 4, 7, 11, 14, 21, and 28 for each treatment. There were 4 observations at each time point used to fit the model, with the ingoing concentration set at the initial concentration. Additionally, for more accurate curve-fitting, an arbitrary data point was added to each treatment, 10 ppm residual nitrite at 90 d, to represent the depletion of nitrite that would expectedly occur over time. This value and time point was based on survey data showing that cured products at retail often have ≤ 10 ppm and 90 d is an acceptable, conservative estimate for shelf life of cured products *(11, 26)*. Depletion rate was analyzed using the central composite design and response surface modeling

in JMP, with ingoing nitrite and ascorbate as main effects and $P < 0.05$. Treatments without ingoing nitrite (treatment 5a and 5b) were omitted from depletion rate analysis.

Model performance. To evaluate the strength of the *L. monocytogenes* growth rate and lag time models, bias and accuracy factors developed by Ross were used *(30).* These two values were calculated by comparing the responses predicted by the models with the observed responses. The first of the two, the bias factor ($10^{(\Sigma \log(\text{predicted/observed}/n)}$), indicates whether the points tend to lie above or below the line of equivalence, on average. This factor also takes into account the distance from that line of equivalence. Thus, if the observed values and predicted values were in perfect agreement, the bias factor would be 1. If the bias factor is greater than 1, for example 1.1, then the predictions exceed the observations by 10%. The second evaluation factor, the accuracy factor ($10^{\Sigma|\log(\text{predicted/observed}|/n)}$), indicates the spread of the results about the prediction, and similar to the bias factor, a value of 1 represents a perfect fit of the model with the observations. An accuracy factor of 2, for example, would indicate that the predictions are different from the observations by a factor of 2, either half as large or twice as large *(30)*.

RESULTS AND DISCUSSION

Production of samples. The factors in this study were ingoing concentrations of sodium nitrite and sodium ascorbate, which were added according to treatment requirement and incorporated during product manufacturing in order to achieve target levels of each factor. Chemical analysis revealed similar composition across all formulations, with average 65.0 ±1.8% moisture, 1.84 ±0.06% NaCl, pH 6.59 ±0.05, and a_w 0.979 ±0.004, respectively.

Objective color analysis. Objective color was measured in this study to determine if nitrite and ascorbate had an impact on cured color intensity and color stability over storage time.

For all treatments, analysis of variance showed that color measurements (*L* a* b** and cured color ratio) did not change over time ($P > 0.05$). Furthermore, the only treatment that differed ($P < 0.05$) was the uncured treatment (treatment 5). Therefore, the color data for all treatments containing nitrite (treatments 1-4 and 6-10) were pooled and compared to the uncured treatment (treatment 5) (Table 4.3). At all sampling points, the cured color ratio for the uncured treatment (0 ppm nitrite + 325 ppm ascorbate) was expectedly less than for those treatments containing nitrite, but ratios did not change over time (data not shown) ($P > 0.05$). The mean ratio for all cured samples was 2.43 ±0.20, as compared to 1.58 ±0.13 for the samples without nitrite. Similar trends in color data were observed for CIE *L* a* b** values. *L** (lightness) and *b** (yellowness) values were similar between cured and uncured over time. However, differences were observed in redness (*a**) values between cured samples and uncured samples, with greater values for cured, as expected.

According to the American Meat Science Association Color Measurement Guidelines, cured color ratios of approximately 1.1 to 1.6 indicate no cured color or moderate fade, and values of approximately 2.2 to 2.6 indicate excellent cured color *(2)*. Therefore, these results indicate that not only did all treatments containing nitrite develop cured color, but that cured color was similar across all factor combinations, at a range of ingoing nitrite from 50 to 352 ppm. These results confirm that 50 ppm of nitrite is a sufficient level to generate cured color *(19)*. Results also suggest that under these storage conditions, a minimum of 50 ppm nitrite and 100 ppm ascorbate are sufficient to maintain cured color over 8 weeks storage time. Light and oxygen are known key contributors to cured color fading *(1, 15)*. Therefore, fading of cured color was not expected in this study, since conditions under which this product was stored

(vacuum packaged and in stored in a dark incubator) minimized product exposure to both light and oxygen.

Residual nitrite. Concentrations of residual nitrite for both the initial inoculation point at d 0 and the delayed inoculation point at d 28 are shown in Table 4.2. The concentration of residual nitrite was different based on inoculation point, and was also dependent upon the ingoing nitrite concentration ($P < 0.05$). Analysis of variance indicated that ascorbate did not affect residual nitrite concentration at d 0 (initial inoculation) but was a significant factor for residual nitrite at d 28 (delayed inoculation) ($P < 0.05$). At initial inoculation, an average of 69% of ingoing nitrite was remaining, with a range of 46 to 91%, whereas an average 45% and range of 28 to 64% was recovered at the point of delayed inoculation. Analysis of variance showed that both ingoing nitrite and the squared term of ingoing nitrite were significant for nitrite depletion rate (Table 4.4) ($P < 0.05$).

Previous studies have reported that up to 50% of ingoing nitrite is lost during processing, including mixing, stuffing, and thermal processing *(10)*. Hustad et al. measured residual nitrite concentrations in vacuum packaged wieners made with 50, 100, 150, 200, and 300 ppm ingoing nitrite and recovered < 50% after thermal processing *(22)*. Furthermore, the authors observed that concentrations continued to decline during storage at 7°C to ≤ 12 ppm after 28 d. Depletion rates in this study agree with previous literature which reported that nitrite concentrations deplete rapidly during processing, heating, and early in shelf life, and the rate of depletion slows during extended storage *(5, 18, 25)*. As compared to literature, the increased concentrations recovered in this study could be attributed to a number of factors, including strict control of processing procedures, such as minimizing holding time before thermal processing, and the use of plastics casings that prevented moisture loss during thermal processing. Additionally, samples to be used

for nitrite analysis were held at -80°C, a temperature that prevents further nitrite-related reactions to occur. The use of the dedicated ENO-20 HPLC system provided increased sensitivity and preliminary studies showed that residual nitrite was not lost during the extraction (sample preparation) procedures (data not shown). This also could have contributed to somewhat higher than expected residual nitrite concentrations.

μ_{max} model analysis. Estimated growth rates (μ_{max}) for *L. monocytogenes* are presented in Table 4.2, along with the design factors and levels for each run. Note that all factor combinations resulted in growth of *L. monocytogenes*, so all runs have $\mu_{max} > 0$. The μ_{max} ranged from 0.194 to 0.913 across both initial and delayed inoculation points. Analysis of variance for μ_{max} indicated that of the 3 main factors (ingoing nitrite, ascorbate, and residual nitrite), only ingoing nitrite significantly impacted the μ_{max} of *L. monocytogenes* ($P < 0.05$) (Table 4.5). The overall model, including coefficients for ingoing nitrite, ascorbate, and residual nitrite, resulted in r^2=0.52 and root mean square error (RMSE) = 0.13. Lack of fit was not significant for this model ($P > 0.05$). The final regression equation (Table 4.6) to predict *L. monocytogenes* growth rate in RTE cooked, cured pork sausage, vacuum packaged and stored at 7°C, is as follows:

Predicted *L. monocytogenes* growth rate (μ_{max})

$$= 0.665951 - 0.000866 \cdot [\text{ingoing } NaNO_2] - 0.000124 \cdot [\text{ascorbate}] - 0.000416 \cdot [\text{residual } NaNO_2]$$

It is not unexpected that increasing concentrations of nitrite would impact growth rates through an inverse relationship. A coefficient of 0.001 would mean that growth rate would be affected by 0.1 log/d for every 100 ppm change in ingoing nitrite, so with the coefficient for ingoing nitrite in this model of 0.000866, the effect is slightly less than that. Therefore, the

magnitude of ingoing nitrite's impact on growth rate observed in this study corresponds to previous reports that a relatively small impact on growth is achieved at concentrations allowed for use in RTE meats. Meanwhile, though not significant factors, residual nitrite concentration had approximately half the impact as ingoing nitrite concentration (coefficients of -0.000416 and -0.000866, respectively) and ascorbate had an even more minor impact.

To evaluate the model performance, a scatter plot was created with predicted versus observed growth rates (Figure 4.1). This figure shows not only the scatter of the data about a line of equivalence, which indicates a perfect fit of the model predictions with the observed data, but also can show if the points tend to lie in a "fail-dangerous" or "fail-safe" zone. For a rate model such as this, an observed rate that exceeds that of the prediction is considered "fail-dangerous" as the model underestimates risk, whereas an observed rate that is less than the predicted rate is considered "fail-safe". Performance indices were calculated based on these data and resulted in a bias factor of 1.03 and accuracy factor of 1.25. A bias factor >1 for a rate model indicates it is a fail-safe model, and in this case, the predicted growth rate exceeds the observed rate by 3% on average. The accuracy factor of 1.25 for this model shows that on average, predicted rates are within 25% of the observed rates.

Lag time model analysis. Along with the estimation of growth rates, lag times for each run were also estimated using DMFit and are shown in Table 4.2. DMFit was able to estimate a lag time for each run, which ranged from 2.2 to 11.5 d for all runs and both inoculation points. The analysis of variance for the lag time model indicated that the squared term of ingoing nitrite and residual nitrite were significant factors in determining lag time ($P < 0.05$) (Table 4.7). The model had an r^2=0.54 and RMSE was 1.65. Similar to the growth rate model, lack of fit was not significant ($P > 0.05$).

The final regression equation (Table 4.8) to predict *L. monocytogenes* lag time in RTE cooked, cured pork sausage, vacuum packaged and stored at 7°C, is as follows:

Predicted *L. monocytogenes* lag time

$$=4.847229 - 0.002307 \cdot [\text{ingoing } NaNO_2] + 0.000570 \cdot [\text{ascorbate}] - 0.000053 \cdot [\text{ingoing } NaNO_2^2] + 0.016884 \cdot [\text{residual } NaNO_2]$$

The scatter plot of predicted lag versus observed lag time is shown in Figure 4.2. As evident from this plot, there is more scatter around the line of equivalence for the lag model than the rate model. Since the bias factor for this model was 0.78, less than 1, this index indicates that the model over predicted lag time, and by a magnitude of approximately 22%, thus making it a fail-safe model. The accuracy factor of 1.55 indicates that the predicted values are within 55% of the observed values.

It is important to note, when evaluating this model, that microbiological enumerations were done twice per week (d 0, 4, 7, 11, 14, etc.) throughout storage time, and as compared to weekly or biweekly sampling, this sampling scheme provides more accurate data for calculating lag time. However, the sampling interval should be kept in mind when considering the precision of this calculation and model for lag time.

Analysis of this study showed that, of ingoing nitrite, ascorbate, and residual nitrite factors tested, ingoing nitrite was the prominent factor impacting growth rate of *L. monocytogenes*, whereas the squared term of ingoing nitrite and the residual nitrite concentration were significant for lag time ($P < 0.05$). Residual nitrite depletion rate did not affect *L. monocytogenes* growth rate or lag time ($P > 0.05$). Previous reports have shown somewhat

conflicting results as to the magnitude of impact ingoing nitrite concentration has on *L. monocytogenes.* An early study on the impact of nitrite on *L. monocytogenes* by Shahamat et al. suggested that nitrite was only inhibitory at pH ≤ 5.5 and > 3% salt *(39)*. However, since then, a number of models have reported that ingoing nitrite concentration is a significant factor in *L. monocytogenes* growth characteristics in RTE meat products *(8, 9, 16, 24)*. Nitrite is known to have less effect on *L. monocytogenes* than on *Clostridium botulinum,* and this less definitive effect may account for the variability in results reported, suggesting that perhaps the levels of nitrite used in RTE meats fall within a precarious area in which the impact on *L. monocytogenes* growth is dependent upon a number of other product parameters *(43)*. A number of studies have proven this hypothesis, and have shown that pH, salt, atmosphere, and temperature interact with nitrite, in different ways. to affect *L. monocytogenes* growth in RTE meats *(9, 24)*.

While the enhancing effect of ascorbate in conjunction with nitrite has been shown in the inhibition of spore formers, literature is less conclusive in regard to *L. monocytogenes* control *(14, 41, 42)*. Farber et al. tested 0 and 200 ppm ingoing nitrite, 0 and 550 ppm sodium erythorbate, 1 and 3% salt, and 4 and 10°C storage temperatures in liver pate inoculated with *L. monocytogenes*, and found that of those factors tested, only temperature significantly impacted growth *(17)*. On the other hand, Duffy et al. found that 420 ppm sodium ascorbate significantly increased the inhibitory effects of nitrite against *L. monocytogenes* in vacuum packaged RTE meats (beef, pork, and poultry sausages) *(16)*. The authors defined lag in this study as the time to a 3 log increase, and reported that vacuum-packed cooked meats with 70 to 140 ppm residual nitrite, at the point of inoculation, extended lag time to twice as long as similar products made without nitrite at 5°C, and this effect was potentiated by the addition of ascorbate *(16)*.

The investigation of residual nitrite at the point of inoculation has been investigated for influencing growth of *C. botulinum*, but has not been as thoroughly examined for *L. monocytogenes (40, 45).* The amount of residual nitrite was shown to be inversely related to the botulinal outgrowth and toxin production in perishable caned cured meat *(13, 44).* Therefore, it was reasonable to investigate whether the remaining residual nitrite at the point of inoculation was important for controlling *L. monocytogenes* growth. In a previous study (Chapter 3 of this dissertation), the residual nitrite concentration was a covariate of the ingoing concentration, not isolated as its own factor, whereas the use of ingoing and delayed inoculation points in this study design allowed for each ingoing combination of nitrite and ascorbate to yield two residual nitrite concentrations at the time of inoculation, with other parameters held constant. The growth rate model showed that the concentration of residual nitrite at inoculation did not impact rate of growth, yet the lag model suggested that it could play a role in determining the time until *L. monocytogenes* begins exponential growth. These combined conclusions suggest that nitrite itself may stress *L. monocytogenes* cells, but it is also plausible that other nitrosated species generated by reactions of nitrite with components of a cured meat system, may play a role by directly interfering with *L. monocytogenes* growth or by eliminating required cofactors for growth *(35, 36).* Much of the mechanistic investigation of nitrite as an antimicrobial has been done with its activity relating to clostridia, and results have pointed to nitric oxide, generated by the reduction of nitrite, as a key active molecule that may inhibit iron-sulfur proteins essential to metabolism *(45, 47, 48).*

Regardless, both ingoing nitrite and residual nitrite were shown by this study to contribute to the control of growth of *L. monocytogenes.* Ingoing ascorbate concentration did not directly contribute to *L. monocytogenes* control, though by influencing residual nitrite

concentration, provided an inadvertent impact on microbial growth. Overall, the results of this study suggest that growth rate of *L. monocytogenes* is largely influenced by ingoing concentrations, and therefore, would imply that maximizing ingoing nitrite concentrations yields peak antimicrobial efficacy of this ingredient. Additionally, results indicate that the amount of residual nitrite in the product at the point of inoculation is important to the onset of exponential bacterial growth. While this study provided reinforces ingoing nitrite concentration as a determining factor in controlling *L. monocytogenes* growth, results additionally demonstrate that the reaction between nitrite and the pathogen is complex and can also be affected by residual nitrite concentrations in RTE meats.

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TABLE 4.1. *Factors and levels for central composite design.*

Factor	Low	Middle	High
	-1	0	1
Sodium nitrite (ppm)	50	175	300
Sodium ascorbate (ppm)	100	325	550

TABLE 4.2. *Design matrix of the central composite design,* L. monocytogenes *growth parameters, and residual nitrite in cooked, cured pork sausage at 7°C.*

Trt[a]	Sodium nitrite[b]	Sodium ascorbate[b]	Sodium nitrite (ppm)[c]	Sodium ascorbate (ppm)[c]	Nitrite depletion rate (ppm/d)[d]	*L. monocytogenes* growth parameters at initial inoculation[e]			*L. monocytogenes* growth parameters at delayed inoculation[e]		
						Growth rate (log/d)[f]	Lag time (d)[f]	Residual nitrite (ppm)[g]	Growth rate (log/d)[f]	Lag time (d)[f]	Residual nitrite (ppm)[g]
1a	-1	-1	50	100	0.1113	0.574	2.2	34	0.712	3.4	24
2a	-1	1	50	550	0.3891	0.445	3.7	35	0.565	4.7	14
3a	1	-1	300	100	0.0356	0.308	10.1	267	0.368	8.5	137
4a	1	1	300	550	0.0498	0.265	11.5	273	0.461	7.7	109
5a	-1.414	0	0	325	ND	0.762	5.4	0	0.555	3.4	0
6a	1.414	0	352	325	0.0403	0.229	6.6	318	0.399	7.2	157
7a	0	-1.414	175	0	0.0284	0.317	5.5	120	0.817	8.2	112
8a	0	1.414	175	643	0.0572	0.379	9.3	123	0.438	7.0	66
9a	0	0	175	325	0.0449	0.340	6.3	151	0.389	6.7	78
10a	0	0	175	325	0.0438	0.556	8.6	151	0.475	7.1	78
1b	-1	-1	50	100	0.1811	0.668	5.1	25	0.704	3.8	23
2b	-1	1	50	550	0.1891	0.913	6.7	23	0.575	3.6	16
3b	1	-1	300	100	0.0287	0.310	11.4	197	0.239	4.2	160
4b	1	1	300	550	0.0434	0.212	9.2	181	0.234	7.6	123
5b	-1.414	0	0	325	ND	0.580	4.9	0	0.453	3.3	0
6b	1.414	0	352	325	0.0336	0.311	7.6	195	0.194	5.3	187
7b	0	-1.414	175	0	0.0345	0.304	10.5	115	0.362	7.1	92
8b	0	1.414	175	643	0.0427	0.260	6.3	100	0.296	5.1	79
9b	0	0	175	325	0.0419	0.358	7.3	103	0.277	5.8	84
10b	0	0	175	325	0.041	0.359	ND	125	0.265	5.6	87

[a]Trt = treatment; a = replication 1, b = replication 2.

[b]Coded statistical design coefficients for RMS analysis.

[c]Uncoded factor levels for ingoing sodium nitrite and sodium ascorbate. Ingredients were added as ppm (mg/kg) on a meat weight basis.

[d]Residual nitrite depletion rate calculated by non-linear regression and first-order kinetic model.

[e]Initial inoculation = d 0; delayed inoculation = d 28. ND = no data.

[f]Growth rate (μ_{max}) and lag time were calculated by DMFit.

[g]Residual nitrite measured on d 0 and d 28.

TABLE 4.3. *Objective color (CIE L*a*b*) and cured color ratio values for vacuum packed, sliced, cooked pork sausage stored at 7°C.*

	Objective color[b]			Cured color
	L*	a*	b*	ratio[c]
Cured[a]	58.51 ±1.35	17.68 ±0.50	5.41 ±0.24	2.43 ±0.20
Uncured	59.82 ±1.31	10.33 ±0.62	6.86 ±0.39	1.58 ±0.13

[a]All treatments containing ingoing nitrite had similar (*P > 0.05)* color values. Means and standard deviations for all 18 runs with ingoing nitrite are combined as Cured and 2 runs without ingoing nitrite were combined as Uncured.

[b]Commission International D'Eclairage (CIE) *L*a*b** where *L**=lightness, *a**=redness, and *b**=yellowness on a 0 to 100 scale.

[c]Cured meat color measurement by reflectance ratio of wavelengths 650/570 nm where no cured color=1.1, moderate fade=1.6, less intense but noticeable cured color=1.7 to 2.0, and excellent cured color=2.2 to 2.6.

TABLE 4.4. *Analysis of variance for residual nitrite depletion rate (ppm/d)*[a].

Source	df	Sum of squares	F Ratio	Probability
Ingoing $NaNO_2$	1	0.061720	23.2097	0.0003
Ascorbate	1	0.008371	3.1479	0.0978
Ingoing $NaNO_2{}^{2}$[b]	1	0.035430	13.3236	0.0026
Total error	14	0.037229		

[a]The model was condensed by eliminating all nonsignificant ($P > 0.05$) two-way interactions and second order terms. r^2=0.74; root mean square error =0.05; lack of fit P =0.25.
[b]Ingoing $NaNO_2{}^{2}$ denotes a second order term for ingoing sodium nitrite.

TABLE 4.5. *Analysis of variance for growth rate (μ_{max}; log/d) of* L. monocytogenes[a].

Source	df	Sum of squares	F Ratio	Probability
Ingoing $NaNO_2$	1	0.079606	4.6800	0.0372
Ascorbate	1	0.024592	1.4458	0.2371
Residual $NaNO_2$	1	0.008899	0.5232	0.4742
Total error	36	0.612354		

[a]The model was condensed by eliminating all nonsignificant ($P > 0.05$) two-way interactions and second order terms. r^2=0.52; root mean square error =0.13; lack of fit P =0.50.

TABLE 4.6. *Regression coefficients for predicting the growth (μ_{max}; log/d) of* L. monocytogenes *in cured meat products.*

Factor	Regression coefficient
Constant	0.665951
Ingoing $NaNO_2$	-0.000866
Ascorbate	-0.000124
Residual $NaNO_2$	-0.000416

TABLE 4.7. *Analysis of variance for lag time (d) of* L. monocytogenes[a].

Source	df	Sum of squares	F Ratio	Probability
Ingoing $NaNO_2$	1	0.560408	0.2053	0.6534
Ascorbate	1	0.519305	0.1902	0.6655
Ingoing $NaNO_2{}^{2}$ [b]	1	14.820742	5.4296	0.0259
Residual $NaNO_2$	1	14.525276	5.3213	0.0273
Total error	34	92.80731		

[a]The model was condensed by eliminating all nonsignificant ($P > 0.05$) two-way interactions and second order terms. r^2=0.54 root mean square error =1.65; lack of fit P =0.17.
[b]Ingoing NaNO2[2] denotes a second order term for ingoing sodium nitrite.

TABLE 4.8. *Regression coefficients for predicting the lag time (d) of* L. monocytogenes *in cured meat products.*

Factor	Regression coefficient
Constant	4.847229
Ingoing $NaNO_2$	0.002307
Ascorbate	0.000570
Ingoing $NaNO_2{}^{2\,a}$	-0.000053
Residual $NaNO_2$	0.016884

[a]Ingoing NaNO2[2] denotes a second order term for ingoing sodium nitrite.

FIGURE 4.1. *Simple regression of observed versus predicted* L. monocytogenes *growth rates (μ_{max}; log/d). The bias factor for this model was 1.03 and the accuracy factor was 1.25.*

FIGURE 4.1

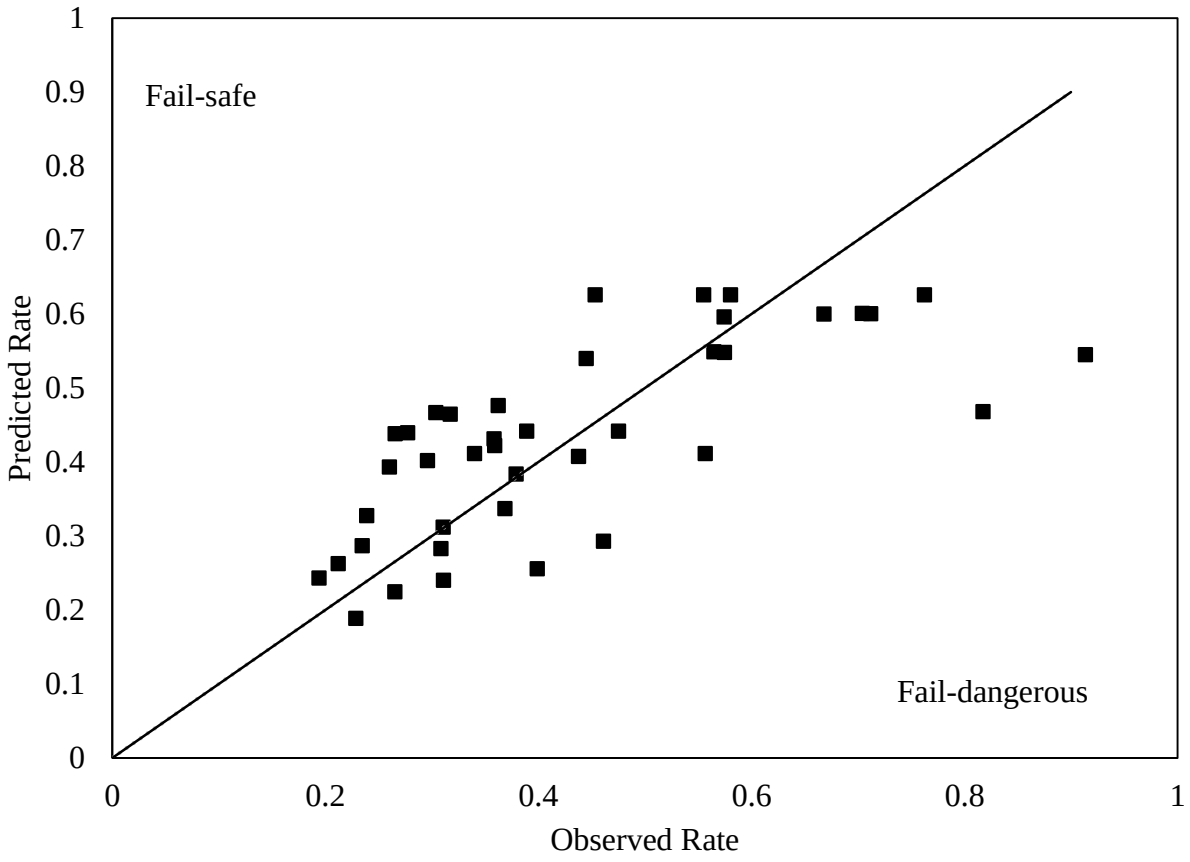
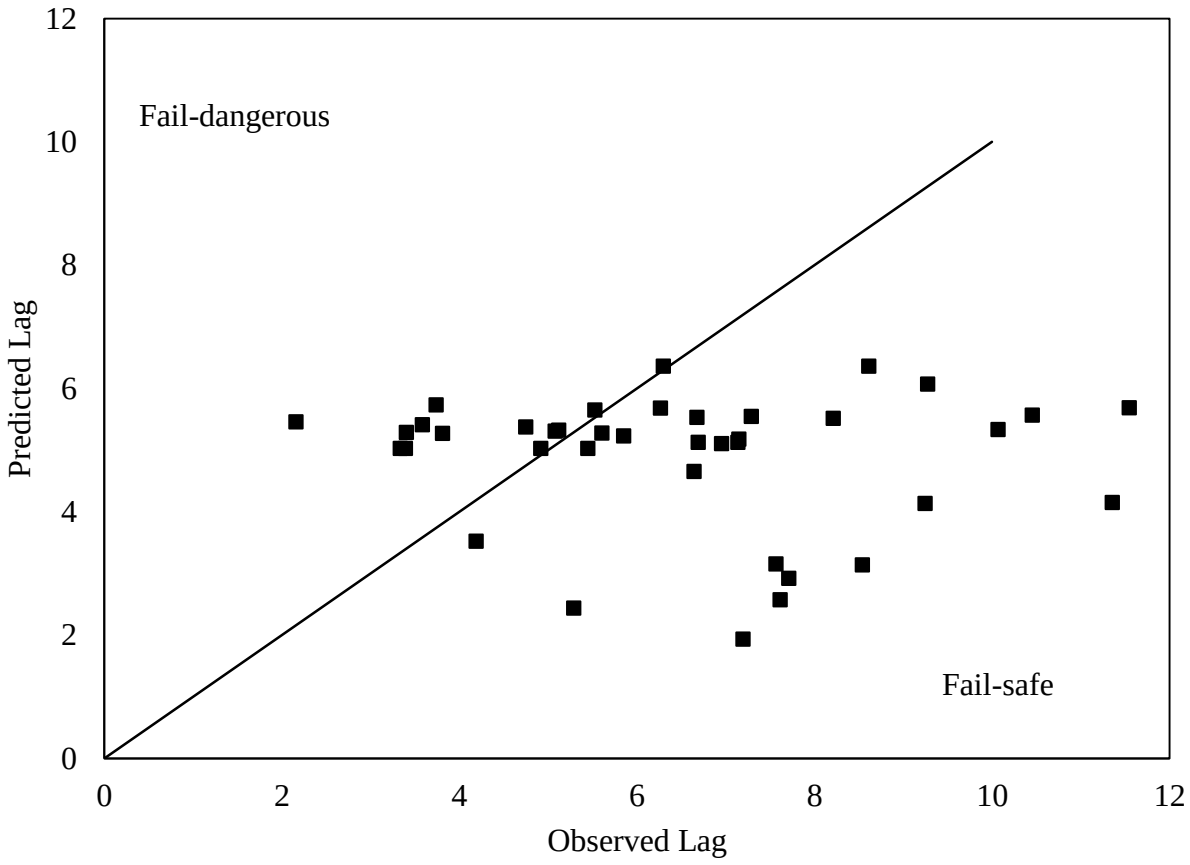

Fail-safe
Fail-dangerous
Predicted Rate
Observed Rate
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
0.2
0.4
0.6
0.8
1

FIGURE 4.2. *Simple regression of observed versus predicted lag time (d) for* L. monocytogenes. *The bias factor for this model was 0.78 and the accuracy factor was 1.55.*

FIGURE 4.2


Fail-dangerous
Fail-safe
Predicted Lag
Observed Lag
0
2
4
6
8
10
12

CHAPTER 5: COMPARISON OF CURING INGREDIENTS DERIVED FROM PURIFIED AND NATURAL SOURCES ON INHIBITION OF *CLOSTRIDIUM PERFRINGENS* OUTGROWTH DURING THE COOLING OF DELI-STYLE TURKEY BREAST

ABSTRACT

The antimicrobial impact of purified and natural sources of both nitrite and ascorbate were evaluated against *Clostridium perfringens* during the post-thermal processing cooling period of deli-style turkey breast. The objective of Phase I was to assess comparable concentrations of nitrite (0 or 100 ppm) and ascorbate (0 or 547 ppm) from both purified and natural sources. Phase II investigated concentrations of nitrite (50, 75, or 100 ppm) from cultured celery juice powder and ascorbate (0, 250, or 500 ppm) from cherry powder to simulate alternative cured formulations. Ground turkey breast (75% moisture, 1.2% salt, pH 6.2) treatments were inoculated with *C. perfringens* spores (three-strain mixture) to yield 2.5 log CFU/g. Individual 50 g portions were vacuum-packaged, cooked to 71.1°C, and chilled from 54.4°C to 26.7°C in 5 hours and from 26.7°C to 7.2°C in 10 additional hours. Triplicate samples were assayed for growth of *C. perfringens* at predetermined intervals by plating on tryptose-sulfite-cycloserine agar; experiments were replicated three times. In Phase I, uncured, purified nitrite, and natural nitrite treatments without ascorbate allowed for 5.3, 4.2, and 4.4 log increases at 15 h, respectively, yet <1 log growth was observed at the end of chilling in treatments containing 100 ppm nitrite and 547 ppm ascorbate from either source. In Phase II, 0, 50, 75, 100 ppm nitrite, and 50 ppm nitrite + 250 ppm ascorbate supported 4.5, 3.9, 3.5, 2.2, and 1.5 log increases, respectively. In contrast, <1 log growth was observed through 15 h in the remaining Phase II treatments supplemented with 50 ppm nitrite and 500 ppm ascorbate or ≥75 ppm nitrite and ≥250 ppm ascorbate. This study confirmed that equivalent concentrations of nitrite, regardless of the source, provide similar inhibition of *C. perfringens* during chilling, and that ascorbate enhances the antimicrobial impact of nitrite against *C. perfringens* at levels commonly used in alternative cured meats.

Clostridium perfringens is a Gram-positive, non-motile, anaerobic bacillus with square ends that forms heat stable spores *(13)*. This organism is widely distributed, as it has been isolated from soil, water, intestines, food, and air. To lead to illness, a person must consume 10^8 to 10^9 vegetative cells; upon exposure to the gastrointestinal environment, cells sporulate and release an enterotoxin, causing symptoms that include abdominal pain, nausea, and diarrhea that generally subside after one to two days *(7, 13)*. While foodborne illness caused by *C. perfringens* results in very few hospitalizations or deaths, it is estimated to cause nearly 1 million cases of illness in the United States each year *(26)*.

As a ubiquitous organism, *C. perfringens* is often a component of the normal intestinal microflora of healthy animals and humans, which can lead to contamination of meat products due to fecal cross contamination during processing. In the United States, 1.6% of raw, whole muscle and 48.7% of raw, ground/emulsified meats were positive for the presence of *C. perfringens*, and 5.3% of the ground/emulsified samples were positive for spores *(36)*. Cooked meat provides a very suitable growth environment, in which *C. perfringens* has been noted for having a remarkably short generation time of less than 10 minutes at temperatures of 43 to 47°C, yet growth has been demonstrated at temperatures ranging from 12 to 50°C *(28)*.

To mitigate the risk of *C. perfringens* germination and outgrowth during cooking and chilling in meat products, the United States Department of Agriculture's Food Safety Inspection Service (USDA, FSIS) has established controlled chilling procedures indicated in 9 CFR 417.5(a)(2) with performance standards published in USDA FSIS Appendix B *(41)*. Appendix B states that maximum internal temperatures of uncured products shall not remain between 54.4°C (130°F) and 26.7°C (80°F) for more than 1.5 hours nor between 26.7°C (80°F) and 4.4°C (40°F) for more than 5 hours. Cured products, defined in Appendix B as those with a minimum 100

ppm ingoing nitrite, are afforded a longer chilling period. According to Appendix B, maximum internal temperatures of these products must not remain between 54.4°C (130°F) and 26.7°C (80°F) for greater than 5 h nor between 26.7°C (80°F) and 7.2°C (45°F) for an additional 10 h. These chilling requirements were designed to limit outgrowth of *C. perfringens* to a maximum 1 log increase during post-thermal processing cooling.

Traditionally, cured meats have been prepared using sodium nitrite, which has been well documented to affect *C. perfringens* outgrowth *(21, 25)*. For example, pork cured with varied concentrations of nitrite, inoculated with 11 spores per gram of *C. perfringens*, held at 1 to 4°C for 2 weeks, 13°C for 2 weeks, and 26°C for 6 weeks, and enumerated after 4, 6, 8 and 10 weeks, showed that increasing concentrations of nitrite decreased the percentage of inoculated spores recovered from the treatments. After 10 weeks, treatments with 0, 50, 100, 150, and 200 ppm sodium nitrite allowed for recovery of 38.0, 12.0, 5.4, 3.6, and 0.9% of inoculated spores, respectively *(25)*. Thus, today's usage rates (maximum allowed) for sodium nitrite in the US, 156 ppm for comminuted products and 200 ppm for immersion cured, massaged, or pumped products regulated by USDA, are well supported and representative of concentrations needed to provide effective control of *C. perfringens* and literature supports the general usage level recommendations near the maximum allowable ingoing concentrations *(40)*.

Due to increased consumer demand for preservative-free, "clean label" processed meat options, alternative cured meats are widely available and are produced without direct addition of purified (a.k.a. synthetic) sodium nitrite, which is considered a preservative. Alternative cured meats can be made with natural sources of nitrite, such as nitrite derived by reducing naturally occurring nitrate in celery powder using specific starter cultures, such as *Staphylococcus carnosus (29, 31)*. However, these processing techniques result in lower concentrations of nitrite

in products than traditional meat curing *(29)*. Because of nitrite's contribution to food safety, these lower concentrations beg the question of whether alternative cured products are equivalent to their traditionally-cured counterparts, from a microbiological safety perspective. A study of commercially prepared alternative cured, natural and organic samples evaluated by Jackson et al. reported decreased *C. perfringens* inhibition in seven of ten frankfurter brands, six of seven ham brands, and four of nine bacon brands relative to traditionally cured controls *(15)*. However, the nitrite concentrations measured at the time of testing were variable (<1 to >65 ppm residual nitrite in 2 brands of commercial frankfurters, for example), which could be explained by variation among manufacturers in the initial amount of formulation nitrite added or the age of the products at the time of testing, since differences in nitrite level at different time points during storage shelf life would be expected as nitrite depletes over time. Similar results have been observed for *Listeria monocytogenes* growth as well as *Clostridium botulinum* growth and toxin production in alternative cured meats compared to traditionally cured controls with purified sodium nitrite *(27, 42)*. In none of these studies was an attempt made to standardize the nitrite levels or to add cure accelerator to the natural versions of the formulations. Currently, products cured with nitrite from natural sources frequently contain less than 100 ppm nitrite and do not qualify for the same extended cooling as meats cured with the direct addition of sodium nitrite *(1)*.

While recent work suggests that nitrite from both purified and natural sources have similar antimicrobial activity against *L. monocytogenes* and *C. perfringens* when used at comparable concentrations, equivalency against *C. perfringens* has not been thoroughly evaluated during extended chilling following the guideline in Appendix B *(9, 10)*. The overall objective of this study was to determine the antimicrobial impact of nitrite from purified and

natural sources for the control of *C. perfringens* outgrowth during a 15 h biphasic chilling curve in deli-style turkey breast. The first phase objective was to determine if purified and natural sources of nitrite and ascorbate, used at equal concentrations, provided similar inhibition of *C. perfringens.* The objective of the second phase was to evaluate the antimicrobial impact of natural nitrite and ascorbate at lower concentrations representative of currently produced alternative cured meats.

MATERIALS AND METHODS

Spore preparation. Three strains of *C. perfringens* (ATCC 13124, 12915, and 12916) were grown individually using a modification of procedures outlined by Kennedy et al. to induce sporulation *(17)*. To enumerate spores, an aliquot of each strain was heat shocked for 20 min at 75°C to kill vegetative cells and appropriate serial dilutions were made in 0.1% peptone and plated on tryptose-sulfate-cycloserine agar (TSC; Oxoid Ltd., Basingstoke, UK) without egg yolk. Once agar was solidified, plates were overlayed with 8 to 10 ml of TSC. Plates were incubated anaerobically for 24 h at 35°C. Spore crops were stored in 0.85% saline at -20°C before preparing spore cocktails for individual experiments. For each meat inoculation, a fresh inoculum was prepared by mixing equivalent levels of the three strains to provide approximately 2.5 log CFU/g of poultry.

Meat preparation and inoculation. Frozen turkey breasts obtained from a commercial supplier were thawed and stored at 2.2 to 4.4°C until use (within 4 d). Turkey was ground through a 4.76 mm plate attached to a grinder (Model 4732, Hobart Corp., Troy, OH). The base formulation for the model turkey breast is shown in Table 5.1. For each treatment, non-meat ingredients were dissolved in distilled water before addition to the ground meat. To ensure

adequate dissolving, the ingredients were added in the following order: sodium tripolyphosphate, salt, modified food starch, nitrite, and ascorbate. The fresh ground turkey and brine were mixed in a mixer (Model AS 200, Hobart Corp.) for 3 min before approximately 800 g of batter was removed to be packaged as uninoculated samples. Uninoculated samples were used for analysis of residual nitrite and proximate composition, as well as temperature monitoring throughout processing. The remaining batter was inoculated with the *C. perfringens* spore mixture to yield approximately 2.5 log CFU/g of poultry.

For each sample, 50 g portions of poultry batter were vacuum sealed in oxygen- and moisture-impermeable bags (3 mil high barrier pouches; oxygen transmission rate, 50-70 cm^3/m^2, 24 h at 25°C and 60% relative humidity; water transmission rate, 6-7.5 g/m^2, 24 h at 25°C and 90% relative humidity; UltraSource, LLC., Kansas City, MO) using a vacuum packaging machine (Multivac AGW, Sepp Haggemuller KG, Wolfertschewenden, Germany). To ensure consistent temperature profiles between samples, the packages were flattened to approximately 3 mm thickness, similar to procedures reported by Kalinowski et al. *(16)*. Samples were held overnight at 4°C before cooking and cooling.

Ingredients and treatment combinations. This study was conducted in two phases. Phases I and II were comprised of 6 and 10 treatments, respectively (Tables 5.2 and 5.3). All usage rates were determined from the manufacturer's specification levels provided for that ingredient and calculated based on a sodium nitrite or sodium ascorbate basis to achieve the target ppm concentration in each treatment. In Phase I, purified ingredients used were sodium nitrite from curing salt (SN; Sure Cure, 6.25% sodium nitrite, 93.75% sodium chloride; Excalibur Seasoning Company, Ltd., Pekin, IL) and sodium ascorbate (SA; Excalibur Seasoning Company). In both phases, natural nitrite was in the form of cultured celery juice powder (PCN;

2.25% sodium nitrite equivalent, Accel™ 2000, Kerry Ingredients and Flavours, Beloit, WI) and natural ascorbate was in the form of cherry powder (CP; 12% ascorbic acid, VegStable™ 515, Florida Food Products, Eustis, FL).

Cooking, cooling and sampling. Before cooking, a thermocouple (digital thermometer and type K probe, Thermo Fisher Scientific, Waltham, MA) was inserted into each of three representative packages through a rubber septum to monitor the internal temperature during cooking and cooling. Temperature data loggers (iButton Temperature Logger DS1922T; Maxim Integrated; San Jose, CA) were placed in the incubator and in four uninoculated packages to continuously record ambient air and meat temperatures during cooking and cooling. Packages were attached to removable incubator racks using small binder clips and immersed in a 75°C water bath until the internal temperature of representative packages reached 71°C, which heat shocked the spores and killed any vegetative cells. The time to target cook temperature (approximately 5 min) was manually recorded. Cooked samples were immediately placed into a programmable air incubator and held at 60°C until all samples were loaded into the incubator (maximum 20 min). This temperature is outside the growth range for the organism and was manually monitored until cooling began. The incubator cooling program was set to cool the product in a biphasic curve that matched the maximum cooling time-temperatures for cured products outlined in USDA FSIS Appendix B (54.4 to 26.6°C in 5 h and 26.6 to 7.2°C in 10 h) (Figure 5.1) *(41)*.

Triplicate samples of each treatment were removed at 0, 2.5, 5, 7.5, 10, 12.5, and 15 h for Phase I and 0, 5, 10, 15 h for Phase II. From each 50 g sample, a representative 25 g portion was removed and diluted with 50 ml Butterfield's phosphate buffer and homogenized for 2 min using a lab blender (Stomacher 400, A.J. Seward, London, UK). Serial dilutions of homogenates were

plated onto TSC plates with an 8 to 10 ml TSC agar overlay and incubated anaerobically at 35°C for 24 h. Each set of experiments was replicated three times using different batches of poultry and spore inocula.

Chemical analysis. Triplicate uninoculated samples for each treatment were analyzed for moisture (5 h, 100°C vacuum oven method), pH (pH measured in a slurry prepared by homogenizing 10 g with 90 ml deionized water), NaCl (measured as % Cl^-, $AgNO_3$ potentiometric titration, Mettler DL22 food and beverage analyzer, Columbus, OH) and water activity (a_w, Decagon AquaLab 4TE water activity meter, Pullman, WA) *(3, 32)*. Residual nitrite was analyzed on duplicate uninoculated meat samples from each treatment, which were frozen at -80°C immediately after cooking. A dedicated HPLC instrument was used to quantify residual nitrite according to modifications of methods previously reported *(5, 19)*. Briefly, samples were powdered in liquid nitrogen and stored at -80°C until analysis. Five g of sample was homogenized with 45 ml of pH 7.4 100 µM phosphate buffer, which was then split into two slurries and centrifuged at 10,000 x *g*, 4°C for 5 min (Avanti J-E with JA-25.50 rotor, Beckman Coulter, Indianapolis, IN). After centrifugation, 400 µl of supernatant from each slurry and 400 µl of methanol were transferred to a 1.5 mL snap cap centrifuge tube. This mixture was vortexed on high speed for 3 to 5 s and allowed to sit at 4°C for at least 10 min to allow the methanol to break down any remaining protein in the sample. The samples were then centrifuged for 8 min at 13,000 x *g* at 4°C (Eppendorf model 5424 centrifuge, Brinkmann Instruments, Inc., Westburg, NY) and the supernatant was transferred to a new 1.5 ml snap cap tube for quantification using the ENO-20 NOx Analyzer (EiCom USA, San Diego, CA). This extraction process yielded 4 subsamples per treatment, which were measured using the ENO-20 NOx Analyzer and Data Processor (EICOM EPC-500) and analyzed in PowerChrom (version 2.3, eDaq Ltd., Denistone

East, New South Wales, Australia). Analyzer settings were: reactor pump 0.22 ml/min, carrier pump 0.66 ml/min, and injection 50 µl. Standards were made from purified $NaNO_2$ powder diluted with MilliQ water into 0, 20, 40, 60, 80, and 100 µM of $NaNO_2$ solution. Standards and samples were analyzed following the same procedure to determine concentration of $NaNO_2$.

Statistical analysis. Three independent replications were performed for each of the two phases. Data were compared with analysis of variance of the Mixed Models procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). The model included fixed main effects of treatment (Phase I, $n = 6$; Phase II, $n = 10$) and replication ($n = 3$). The random effect was the interaction of treatment by replication. All least significant differences were found using the Tukey-Kramer pairwise comparison method with significance levels determined at $P < 0.05$.

RESULTS AND DISCUSSION

Measurements of moisture, salt, pH, and a_w confirmed consistency of formulation and manufacturing among treatments. All treatments in Phase I had an average moisture of 74.2 ±1.8%, 1.31 ±0.14% NaCl, 6.31 ±0.05 pH, and 0.981 ±0.003 a_w. Residual nitrite was analyzed in only the 4 treatments that included nitrite in the formulations; average residual nitrite was 73.1 ±15.7 and 78.6 ±7.0 ppm for 100 ppm ingoing nitrite from purified and natural sources, respectively. When purified or natural sources of ascorbate were added to formulations, the residual nitrite levels were 67.8 ±17.1 and 64.9 ±16.3 ppm, respectively. However, the addition of ascorbate did not significantly reduce ($P > 0.05$) residual nitrite concentrations as compared to treatment without ascorbate.

Proximate analyses for Phase II treatments were similarly consistent, with an average 76.6 ±0.4% moisture, 1.19 ±0.03% NaCl , pH 6.22 ±0.03, and a_w 0.980 ±0.002. Phase II results

showed that residual nitrite concentration was dependent upon ingoing nitrite level, but was not affected by use or level of ascorbate (Table 5.4). Treatments with 50 ppm ingoing nitrite had 37.7 to 38.7 ppm residual nitrite. Treatments with 75 ppm ingoing nitrite had 54.7 to 59.6 ppm, and treatments with 100 ppm ingoing nitrite had 77.2 to 80.5 ppm residual nitrite. The residual nitrite results for both phases indicated that the cure accelerating function of ascorbate did not have an appreciable effect on nitrite depletion during the time allowed in this product and process. In commercial meat products, it is estimated that less than 50% of the originally formulated nitrite is recoverable after thermal processing and that cure accelerators significantly affect residual nitrite levels *(18, 22, 30)*. However, it is possible that the artificially short time these products were exposed to high temperatures for thermal processing (approximately 5 min) limited the extent to which nitrite depletion occurred, and minimized the difference in depletion between treatments with ascorbate and those without, which would be more pronounced in a longer commercial cooking process.

Changes in populations from the initial concentration of *C. perfringens* versus temperature over 15 hours are shown in Figure 5.1 (Phase I). Approximately 2.5 log CFU/g *C. perfringens* were recovered after the initial cooking (heat shock) and before chilling. Recovered populations after heat shocking were not different among treatments ($P > 0.05$), indicating the consistency of the inoculation procedure. As expected, the uncured control provided a very suitable growth environment for this organism, with an average 4.36 ±0.80 log increase observed within the first 5 h when the meat temperature was in the known optimal growth range for *C. perfringens*. The treatment containing purified ascorbate in the absence of nitrite supported similar growth as the control (4.24 ±0.31 log increase; $P > 0.05$), confirming that ascorbate alone does not inhibit *C. perfringens*. Both treatments containing 100 ppm nitrite, from purified or

natural sources, but without a cure accelerator, grew similarly to each other and the uncured control ($P > 0.05$), with only a slightly greater inhibition (<1 log difference) than the control. As noted with the control and ascorbate-only treatments, the majority of growth occurred within the first 7.5 h, with populations remaining relatively unchanged ($P > 0.05$) during 7.5 to 15 h chilling, when temperatures ranged from 20.2 to 7.2°C according to the chilling cycle. In contrast, the treatments containing 100 ppm nitrite plus 547 ppm ascorbate, derived from either purified or natural sources, limited the outgrowth of *C. perfringens* throughout this chilling curve to less than 1 log and final populations were not significantly different from the initial populations ($P > 0.05$). Purified nitrite with ascorbate and natural nitrite plus ascorbate resulted in log decreases of 0.25 ±1.07 and 0.36 ±0.94 log, respectively. These data confirm that when used at equal concentrations, the purified forms and natural forms of nitrite and ascorbate provided equivalent microbial inhibition ($P < 0.05$). Furthermore, the combination of 100 ppm nitrite and 547 ppm ascorbate provides significantly greater inhibition than 100 ppm nitrite alone ($P < 0.05$).

Although no differences were observed in the antimicrobial effect of 100 ppm nitrite and 547 ppm ascorbate derived from natural vs. purified sources, further testing was necessary to validate the safety of products made with lower concentrations which are relevant to current industry practice. While conventionally cured meats commonly contain both a cure accelerator (e.g. ascorbate or erythorbate) and >100 ppm sodium nitrite, alternative cured meats often are made with natural sources of nitrite that result in <100 ppm ingoing sodium nitrite and without a curing accelerator such as ascorbate *(31, 33, 34)*. The hypothesis of the second phase of this study was that natural nitrite and ascorbate used at levels representative of current alternative

curing formulations could inhibit *C. perfringens* outgrowth during a 15 h biphasic chilling curve outlined in Appendix B.

Changes in *C. perfringens* populations over time versus temperature are presented in Figure 5.2 with the 0 value on the y-axis representing the recovered populations after heat shock. Populations of *C. perfringens* after heat shocking were not different among treatments ($P > 0.05$). Results from Phase II confirmed >4 log increase of *C. perfringens* in the uncured control whereas nitrite alone, though concentration dependent, provided limited additional inhibition of *C. perfringens* outgrowth at 50, 75, or 100 ppm (Figure 5.2A). However, nitrite was shown to provide a dose-dependent effect, and as nitrite concentration increased, *C. perfringens* growth decreased; nonetheless, even 100 ppm ingoing nitrite alone was insufficient to limit outgrowth to < 1 log CFU/g. After 15 h chilling, growth in the 50 ppm nitrite treatment was not significantly different from the uncured control (3.90 ±0.33 and 4.46 ±0.34 log increase, respectively; $P > 0.05$). Growth in the 75 ppm nitrite treatment (3.47 ±0.40 log increase) was significantly less than the uncured control ($P < 0.05$) but not different from 50 ppm; whereas growth in the 100 ppm nitrite formulation (2.24 ±0.59 log increase) was significantly less than the control as well as the 50 or 75 ppm nitrite treatments ($P < 0.05$).

Supplementing treatments with 50 ppm nitrite with 250 ppm ascorbate increased the inhibition of *C. perfringens* growth compared to the uncured control ($P < 0.05$; Figure 5.2B). Notably, the only nitrite plus ascorbate treatment to allow for *C. perfringens* growth relative to the initial level was the treatment with 50 ppm nitrite and 250 ppm ascorbate, which supported a 1.46 ±0.92 log increase during the entire chilling period. However, increasing ascorbate levels to 500 ppm in combination with 50 ppm nitrite were effective in limiting growth to <1 log during chilling. The average populations of *C. perfringens* decreased in all of the remaining treatments

containing ≥75 ppm nitrite plus ≥250 ppm ascorbate during chilling ($P < 0.05$). Treatments with 75 ppm nitrite plus 250 or 500 ppm ascorbate decreased 0.65 ±0.66 log and 1.4 ±0.23 log, respectively, whereas 100 ppm nitrite with 250 or 500 ppm ascorbate decreased 1.16 ±0.76 and 1.26 ±0.40 log, respectively.

These results confirmed that equivalent concentrations of nitrite, regardless of the source, provided similar inhibition of *C. perfringens* during chilling and that greater inhibition existed when nitrite was combined with ascorbate. However, this study also suggests that 100 ppm sodium nitrite alone, excluding a cure accelerator such as ascorbate or erythorbate, may be insufficient to prevent outgrowth even when the cooling profile of a high-moisture turkey product meets the extended chilling option of Appendix B for a cured product. The Perfringens Predictor model, part of the Combase online modeling system (http://modelling.combase.cc/ComBase_Predictor.aspx), estimates that an uncured product with pH 6.2, 1.2% salt, and the chilling temperatures similar to those followed in this study would allow for a 3.8, 4.7 and 5 log increase in *C. perfringens* at 5, 7.5 and 15 h, respectively. Under the same product parameters and chilling data, the model predicts that a cured product with 100 ppm ingoing sodium nitrite would support a 2.5, 3.4 and 3.6 log increase by 5, 7.5 and 15 h, respectively. The model accurately predicted the observed growth in this study for a product with a 6.2 pH, 1.2% salt, with or without 100 ppm nitrite but did not take into consideration the synergistic effect by ascorbate during chilling. The uncured treatment varied slightly from Phase I to Phase II, with Phase I allowing for 0.84 log more growth than Phase II, for the uncured product. Similarly, 100 ppm nitrite alone in Phase I treatments permitted 1.94 to 2.14 log greater increases than the 100 ppm nitrite alone in Phase II. Overall, product composition was comparable between the two Phases, though a slightly lower pH in Phase II (6.2 versus 6.3)

could have some limiting effect on total outgrowth. Roberts et al. reported effects of pH on *C. perfringens,* showing that at pH 6.8 and 6.2, growth was observed in 1 week at 15°C in broth, whereas decreasing pH to 5.6 inhibited growth until 4 weeks *(8).* Additionally, though chilling procedures in this study were the same for both phases, heavier loading of the incubator for Phase II decreased the rates at which the incubator could remove heat at high temperatures early in the chilling cycle, so that Phase II samples took approximately 0.5 h longer to reach 50°C, the reported maximum growth temperature for the organism *(13)*. It is plausible that the combination of these two factors are the cause for slightly decreased growth levels observed in Phase II compared to Phase I. Regardless, uncured treatments in both phases showed substantial outgrowth, and 100 ppm nitrite alone treatment was insufficient in either phase to limit outgrowth to <1 log.

It is important to recognize that only products cured with the direct addition of purified sodium nitrite currently qualify for the extended cooling option outlined in Appendix B. Furthermore, traditional curing formulations normally contain near the maximum allowable ingoing concentration of sodium nitrite (156 to 200 ppm) and almost universally include ascorbate or its stereoisomer, erythorbate, as a cure accelerator. Traditionally, cure accelerators are utilized for quality impacts, most notably maximizing the usage of added nitrite, increasing the formation of cured pigment, and maintaining cured color during storage *(20)*. Therefore, products that currently qualify for this extended chilling option (15 h) would not only contain >100 ppm nitrite, but also would likely include ascorbate or erythorbate near the maximum regulatory limit of 547 ppm, and according to this study, can be safely chilled over this curve. Meanwhile, in alternative cured formulations, ingoing nitrite concentrations are commonly less than 100 ppm, and ascorbate, available in a natural form as cherry powder, can often be omitted

from products, and as such, formulations without ascorbate may be considered inherently less safe when cooled over this chilling curve *(29)*.

While the mechanism of nitrite's antimicrobial action is not entirely clear, these results confirm previous findings that increasing the concentration of nitrite provides a greater level of protection against pathogenic growth *(25, 35)*. However, the minimum nitrite concentration to exhibit a consistent antimicrobial impact is not entirely clear. To prevent toxin production by *C. botulinum* in frankfurters, Hustad et al. determined that 50 ppm ingoing nitrite was sufficient *(12)*. Recently, however, Jackson et al. showed *C. perfringens* grew similarly in frankfurters and hams indirectly cured with a natural nitrite source at approximately 50 ppm nitrite and when stored at 20°C for 10 days as compared to an uncured control with no nitrite *(14)*. Our results confirm these findings, but suggest that lower concentrations of nitrite representative of alternative cured meat formulations (e.g. 50 or 75 ppm) are effective if supplemented with sufficient levels of a cure accelerator such as the ascorbate included in this study.

There is no clear agreement about the synergistic effect of ascorbate and nitrite in inhibiting *Clostridium* sp. in meat products. Several studies have reported that ascorbate or erythorbate can enhance the antimicrobial effect of nitrite against clostridia *(2, 38, 39)*. Though a direct comparison between treatments with and without nitrite was not made in those studies, a recent study reported that large, intact hams cured with 200 ppm sodium nitrite and 540 ppm sodium erythorbate, inoculated with *C. perfringens,* did not support growth over a similar chilling curve of that used in this experiment and even inhibited growth over 23 h chilling from 54.4 to 7.2°C *(11)*. Johnston and Loynes reported that the inclusion of reducing agents, ascorbate or cysteine, greatly reduced the amount of nitrite required to inhibit outgrowth of *C. botulinum* in a meat suspension *(6)*. In perishable canned cured meats, ascorbate was reported to

enhance the antimicrobial impact of both 50 ppm and 156 ppm sodium nitrite *(38)*. Researchers hypothesized that this effect was not attributed to the reducing capacity or the antioxidant effects of ascorbate, but instead was due to cell damage by nitric oxide reduced from nitrite, followed by the chelation of a cation essential for recovery by ascorbate *(37)*. Tompkin et al. reported that 200 ppm isoascorbate used with 50 ppm sodium nitrite provided similar inhibition of *C. botulinum* to 156 ppm sodium nitrite alone in perishable canned meat *(39)*. Our results suggest that 50 ppm sodium nitrite with 250 ppm ascorbate may be just below the minimum threshold needed to control *C. perfringens* outgrowth in meat products during a 15 h chilling, but emphasizes the critical role that ascorbate plays in conjunction with nitrite to prevent outgrowth.

Meanwhile, some studies have concluded that ascorbate and erythorbate do not enhance the antimicrobial activity of nitrite at all *(4, 23, 25)*. Bowen et al. *(4)* formulated wieners with 0, 15, 30, 50, 100, and 150 ppm nitrite and 0, 105, and 655 ppm ascorbate. Results showed that at 28°C, inoculated *C. botulinum* began to produce toxin by 7 days with ≤50 ppm nitrite, while ≥100 ppm did not develop toxin within 56 days of testing. Furthermore, the authors saw no enhancement or detriment to the nitrite effect on toxin prevention when ascorbate was included in formulations. Redondo-Solano et al. reported that ham formulated with 547 ppm erythorbate with 50 or 100 ppm nitrite actually supported greater increases of *C. perfringens* than the same levels of nitrite devoid of erythorbate during exponential cooling over 15 h *(23)*. It is likely that this contradictory literature has prevented the general, industry-wide acceptance of cure accelerators as contributing factors to add to a multiple hurdle approach to food safety.

CONCLUSION

First and foremost, the importance of the concentration of nitrite, not the source, impacting the microbiological safety of meat products was made evident in this study. At equal concentrations of nitrite, purified sodium nitrite and natural nitrite from cultured celery juice powder were shown to provide an equivalent antimicrobial impact. Since formulations of alternative cured products often contain lower concentrations of natural nitrite than products conventionally cured by direct addition of purified sodium nitrite, it is critical to evaluate the complete formulation, including curing ingredient concentrations, to ensure utmost product safety. This study also revealed that the inclusion of ascorbate can greatly contribute to product safety in regard to outgrowth of *C. perfringens*. Since ascorbate and erythorbate function in identical ways, it would be appropriate to expect the same results if used interchangeably. Similar to nitrite, both purified and natural sources of ascorbate, when used at equal concentrations, provide the same level of enhancement of the antimicrobial activity of nitrite. The interaction of the two ingredients is evident in this study, and furthermore, the true margin of safety at minimum regulatory limits of 100 ppm ingoing nitrite as the only point of differentiation for chilling requirements is worth attention, since 100 ppm ingoing nitrite did not limit growth to <1 log when used in the absence of ascorbate in this study. Further, this study has shown combined usage of concentrations of nitrite and ascorbate of 50 and 500, or as low as 75 and 250, respectively, are effective in controlling the outgrowth of *C. perfringens* during a 15 h cooling period. While nitrite and ascorbate concentrations are critical in predicting product safety for chilling processed meat products, it is still necessary to consider overall product composition and chilling times and temperatures to ensure safe chilling of cured meats.

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TABLE 5.1. *Base formulation for manufacture of deli-style turkey breast*[a].

Ingredient	%	gram
Turkey breast	100.0	2268
Water/ice (50/50 mix)	20.0	454
Salt (sodium chloride)	1.4	31.8
Modified food starch	2.0	45.4
Sodium tripolyphosphate	0.4	9.1
Total batch		2807.7

[a]Formulated ingredients reported as ingoing percentage based on poultry weight basis.

TABLE 5.2. *Definitions of treatments used to evaluate equal concentrations of purified and natural sources of nitrite and cure accelerator to inhibit outgrowth of* C. perfringens *during a 15-hour cooling period (Phase I).*

	Ingredient[a]	
Treatment[b]	100 ppm Nitrite[cd]	547 ppm Ascorbate[ef]
TRT 1: uncured	NA	NA
TRT 2: SA	NA	0.0547% sodium ascorbate
TRT 3: SN	0.16% purified curing salt	NA
TRT 4: PCN	0.44% cultured celery juice powder	NA
TRT 5: SN+SA	0.16% purified curing salt	0.0547% sodium ascorbate
TRT 6: PCN+CP	0.44% cultured celery juice powder	0.32% cherry powder

[a]ppm added based on concentration provided in ingredient specifications and % of formulation on poultry weight basis. NA, not added.
[b]Treatments: uncured=no nitrite from any source; SN=purified sodium nitrite; PCN=natural preconverted nitrite from cultured celery juice powder; SA=purified sodium ascorbate; CP=natural ascorbate from cherry powder.
[c]Sodium nitrite from curing salt containing 6.25% sodium nitrite.
[d]Natural nitrite from cultured celery juice powder (PCN) containing 2.25% sodium nitrite equivalent.
[e]Sodium ascorbate from pure chemical source.
[f]Natural ascorbate from cherry powder (CP) containing 12% ascorbate.

TABLE 5.3. *Definitions of treatments used to evaluate combinations of natural sources of nitrite and cure accelerator to inhibit outgrowth of* C. perfringens *during a 15-hour cooling period (Phase II).*

	Ingredient[a]			
	ppm		%	
Treatment[b]	Nitrite[c]	Ascorbate[d]	PCN	CP
TRT 1: 0/0	0	0	0	0
TRT 2: 50/0	50	0	0.22	0
TRT 3: 50/250	50	250	0.22	0.21
TRT 4: 50/500	50	500	0.22	0.41
TRT 5: 75/0	75	0	0.34	0
TRT 6: 75/250	75	250	0.34	0.21
TRT 7: 75/500	75	500	0.34	0.41
TRT 8: 100/0	100	0	0.45	0
TRT 9: 100/250	100	250	0.45	0.21
TRT 10: 100/500	100	500	0.45	0.41

[a]ppm added based on concentration provided in ingredient specifications and calculated on poultry weight basis.
[b]Treatments defined by concentrations of natural nitrite and ascorbate in formulation (ppm nitrite/ppm ascorbate).
[c]Naturally generated nitrite from cultured celery juice powder (PCN) containing 2.25% sodium nitrite equivalent.
[d]Natural ascorbate from cherry powder (CP) containing 12% ascorbate.

TABLE 5.4. *Residual nitrite measured in Phase II poultry samples containing varied levels of nitrite from pre-converted celery powder and ascorbate from cherry powder*[a].

Treatment[b]	Residual nitrite	
TRT 1: 0/0	NT	
TRT 2: 50/0	38.7 ±3.2	C
TRT 3: 50/250	37.9 ±3.6	C
TRT 4: 50/500	37.7 ±3.3	C
TRT 5: 75/0	59.6 ±8.9	B
TRT 6: 75/250	59.1 ±6.0	B
TRT 7: 75/500	54.7 ±4.1	B
TRT 8: 100/0	80.5 ±5.0	A
TRT 9: 100/250	78.9 ±6.3	A
TRT 10: 100/500	77.2 ±7.5	A

[a]Means within a column bearing a common letter are not significantly different ($P > 0.05$). NT, not tested.
[b]Treatments defined by concentrations of natural nitrite from pre-converted cultured celery juice powder and natural ascorbate from cherry powder (ppm nitrite/ppm ascorbate).

FIGURE 5.1. Log change (log CFU/g at sampling point minus initial) in counts of *C. perfringens* during 15 h cooling of ground turkey breast formulated with purified nitrite or nitrite from cultured celery juice powder and purified ascorbate or ascorbate from cherry powder (Phase I). Data points represent the mean of three independent replications and error bars represent the standard deviation.

FIGURE 5.1

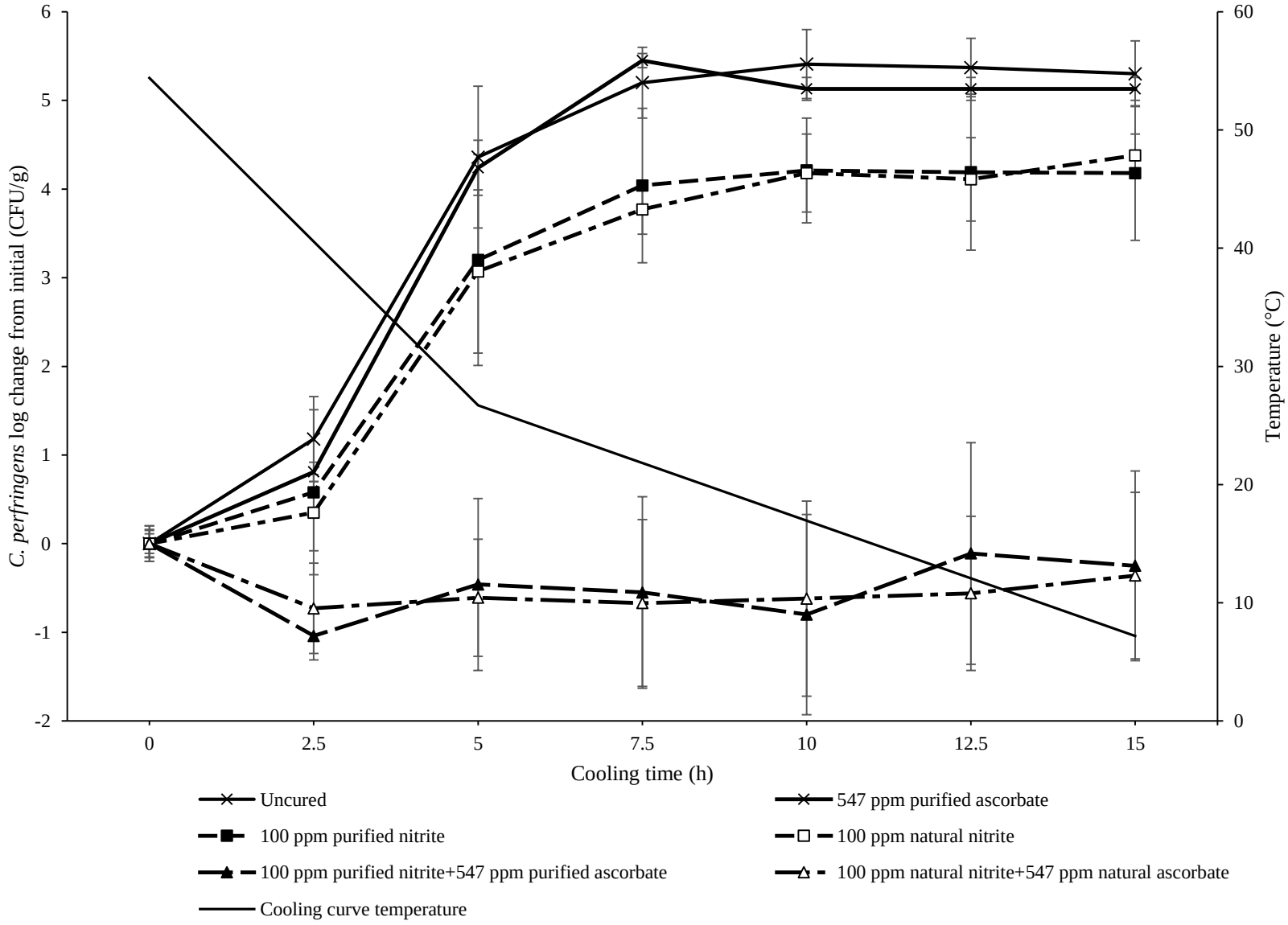
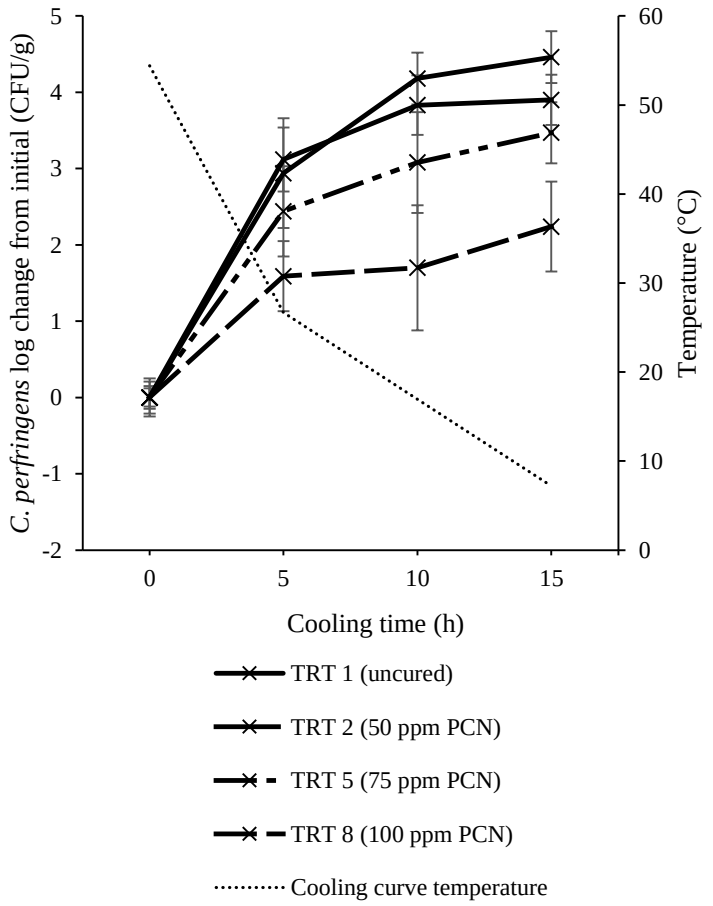

C. perfringens log change from initial (CFU/g)
Temperature (°C)
Cooling time (h)
6
5
4
3
2
1
0
-1
-2
60
50
40
30
20
10
0
0
2.5
5
7.5
10
12.5
15
Uncured
547 ppm purified ascorbate
100 ppm purified nitrite
100 ppm natural nitrite
100 ppm purified nitrite+547 ppm purified ascorbate
100 ppm natural nitrite+547 ppm natural ascorbate
Cooling curve temperature

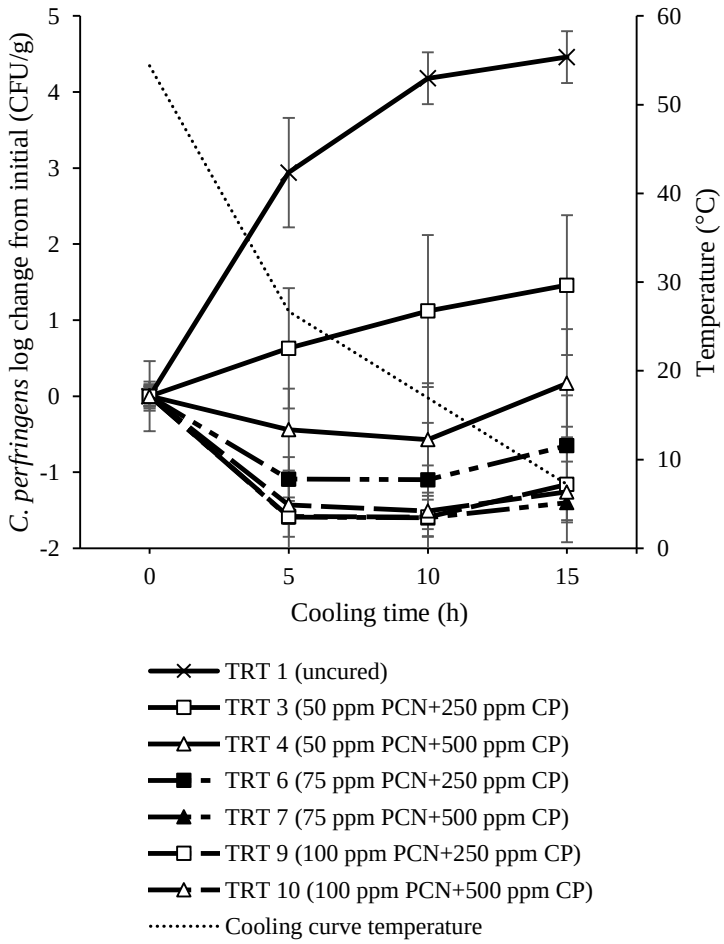
FIGURE 5.2. Log change (log CFU/g at sampling point minus initial) of *C. perfringens* during 15 h cooling of ground turkey breast. A) Treatments with natural nitrite from cultured celery juice powder (PCN). B) Treatments with PCN and natural ascorbate from cherry powder (CP) (Phase II). Data points represent the mean of three independent replications and error bars represent the standard deviation.

FIGURE 5.2

A


C. perfringens log change from initial (CFU/g)
Temperature (°C)
Cooling time (h)
TRT 1 (uncured)
TRT 2 (50 ppm PCN)
TRT 5 (75 ppm PCN)
TRT 8 (100 ppm PCN)
Cooling curve temperature

B


C. perfringens log change from initial (CFU/g)
Temperature (°C)
Cooling time (h)
TRT 1 (uncured)
TRT 3 (50 ppm PCN+250 ppm CP)
TRT 4 (50 ppm PCN+500 ppm CP)
TRT 6 (75 ppm PCN+250 ppm CP)
TRT 7 (75 ppm PCN+500 ppm CP)
TRT 9 (100 ppm PCN+250 ppm CP)
TRT 10 (100 ppm PCN+500 ppm CP)
Cooling curve temperature

CHAPTER 6: IMPACT OF CLEAN LABEL ANTIMICROBIALS AND NITRITE DERIVED FROM NATURAL SOURCES ON OUTGROWTH OF *CLOSTRIDIUM PERFRINGENS* DURING THE COOLING OF DELI-STYLE TURKEY BREAST

ABSTRACT

Organic acids and sodium nitrite have long been shown to provide antimicrobial activity during chilling of cured meat products. However, neither purified organic acids nor sodium nitrite are permitted in products labeled natural and are generally avoided in clean label formulations; efficacy of their replacement is well understood. Natural and clean label antimicrobial alternatives were evaluated in both uncured and in alternative cured (a process that utilizes natural sources of nitrite) deli-style turkey breast to determine inhibition of *Clostridium perfringens* outgrowth during 15 h chilling. Ten treatments of ground turkey breast (76% moisture, 1.2% salt) included a control and 4 antimicrobials (FE-1.0% fruit extracts, DV-0.7% dried vinegar, CSV-1.0% cultured sugar/vinegar, and LV-2.0% lemon/vinegar), each formulated without (uncured) and with nitrite (PCN-50 ppm sodium nitrite from cultured celery juice powder). Treatments were inoculated with *C. perfringens* spores (three-strain mixture) to yield 2.5 log CFU/g. Individual 50 g portions were vacuum-packaged, cooked to 71.1°C and chilled from 54.4°C to 26.7°C in 5 hours and 26.7°C to 7.2°C in 10 additional hours. Triplicate samples were assayed for growth of *C. perfringens* at predetermined intervals by plating on tryptose-sulfite-cycloserine agar. Uncured control and PCN-only treatments allowed for 4.6 and 4.2 log increases at 15 h, respectively, and while all antimicrobial treatments allowed less outgrowth than uncured and PCN, the degree of inhibition varied. 1.0% FE and 1.0% CSV were effective at controlling populations at or below initial levels, whether or not PCN was included. Without PCN, DV and LV allowed for 2.0 and 2.5 log increases, respectively, and approximately 1.5 log increases with PCN. Results suggest using clean label antimicrobials can provide for safe cooling following the study parameters, and greater inhibition of *C. perfringens* may exist when antimicrobials are used with nitrite.

Clostridium perfringens causes nearly 1 million cases of foodborne illness in the United States annually, triggering generally self-limiting gastrointestinal symptoms due to a toxicoinfection resulting from ingestion of a relatively high dose of *C. perfringens* cells (10^8) *(5, 8, 28).* *C. perfringens* is a Gram-positive, non-motile, anaerobic bacillus that forms heat stable spores, and is widely distributed in the environment, found in soil, water, and intestinal tracts of healthy animals and humans *(8).* Therefore, this organism can be a concern in the production of ready-to-eat (RTE) meat products if the spores are present on raw materials and adequate conditions for growth exist during processing. While commercial thermal processing normally destroys vegetative cells, it can also activate spores to germinate. Meat products can provide a very suitable growth environment, in which *C. perfringens* has been noted for having a particularly short generation time of less than 10 minutes at temperatures of 43 to 47°C, although growth has been shown in a wider temperature range from 12 to 50°C *(30).* Therefore, if temperature abuse or insufficient chilling occurs during manufacturing of RTE meat products, there is a risk for significant outgrowth of *C. perfringens.*

Chilling of RTE meat products after thermal processing presents the highest level of risk for growth of *C. perfringens*, as product temperatures may remain in a range permissive of rapid growth for extended periods of time. Therefore, the United States Department of Agriculture's Food Safety Inspection Service (USDA FSIS) has established controlled chilling procedures outlined in 9 CFR 417.5(a)(2) with performance standards published in a guidance document referred to as Appendix B *(38).* To limit outgrowth to <1 log during chilling, this guidance document states that maximum internal temperatures not remain between 54.4°C (130°F) and 26.7°C (80°F) for more than 1.5 hours nor between 26.7°C (80°F) and 4.4°C (40°F) for more than 5 hours in uncured products, those not containing sodium nitrite. Cured products, defined

as products with a minimum 100 ppm ingoing purified sodium nitrite, are afforded a longer chilling period, and must not remain between 54.4°C (130°F) and 26.7°C (80°F) for greater than 5 h nor between 26.7°C (80°F) and 7.2°C (45°F) for an additional 10 h. At present, alternative cured products (those containing natural sources of nitrite) must be chilled using the shorter cooling parameters (6.5 total hours) of USDA FSIS Appendix B; however, longer cooling options, similar to those used for traditionally cured products with 100 ppm purified sodium nitrite, would significantly facilitate post-thermal processing chilling, particularly for large diameter products or in cases of cooling deviations.

Sodium nitrite, used to manufacture cured meats, has long been known to contribute to product quality and safety *(37)*. However, due to consumer interest in reducing dietary consumption of preservatives, of which sodium nitrite is classified, alternative curing methods have been developed to produce products with similar quality characteristics to those of conventionally cured, but without utilizing direct addition of purified sodium nitrite *(31, 32)*. Original techniques for alternative curing involved using a natural source of nitrate, usually celery juice powder, together with a nitrate-reducing bacterial starter culture, such as *Staphylococcus carnosus*, for nitrate-to-nitrite conversion during an incubation period before thermal processing. More recently, these methods have been streamlined and ingredient manufacturers now incubate the nitrate source with a starter culture, supplying meat processers with a standardized "cultured celery juice powder" composed of a high concentration of nitrite. Regardless of the method, natural sources of nitrite are nearly always added at levels that deliver less nitrite in a formulation than conventionally cured products that use a purified (aka. synthetic) form of sodium nitrite. While conventionally cured products are formulated to contain near the maximum allowable limit of 156 or 200 ppm ingoing nitrite, or 120 ppm for bacon, the

ingoing concentration in alternative cured formulations is typically closer to 40 to 60 ppm *(31)*. Lower levels are used due to ingredient cost and product/ingredient functionality limitations since, besides nitrite, cultured celery juice powder and other natural nitrite sources also contain a number of organic compounds that can adversely affect color and flavor when added at high (equivalent to purified nitrite) levels. To avoid detrimental quality impacts, natural nitrite is used at rates that provide sufficient nitrite to impart cured color and flavor, but not so high as to cause off-flavors or colors. However, the decreased nitrite level in these products contributes to concerns regarding the microbiological safety concerns in alternative cured products *(31)*.

The effect nitrite exhibits against *C. perfringens* growth has been well documented and has been shown to be dependent upon nitrite concentration *(23, 27)*. Pork cured with increasing concentrations of nitrite (0, 50, 100, 150, and 200 ppm) allowed for decreasing recovery of inoculated spores (38.0, 12.0, 5.4, 3.6 and 0.9%, respectively) at 10 weeks, after storing at 1 to 4°C for 2 weeks, 13°C for 2 weeks, and 26°C for 6 weeks *(27)*. Further, a study conducted by Jackson et al. reported greater *C. perfringens* growth in commercial alternative cured products than conventionally cured controls *(10)*. For example, during 10 days of storage at 20°C, alternative cured treatments of frankfurters (ingoing nitrite concentration not known) allowed for approximately 4.7 log increase, whereas conventionally cured controls (156 ppm ingoing nitrite) permitted approximately 1.7 log increase *(10)*. Furthermore, a recently completed study in our laboratory investigating *C. perfringens* outgrowth during 15 h chilling revealed that treatments with nitrite concentrations of 50, 75, or 100 ppm from cultured celery juice powder (with no adjunct curing ingredients) provided increased inhibition as nitrite concentration increased, but none were sufficient to restrict outgrowth to <1 log during chilling *(16)*. That study, however, also demonstrated that the combining low levels of nitrite (50 ppm) from cultured celery juice

powder with 500 ppm ascorbate from cherry power, or treatments with ≥75 ppm nitrite from cultured celery juice powder and ≥250 ppm ascorbate from cherry powder were capable of limiting *C. perfringens* outgrowth to <1 log during that same 15 h biphasic chilling *(16)*.

One avenue for enhancing the level of protection provided by nitrite against *C. perfringens* germination and outgrowth is to include antimicrobials in product formulations. Weak organic acids, such as calcium, sodium, and potassium salts of lactate, diacetate and citrate, all generally recognized as safe (GRAS) and approved for use in meat products, have been shown to be effective antimicrobials to control *C. perfringens* in uncured products *(15, 24, 25, 35)*. However, purified organic acids are typically considered preservatives and therefore are not allowed in natural/organic labeled products, drawing further attention to the need for identification and characterization of alternative antimicrobial agents that are acceptable within a natural, organic, or clean label product.

For applications in natural/organic or clean label formulations, a number of naturally-derived compounds with antimicrobial properties can be isolated from plant extracts, essential oils, animal-produced proteins, or bacterial peptides, to name a few *(20)*. At present, many commonly used clean label bacteriostatic antimicrobial ingredients are derived from microbial fermentations, which produce weak organic acids as byproducts, providing replacements for purified organic acids in foods *(26, 29, 34, 36)*. Further, ingredients containing these naturally-derived organic acids, such as lactic, acetic or propionic acid, can be used individually, or in many cases, various combinations are often blended in order to offset potential undesirable organoleptic impacts that could occur with high concentrations of a single ingredient or improve effectiveness of the blend *(17, 36)*. Though commercial availability of these naturally-derived antimicrobials continues to increase, minimal research has been published regarding the use of

currently available natural or clean label antimicrobials against *C. perfringens* outgrowth during chilling of meat products. In uncured turkey and roast beef products, lemon juice and vinegar, which are natural sources of citric and acetic acids, respectively, have been shown to inhibit outgrowth during chilling *(18, 39)*. Jackson et al. investigated the use of natural antimicrobial ingredients in alternative cured frankfurters and ham, but the study was conducted during temperature abuse, 10 days at 20°C, as opposed to during a post-thermal processing chilling period *(9)*. Results showed that frankfurters and ham manufactured with a vinegar/lemon/cherry blend or a cultured sugar/vinegar blend in conjunction with naturally-generated nitrite (<100 ppm ingoing), allowed for greater increases in *C. perfringens* than conventionally cured controls (156 ppm ingoing sodium nitrite). Further, the results were highly variable between the two natural antimicrobial ingredients tested. Overall, the authors concluded that the combinations of antimicrobials and natural sources of nitrite in the study had potential to prevent *C. perfringens* outgrowth, yet the alternative cured treatments were generally not as safe as conventionally cured sodium nitrite treatments *(9)*.

While a number of commercially available natural or "clean-label" antimicrobials are currently utilized in RTE meat formulations as growth inhibitors of *Listeria monocytogenes* during refrigerated storage, it is feasible that the same ingredients could supplement the antimicrobial activity of the lower levels of nitrite in alternative cured products and result in a multiple hurdle approach that provides for safe chilling of these products over a longer time period (15 h) than currently allowed according to Appendix B (6.5 h) *(17, 20, 41, 42)*. Therefore, the objective of this study was to evaluate commercially available clean label antimicrobials as inhibitors of *C. perfringens* during a 15 h biphasic cooling period of deli-style

turkey breast with nitrite concentrations representative of those currently found in alternative cured processed meats.

MATERIALS AND METHODS

Spore preparation. Three strains of *C. perfringens* (ATCC 13124, 12915, and 12916) were grown individually using a modification of procedures as reported by Kennedy et al. to induce sporulation *(11).* To enumerate spores, an aliquot of each strain was heat shocked for 20 min at 75°C to kill vegetative cells and appropriate serial dilutions were made in 0.1% peptone and plated on tryptose-sulfate-cycloserine (TSC; Oxoid Ltd., Basingstoke, UK) agar without egg yolk. Once the agar was solidified, plates were overlayed with 8 to 10 ml of TSC. Plates were incubated anaerobically for 24 h at 35°C. Spore crops were stored in 0.85% saline at -20°C before preparing spore cocktails for individual experiments. For each meat inoculation, a fresh inoculum was prepared by mixing equivalent levels of the three strains to provide approximately 2.5 log CFU/g of meat.

Meat preparation and inoculation. Frozen turkey breasts obtained from a commercial supplier were thawed and stored at 2.2 to 4.4°C until use (within 4 d). Turkey was ground through a 4.76 mm plate attached to a grinder (Model 4732, Hobart Corp., Troy, OH). The base formulation for the model turkey breast is shown in Table 6.1. For each treatment, non-meat ingredients were dissolved in distilled water before addition to the ground meat. To ensure adequate dissolving, the ingredients were added in the following order: sodium tripolyphosphate, sodium chloride, modified food starch, and cultured celery juice powder. The fresh ground turkey and brine were mixed in a mixer (Model AS 200, Hobart Corp.) for 3 min before approximately 800 g of batter was removed to be packaged as uninoculated samples.

Uninoculated samples were used for analysis of residual nitrite and proximate composition, as well as temperature monitoring throughout processing. The remaining batter was inoculated with the *C. perfringens* spore mixture to yield approximately 2.5 log CFU/g of meat.

For each sample, 50 g portions of meat batter were vacuum sealed in oxygen- and moisture-impermeable bags (3 mil high barrier pouches; oxygen transmission rate, 50-70 cm^3/m^2, 24 h at 25°C and 60% relative humidity; water transmission rate, 6-7.5 g/m^2, 24 h at 25°C, and 90% relative humidity; UltraSource, Kansas City, MO) using a vacuum packaging machine (Multivac AGW, Sepp Haggemuller KG, Wolfertschewenden, Germany). To ensure consistent temperature profiles between samples, the packages were flattened to approximately 3 mm thickness, similar to procedures reported by Kalinowski et al. *(14)*. Samples were held overnight (12 to 16 h) at 4°C before cooking and cooling.

Ingredients and treatment combinations. The treatments used in this study are defined in Table 6.2. Antimicrobials were selected to represent a diverse range of antimicrobial ingredients that are commercially available for use in natural or clean label formulations, including plant extracts and naturally-derived organic acids. Usage rates for these antimicrobial ingredients were based on manufacturer recommendations and were calculated on a total batch weight basis. The natural nitrite used was a cultured celery juice powder (PCN; 2.25% sodium nitrite, Accel™ 2000, Kerry Ingredients and Flavours, Beloit, WI). Antimicrobials investigated included a blend of citrus and tropical fruit extracts (FE; 1.0%) and a group of different fermentates designed to contain various types and concentrations of naturally-generated/present organic acids. These included dried vinegar (DV; 0.7%), a blend of cultured sugar and vinegar (CSV; 1.0%), and a blend of lemon juice concentrate and vinegar (LV; 2.0%). To determine if nitrite offered an additive or synergistic effect when used with these antimicrobials, each

antimicrobial was tested alone and in combination with 50 ppm nitrite from cultured celery juice powder, a usage level typically found in alternative cured formulations and shown to provide minimal growth inhibition when used alone *(16).*

Cooking, cooling and sampling. Before cooking, a thermocouple (digital thermometer and type K probe, Thermo Fisher Scientific, Waltham, MA) was inserted into each of three representative packages through a rubber septum to monitor the internal temperature during cooking and cooling. Temperature data loggers (iButton Temperature Logger DS1922T; Maxim Integrated; San Jose, CA) were placed in the incubator and in four uninoculated packages to continuously record ambient air and meat sample temperatures during cooking and cooling. Packages were attached to removable incubator racks using small binder clips and immersed in a 75°C water bath until the internal temperature of representative packages reached 71°C, which heat shocked the spores and killed any vegetative cells. The time to target cook temperature (approximately 5 min) was manually recorded. Cooked samples were immediately placed into a programmable air incubator and held at 60°C until all samples were loaded into the incubator (maximum 20 min). This temperature is outside the growth range for the organism and was manually monitored until cooling began. The program was set to cool the product samples in a biphasic curve that matched the maximum cooling time-temperatures for cured products outlined in USDA FSIS Appendix B (54.4 to 26.6°C in 5 h and 26.6 to 7.2°C in 10 h) *(38).*

Triplicate samples of each treatment were removed for enumerations at 0, 5, 10, and 15 h. From each 50 g sample, a representative 25 g sample was removed and diluted with Butterfield's phosphate buffer and homogenized for 2 min using a lab blender (Stomacher 400, A.J. Seward, London, UK). Serial dilutions of homogenates were plated onto TSC agar with an 8 to 10 ml

TSC agar overlay and were incubated anaerobically at 35°C for 24 h. Each set of experiments was replicated three times using different batches of meat and spore inocula.

Proximate analysis. Triplicate cooked samples for each treatment were analyzed for moisture (5 h, 100°C vacuum oven method), pH (measured in slurry prepared by homogenizing 10 g sample with 90 ml deionized water), NaCl (measured as % Cl^-, $AgNO_3$ potentiometric titration, Mettler DL22 food and beverage analyzer, Columbus, OH), and water activity (a_w, Decagon AquaLab 4TE water activity meter, Pullman, WA) *(2, 33)*. Residual nitrite was analyzed in duplicate meat samples from each treatment, which were immediately frozen and stored at -80°C after cooking and before cooling commenced. A dedicated HPLC instrument was used to quantify residual nitrite according to methods previously reported *(4, 22)*. Samples were powdered in liquid nitrogen and stored at -80°C until analysis. Briefly, 5 g of sample was homogenized with 45 ml of pH 7.4 100 µM phosphate buffer, which was then split into 2 slurries and centrifuged at 10,000 x *g*, 4°C for 5 min (Avanti J-E with JA-25.50 rotor, Beckman Coulter, Indianapolis, IN). After centrifugation, 400 µl of supernatant from each slurry and 400 µl of methanol were transferred to a 1.5 mL snap cap centrifuge tube. This mixture was vortexed on high speed for 3 to 5 s and allowed to sit at 4°C for at least 10 min to allow methanol to break down any remaining protein in the sample. The samples were then centrifuged for 8 min at 13,000 x *g* at 4°C (Eppendorf model 5424 centrifuge, Brinkmann Instruments, Inc., Westburg, NY) and supernatant was transferred to a new 1.5 ml snap top tube for quantification using the ENO-20 NOx Analyzer (EiCom USA, San Diego, CA). This extraction process yielded 4 subsamples per treatment, which were measured using the ENO-20 NOx Analyzer and Data Processor (EICOM EPC-500) and analyzed in PowerChrom (version 2.3, eDaq Ltd., Denistone East, New South Wales, Australia). Analyzer settings were: Reactor pump 0.22 ml/min, carrier

pump 0.66 ml/min, and injection 50 µl. Standards were made from purified $NaNO_2$ powder diluted with MilliQ water into 0, 20, 40, 60, 80, and 100 µM of $NaNO_2$ solution. Standards and samples were analyzed following the same procedure to determine concentration of $NaNO_2$.

Statistical analysis. Three independent replications of this study were completed. Data were compared with analysis of variance using the Mixed Models procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). The model included the fixed main effects of treatment (n = 10) and replication (n = 3). The random effect was the interaction of treatment by replication. All least significant differences were found using the Tukey-Kramer pairwise comparison method with significance levels determined at $P < 0.05$.

RESULTS AND DISCUSSION

Results of proximate analysis are presented in Table 6.3. All treatments had an average 1.18 ±0.04 % NaCl, 75.6 ±0.4 % moisture, and a_w 0.973 ±0.002. Because the pH was not adjusted in formulations during product manufacturing in this study, the antimicrobials used influenced the pH of the treatments ($P < 0.05$). The treatments without antimicrobials, uncured and PCN, had pH values of 6.31 ±0.06 and 6.30 ±0.03, respectively. Most notably, FE and FE+PCN treatments were significantly lower ($P < 0.05$) than the remaining treatments, with pH values of 5.89 ±0.06 and 5.87 ±0.09, respectively. According to specifications provided by the ingredient manufacturer, the FE antimicrobial ingredient used had a pH of 4.0 to 5.0, which was concluded to account for the observed decrease in pH. The remaining treatments formulated with DV, CSV, and LV had average pH values between 6.16 and 6.25, slightly less than the uncured and PCN treatments.

Residual nitrite was measured only in treatments containing nitrite in the formulations, and was significantly lower ($P < 0.05$) in FE+PCN (23.6 ±4.7 ppm) than the remaining nitrite containing treatments, which ranged from 33.6 to 37.2 ppm. This was likely due to the lower pH observed in the FE+PCN treatment, as a decrease in meat system pH has been reported to greatly accelerate nitrite conversion to nitric oxide during product manufacture and thus decrease the amount of residual nitrite *(21)*.

Populations of *C. perfringens* after heat shocking/cooking and after chilling are presented in Figure 6.1. All treatments had recovery of approximately 2.3 log CFU/g after cooking and before chilling began. In the treatments that supported overall growth during 15 h chilling, the majority of that growth occurred within the first 5 h, when temperatures were between 54.4 and 26.7°C (data not shown). For example, when compared to populations after cooking, uncured and PCN treatments increased by 3.50 ±0.41 and 3.18 ±0.71 log at 5 h, and 4.50 ±1.06 and 4.29 ±1.11 log at 10 h, respectively. By the end of 15 h chilling, the uncured and PCN treatments allowed for significant population increases ($P < 0.05$) (4.62 ±0.41 and 4.22 ±0.34 log increases, respectively), demonstrating that both treatments supported significant growth and the low nitrite level (50 ppm) in the PCN treatment provided minimal inhibitory effect ($P > 0.05$) when compared to the uncured treatment. The uncured control was expected to, and did, allow for substantial growth under the conditions evaluated in this study and was included to help elucidate any inhibitory effects displayed by the other treatments. Based on a commonly used online predictive modeling program, the Combase Perfringens Predictor (http://modelling.combase.cc/ComBase_Predictor.aspx), the 4.62 log increase observed over 15 h in the uncured control was in accordance with the model's estimated 4.7 log increase, under the product parameters and temperature profile followed in this study.

While all antimicrobials evaluated in this study provided some degree of inhibition, FE and CSV were found more effective than DV and LV ($P < 0.05$). FE and CSV inhibited growth and maintained populations throughout chilling similar to after heat shocking, therefore meeting performance standards set forth in Appendix B (<1 log growth) ($P > 0.05$). Slight decreases in populations (≤1 log) were observed at the 5 h sampling point for FE and CSV treatments, and populations subsequently remained relatively constant through 15 h (-0.11 ±0.2 and -0.04 ±0.46 log change at 15 h, respectively). On the other hand, though DV and LV treatments allowed for approximately 2 log less growth than the uncured and PCN treatments after 15 h, populations for treatments containing DV and LV were still well outside the <1 log performance standards noted in Appendix B. At 5 h, populations in DV and LV treatments increased by 1.29 ±0.31 and 1.86 ±0.51 log, and at 10 h, increased by 1.60 ±1.23 and 1.95 ±1.13, respectively (data not shown). At the end of 15 h, DV and LV permitted 1.98 ±0.49 and 2.52 ±0.56 log increases, respectively.

In this study, significant differences in *C. perfringens* populations were observed for some, but not all of the antimicrobials tested when comparing the antimicrobial + nitrite against the antimicrobial alone. LV+PCN and FE+PCN treatments displayed greater inhibition when combined with 50 ppm sodium nitrite than when the antimicrobial ingredients were used alone ($P < 0.05$). An approximate 1 log difference was observed between LV+PCN and LV treatments (1.46 ±0.42 and 2.52 ±0.56 log increases, respectively) ($P < 0.05$). While FE alone prevented populations from increasing over 15 h, FE+PCN actually caused a decrease of 1.02 ±0.42 log during chilling ($P < 0.05$). Meanwhile, the other 2 antimicrobials tested did not demonstrate improved effectiveness when used in combination with nitrite, even though populations at the end of chilling tended to be slightly lower compared to the treatments containing nitrite. The DV+PCN treatment was not different than the treatment with DV (1.61 ±0.49 and 1.98 ±0.34 log

increases, respectively) and a similar relationship was shown between the CSV+PCN and CSV treatments (0.30 ±0.51 and 0.04 ±0.99 log decreases, respectively) ($P > 0.05$). These results indicate that the natural and clean label antimicrobials tested provided variable contribution to the inhibition of *C. perfringens* outgrowth during cooling, but they tended to be enhanced by the inclusion of 50 ppm nitrite. Conversely, the antimicrobial impact observed with the relatively low nitrite concentration included in this study can be increased with the addition of an antimicrobial. It is important to note that the treatments evaluated in this study did not contain ascorbate, which likely would have further enhanced the observed inhibition in treatments containing nitrite *(16)*.

Jackson et al. also reported variability between clean label antimicrobial ingredients, based on their inhibitory effects against *C. perfringens* when used in formulations with natural sources of nitrite *(9)*. After inoculated frankfurters and ham were stored for 10 days at 20°C, results showed both the uncured (0 ppm nitrite) treatments and the treatments cured with a natural source of nitrite (equivalent to approximately 50 ppm ingoing) had reached nearly 7 log CFU/g of *C. perfringens*, indicating, as in this study, the relatively low nitrite concentration contributes negligible antimicrobial effect as a sole ingredient inhibitor. Jackson et al. also evaluated two antimicrobials, a lemon/cherry/vinegar blend (1.4% in frankfurters and 0.7% in ham) and a cultured sugar/vinegar blend (unreported usage in frankfurters and 3% in ham), tested in combination with naturally generated nitrite (approximately 50 ppm) and showed varied degrees of inhibition of *C. perfringens* growth. In inoculated frankfurters, for example, 1.4% vinegar/lemon/cherry blend with natural nitrite resulted in 0.49 ±0.24 log *C. perfringens* across sampling days, whereas populations of 1.49 ±0.29 log were observed in frankfurters containing a cultured sugar/vinegar blend with natural nitrite. The authors concluded the alternative cured

formulations, even those including antimicrobials as additional inhibitors, were generally not as effective as the conventionally cured treatments containing maximum levels of nitrite (156 ppm), sodium ascorbate (550 ppm), and 2.5% lactate-diacetate, a purified organic acid blend *(9)*.

The functionality of many natural and clean label antimicrobial alternatives is likely due primarily to the presence of naturally occurring or naturally generated weak organic acids including lactic, acetic, propionic, citric, or butyric acids, to name a few, or to other natural compounds such a flavonoids and phenolic compounds *(36)*. Though the exact composition of the antimicrobials investigated in these experiments was not known, it is likely that DV, or vinegar, is a natural source of acetic acid; CSV (cultured sugar/vinegar blend) contains varying amounts of acetic, lactic, and propionic acids; and LV (lemon/vinegar blend) contains acetic acid along with citric acid from the addition of lemon. According to literature citing the inhibitory effects of similar organic acids and their respective salts, it was reasonable to expect ingredients such as DV, CSV, and LV to impact *C. perfringens* growth *(13, 40)*. Previously, in marinated, ground turkey breast, 1% sodium lactate and sodium diacetate (salts of lactic and acetic acids) controlled *C. perfringens* outgrowth during 15 h cooling, and that control could be extended to 21 h cooling if 1% buffered sodium citrate (salt of citric acid) was combined with the lactate and diacetate blend *(13)*. In a ground turkey product, clean label antimicrobials were assessed, and both buffered vinegar (2.5%) and a blend of buffered vinegar and lemon juice powder (3.5%) inhibited *C. perfringens* outgrowth during 21 h cooling *(39)*. The inhibition provided by natural and clean label antimicrobials in this study and in previous work supports the potential for natural sources of organic acids, fermentates, and other similar functioning compounds as efficacious bacteriostatic antimicrobial agents in RTE meats.

Meanwhile, unlike the aforementioned organic acids generated from microbial fermentation, an ingredient such as FE (fruit extracts) used in this study is slightly ambiguous in its composition, making it more challenging to hypothesize its active ingredients. Citrus fruits like lemon, lime, and grapefruit have been shown to have antimicrobial activity, partially attributed to their high citric acid content, and some combination of these fruits is reasonably likely to be found in such an ingredient *(7)*. The activity of citric acid, as previously been mentioned in this discussion, functions similarly to other organic acids and has been shown to inhibit *C. perfringens* during chilling *(13, 39)*. For example, Juneja et al. reported that 200 ppm of a grapefruit extract added to sous-vide chicken products inhibited growth of *C. perfringens* during storage at 19 and 25°C *(12)*. Another fruit, pomegranate, was also identified as having antimicrobial activity against Gram-positive organisms such as *L. monocytogenes* and *Staphylococcus aureus*, both *in vitro* as well as in foods, and could also be present as part of the FE ingredient *(1)*. While difficult to ascertain with certainty, it is rational to assume that this FE ingredient is comprised of a unique blend of multiple fruit extracts that contribute primarily citric acid as an active antimicrobial agent.

Along with its clear inhibition of *C. perfringens* outgrowth, FE was also observed to decrease the pH of the treatments (Table 6.3) in which it was used, which could have had considerable impact on the *C. perfringens* population changes observed, solely from a pH/antimicrobial effect. Gibson et al. reported a small pH effect on *C. perfringens* growth in broth, as results showed that visible growth (turbidity) was observed in 4 weeks at pH 5.6, as compared to 1 week at pH 6.2 and 6.8 *(6)*. Furthermore, assuming part of the bioactivity of FE was due to citric acid or other weak organic acids, the activity could be pH dependent. Organic acids exist in both undissociated and dissociated forms, based on the pK_a of the acid and the pH

of the meat system. At lower pH, the undissociated form is more prevalent and is primarily responsible for the antimicrobial effect. As the undissociated molecule crosses the cell membrane into a bacterial cell and is exposed to the higher intracellular pH, it dissociates, releasing charged anions and protons that disrupt cell metabolism *(3)*.

Since the antimicrobial activity of organic acids is dependent upon pH, a follow up experiment was designed to explore the extent to which the antimicrobial effect we observed was due to the pH decrease caused by the addition of the FE ingredient. For this experiment, the pH of the FE treatment was adjusted to match that of an uncured control (no antimicrobial included) and similar testing procedures were followed as previously outlined, except samples were enumerated at 0, 5, and 10 h following the same 15 h cooling curve. In previous studies, little population change was observed between 10 and 15 h, and the objective of this study was to elucidate differences between the treatments, not to validate the 15 h cooling curve *(16)*. The treatments, analyzed pH values, and changes in *C. perfringens* populations during chilling are presented in Table 6.4. During treatment development, pH adjustments were made by measuring the unadjusted brine pH for the Uncured high pH (brine pH 8.2) and FE low pH (brine pH 4.7) treatments, and adding appropriate amounts of 5N hydrochloric acid or 5N sodium hydroxide to attain target pHs in brines used for the FE high pH and Uncured low pH treatments. Though the pH was the same (pH 4.7) for Uncured low pH and FE low pH treatment brines, final cooked pH for these treatments was different (6.2 and 6.0, respectively), suggesting that the FE ingredient provided greater buffering capacity than expected which prevented the desired meat system pH change after addition. Results (Table 4) showed that the 0.2 pH unit difference between the Uncured high pH and Uncured low pH treatment accounted for <1 log difference in overall outgrowth of *C. perfringens*, which was not significant ($P > 0.05$). At equal pH (6.4), FE

provided greater inhibition of growth than the Uncured control (1.56 ±0.57 and 3.82 ±0.15 log change, respectively) ($P < 0.05$). Furthermore, with a lower pH (approximately 0.4 pH units), the FE low pH treatment populations were approximately 1 log less than those observed in the FE high pH treatment. Overall, the results of this follow up experiment suggest that within the pH range investigated (pH 6.0 to 6.4), the lower pH observed in the previous experiment would not be expected to account for the inhibition of *C. perfringens* that was previously reported. While decreased pH appeared to contribute to the efficacy of the FE ingredient as an antimicrobial, this likely wasn't the explanation for antimicrobial activity originally observed, since FE also provided inhibition at increased pH. High ascorbic acid contents have been reported in some tropical fruits (130 mg/100 g in guava and 100 mg/100 g in papaya) and the chelating capacity of fruits such as star fruit and papaya were also reported *(19)*. If these fruits make up part of the FE blend, they could have provided similar antimicrobial activity as previously demonstrated when nitrite and ascorbate were used in combination *(16)*.

CONCLUSION

While the antimicrobials tested in this study were variable in their effectiveness at controlling outgrowth of *C. perfringens* during 15 h chilling, it is important to note that all antimicrobials, with or without the use of nitrite, did limit population increases as compared to the Uncured formulation devoid of both nitrite and antimicrobials. FE and CSV ingredients effectively controlled *C. perfringens* outgrowth at 1.0% usage rates, when used with or without 50 ppm sodium nitrite from cultured celery juice powder. In addition, results of this study validate that ≥50 ppm nitrite + 1.0% FE or CSV provided sufficient inhibition of *C. perfringens* to allow for safe chilling of deli-style turkey breast over 15 h, following the critical parameters

outlined in USDA FSIS Appendix B. Supplementing purified or natural nitrite with ascorbate, a cure accelerator from purified and natural (cherry powder) sources, has been demonstrated to be a viable option to inhibit growth of *C. perfringens* during extended chilling *(16)*. Along with ascorbate, the results of this study provide additional options for meat processors to incorporate adjunct ingredients and safely chill alternative cured products. The combined use of multiple layers of inhibition, also referred to as the multiple hurdle approach, is a common strategy to control biological hazards within a food or meat system. This study reinforces that, while a "magic bullet" has yet to be identified to prevent pathogen presence or growth in processed meat and poultry products, nitrite and a variety of antimicrobial ingredients can be utilized alone or in tandem to enhance food safety.

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TABLE 6.1. *Base formulation for manufacture of deli-style turkey breast*[a].

Ingredient	%	gram
Turkey breast	100.0	2268
Water/ice (50/50 mix)	20.0	454
Salt (sodium chloride)	1.4	31.8
Modified food starch	2.0	45.4
Sodium tripolyphosphate	0.4	9.1
Total batch		2807.7

[a]Formulated ingredients reported as ingoing percentage on a poultry weight basis.

TABLE 6.2. *Treatments used to evaluate combinations of clean label antimicrobials and naturally generated nitrite from cultured celery juice powder to inhibit outgrowth of* C. perfringens *during extended cooling.*

Treatment (TRT)	Description[abc]
TRT 1 (Uncured)	Uncured, no nitrite added
TRT 2 (PCN)	50 ppm natural nitrite
TRT 3 (FE)	1.0% fruit extracts
TRT 4 (FE+PCN)	1.0% fruit extracts + 50 ppm natural nitrite
TRT 5 (DV)	0.7% dried vinegar
TRT 6 (DV+PCN)	0.7% dried vinegar + 50 ppm natural nitrite
TRT 7 (CSV)	1.0% cultured sugar/vinegar blend
TRT 8 (CSV+PCN)	1.0% cultured sugar/vinegar blend + 50 ppm natural nitrite
TRT 9 (LV)	2.0% lemon/vinegar blend
TRT 10 (LV+PCN)	2.0% lemon/vinegar blend + 50 ppm natural nitrite

[a]ppm sodium nitrite added based on concentration provided in supplier ingredient specifications and calculated on poultry weight basis.
[b]PCN = natural nitrite from cultured celery juice powder standardized to 2.25% sodium nitrite equivalent.
[c]Antimicrobials formulated on total batch weight (w/w) basis.

TABLE 6.3. *pH and residual nitrite measured in deli-style turkey breast treatments containing clean label antimicrobials and/or nitrite from cultured celery juice powder (PCN)*[a].

	Parameter[b]				
Treatment[c]	pH			Residual nitrite	
TRT 1 (Uncured)	6.31 ±0.06	A		NT[d]	
TRT 2 (PCN)	6.30 ±0.03	A		33.6 ±2.9	A
TRT 3 (FE)	5.89 ±0.06	D		NT	
TRT 4 (FE+PCN)	5.87 ±0.09	D		23.6 ±4.7	B
TRT 5 (DV)	6.21 ±0.04	BC		NT	
TRT 6 (DV+PCN)	6.19 ±0.04	BC		35.7 ±6.8	A
TRT 7 (CSV)	6.25 ±0.08	AB		NT	
TRT 8 (CSV+PCN)	6.25 ±0.04	AB		37.2 ±4.9	A
TRT 9 (LV)	6.17 ±0.06	C		NT	
TRT 10 (LV+PCN)	6.16 ±0.05	C		36.4 ±2.4	A

[a]Means within a column bearing a common letter are not significantly different ($P > 0.05$). NT, not tested.
[b]The percent moisture, water activity, and percent NaCl were not different across treatments ($P > 0.05$). Moisture = 75.6 ±0.4%; water activity = 0.973 ±0.002; NaCl = 1.18 ±0.04%.
[c]Treatments defined by presence of 50 ppm nitrite from cultured celery juice powder (PCN) on a poultry weight basis and antimicrobial on a total batch weight (w/w) basis: FE = 1.0% fruit extracts; DV = 0.7% dried vinegar; CSV = 1.0% cultured sugar/vinegar blend; LV = 2.0% lemon/vinegar blend.
[d]NT = not tested.

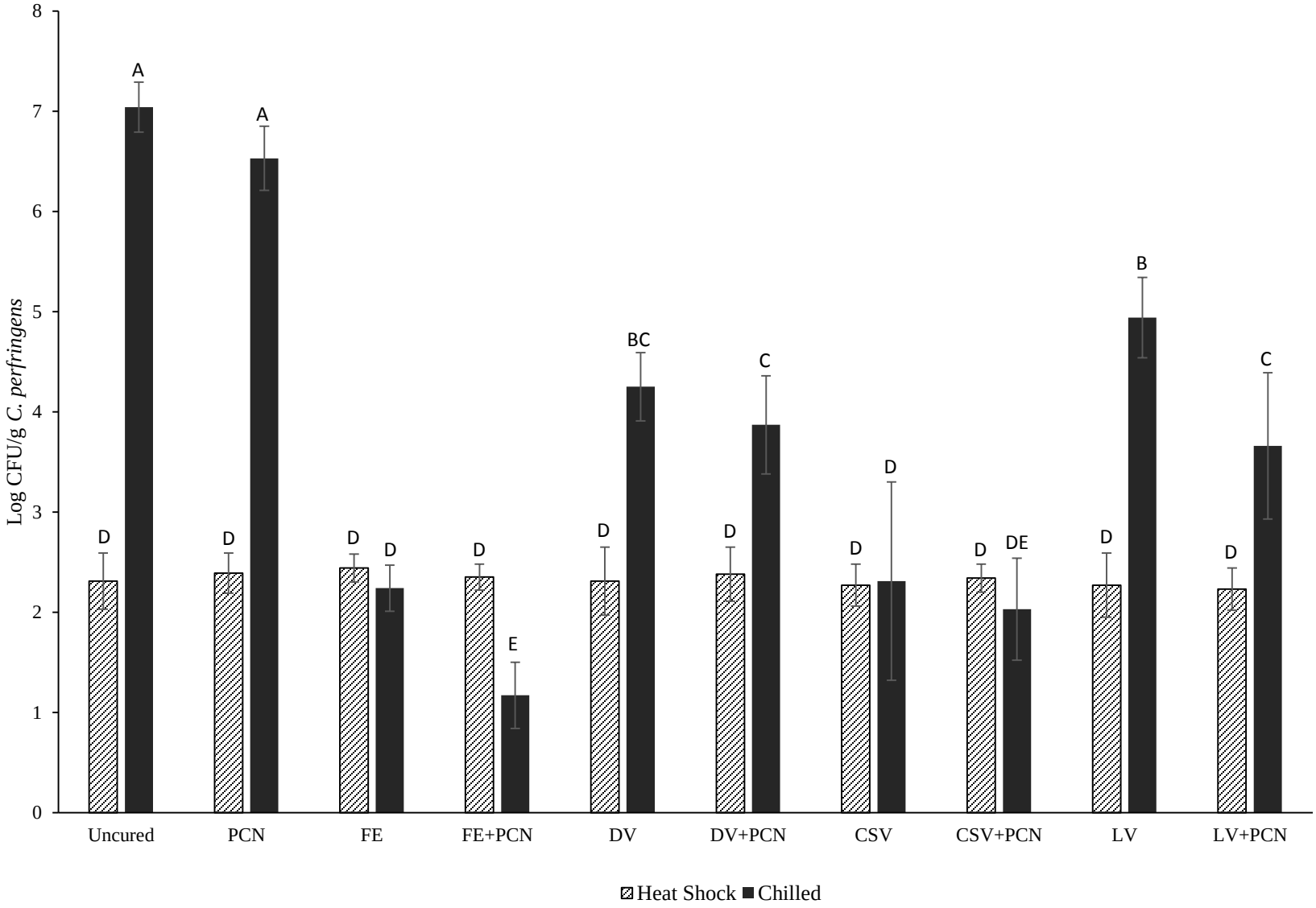
TABLE 6.4. *Treatments, analyzed pH, and changes in C. perfringens populations during chilling of pH adjusted, uncured and FE (1.0% fruit extracts) treatments of deli-style turkey breast.*

		Log change of *C. perfringens*[c]			
Treatment	pH[a]	5 h		10 h	
Uncured high pH	6.39 ±0.01	3.17 ±0.54	A	3.82 ±0.15	A
Uncured low pH	6.20 ±0.01	2.66 ±0.33	AB	3.06 ±0.15	A
FE high pH	6.37 ±0.01	1.69 ±0.38	BC	1.56 ±0.57	B
FE low pH	6.02 ±0.00	0.72 ±0.21	C	0.47 ±0.16	C

[a]pH measured in cooked samples.
[b]Log change = population of *C. perfringens* measured at 5 and 10 h chilling minus the initial population after cooking (0 time). Values are means of triplicate samples with standard deviations.
[c]Means bearing common letters are not different ($P < 0.05$).

FIGURE 6.1. Means of *C. perfringens* populations (log CFU/g) in deli-style turkey breast containing clean label antimicrobials (1.0% FE – fruit extracts, 0.7% DV – dried vinegar, 1.0% CSV – cultured sugar/vinegar blend, 2.0% LV – lemon/vinegar blend) and 50 ppm nitrite from cultured celery juice powder (PCN) after heat shock and after 15 h biphasic cooling (54.4 to 26.7°C in 5 h and 26.7 to 7.2°C in 10 h). Bars represent the mean of three independent replications and lines represent the standard deviation. Different letters on the bars represent statistical differences ($P < 0.05$).

FIGURE 6.1


Log CFU/g C. perfringens
0
1
2
3
4
5
6
7
8
A
A
D
D
D
D
D
E
D
BC
D
C
D
D
D
DE
D
B
D
C
Uncured
PCN
FE
FE+PCN
DV
DV+PCN
CSV
CSV+PCN
LV
LV+PCN
Heat Shock
Chilled

CHAPTER 7: GENERAL CONCLUSIONS

The enduring controversy surrounding the use of sodium nitrite as an ingredient in RTE meats has continued for years and does not show signs of waning. Because a replacement for nitrite has yet to be discovered or developed, and consumers continue to demand products with the distinctive characteristics imparted by this "magic" ingredient, continued advances in processing technologies are needed to meet demands for high quality, wholesome, and most importantly safe RTE products. Pathogens such as *Clostridium botulinum, Clostridium perfringens,* and *Listeria monocytogenes* are continued risks in RTE products, but can be controlled using nitrite and an array of adjunct ingredients. As the industry experiences substantial growth in the production of alternative cured products, emphasis can still be placed on understanding the impact of nitrite on these pathogens. Furthermore, it is necessary to identify adjunct ingredients with food safety impact that can be used in conjunction with nitrite to amass multiple hurdles and prevent the growth of pathogens, while maintaining clean labels and minimal preservatives. To inhibit *L. monocytogenes*, nitrite concentration has been shown to be the paramount factor, while source (purified or natural) is irrelevant. As hypothesized, nitrite does not inhibit *L. monocytogenes* growth in the absence of additional inhibitors, but can improve safety as concentrations increase. Concentrations of nitrite found in alternative cured products (50 to 100 ppm) have a negligible effect, but results indicate that maximizing ingoing concentrations is the most effective way to utilize this ingredient to slow the growth of *L. monocytogenes*.

As with its inhibitory effects on *L. monocytogenes*, nitrite prevents outgrowth of *C. perfringens* based on concentration, not source. In this research, regulated levels of nitrite (100

ppm) were unable to achieve performance standards for *C. perfringens* during chilling when used alone, but were significantly more effective in the presence of ascorbate, another ingredient whose activity was based on concentration but not on source (purified or natural). The combined antimicrobial impact of nitrite and ascorbate held true at ingredient concentrations commonly found in alternative cured products. The necessity of adjunct ingredients to enhance nitrite inhibition of *C. perfringens* prompted the evaluation of commercially available natural and clean label antimicrobials for improved safety of these products. Fruit extracts and a blend of cultured sugar and vinegar worked synergistically with low concentrations of added nitrite to prevent growth of *C. perfringens* during chilling. Collectively, this research demonstrated that equivalent safety can be achieved in alternative cured RTE meats without the use of preservatives.

APPENDICES

APPENDIX 1: Nitrites in Cured Meat – Colorimetric Method; Alkaline Modification

Association of Official Analytical Chemists. 1990. Nitrites in cured meat. In: Official Methods of Analysis. 15th ed. Arlington, VA: AOAC 973.31.

Preliminary steps (reagents and apparatus):

*use MilliQ water for all reagents and solutions

1. Make up 1000 ml or 500 ml of the 15% CH3COOH (acetic acid) solution

2. Make up NED reagents and sulfanilimide reagents as described in AOAC.

    a. Store them in brown glass bottles in dark cabinet.

3. Make nitrite standard solutions (stock, intermediate and working).

    a. Stock solution – dissolve 1.000 g of sodium nitrite in a 1000 ml. volumetric and shake it up thoroughly diluting with distilled, deionized water.

    b. Intermediate solution – take 100 ml. of the stock solution add it to another 1000 ml. volumetric and dilute with distilled, de-ionized water.

    c. Working solution – take 10 ml. of the Intermediate solution and put in a 1000 ml. volumetric and dilute to volume with distilled, deionized water.

Procedure:

1. Heat distilled, de-ionized water in big beakers or Erlenmeyer flasks and put them onto a warming plate approximately 30-50 minutes before beginning experiment. The water will need to be 80°C before use.

2. Weigh 5 g of finely chopped sample into a 50 ml beaker. If you want to do duplicates you will need to weigh up two samples per treatment.

3. After the samples are weighed add roughly 40 ml of the hot water to the sample and stir vigorously with a glass stir rod. Be careful not to slop the sample out of the beaker.

4. Add 4 ml of saturated sodium borate solution to the beaker with the sample and 40 ml of hot water.

5. After the sample has been stirred with a glass rod, transfer the beaker contents to a 500 ml volumetric flask. You will need to add some more hot water to the beaker to clean out all the sample and what sample is left on the funnel you will use. Be very liberal with the hot water. It is acceptable to use 100-200 ml to get all of the sample cleaned off of the beaker and into the flask.

6. After all samples in the beakers are transferred to their own 500 ml flask make sure that each flask has about 350 ml of solution in it by adding hot water.

7. Cover the flasks with a small piece of aluminum foil. Put the flasks into the steam cabinet and crank the steam full blast. Swirl the flasks every 30 minutes to aid in extraction. Be careful handling the hot flasks and be sure to let the pressure out of the flask before shaking.

8. After 2 hours take the flasks out of the steam and set them on the bench top to cool to room temperature (approx 2 hours).

9. Add 4 ml of 30% (w/v) of zinc sulfate to the cooled flasks. Then fill the flask to volume with room temperature distilled water. If you add the water before the flask has cooled, the volume may change once temperature equilibration has been done.

10. Filter about 35 ml of the flask contents into each of 2 separate 50 ml. volumetric flasks. You could also use 1, but I preferred splitting the sample in two at this point so that any discrepancies on the spec would show up right away.

11. Add 2.5 ml of sulfanilamide solution and wait 5 min. Use a pipette set for 2.5 ml.

12. Pipette 2.5 ml of NED reagent and then fill the flask to volume with some more filtered sampler.

13. Let the sample set for 15 min. to allow the color to develop.

14. After color development, use disposable transfer pipets to transfer sample to disposable cuvettes. Run samples on spectrophotometer. Set the spec at 540 nm as described in the method.
15. Prepare a blank right along with your other samples, 2.5 ml sulfanilamide, 45 ml water, and 2.5 ml NED then standardize the spec with the blank.

APPENDIX 2: Nitrites in Cured Meat – HPLC Method

Bryan, N. S., and M. B. Grisham. 2007. Methods to detect nitric oxide and its metabolites in biological samples. Free Radic. Biol. Med. 43:645-657.

Nuñez De González, M. T., W. N. Osburn, M. D. Hardin, M. Longnecker, H. K. Garg, N. S. Bryan, and J. T. Keeton. 2012. Survey of residual nitrite and nitrate in conventional and organic/natural/uncured/indirectly cured meats available at retail in the United States. J. Agric. Food Chem. 60:3981-3990.

Extraction Procedure:

1. Blend sample in Waring blender with liquid nitrogen to a homogenous powder. (~1-2 min)
2. Weigh 5 g of the meat powder directly into a 50 mL snap cap conical tube.
3. Bring solution to 50 mL total volume with pH 7.4 100 µM phosphate buffer (use graduated marks on tube).
4. Vortex the slurry for 30 s.
5. Pour slurry into two 50 mL polycarbonate centrifuge tubes. Each tube should be ~25 g. To achieve homogenous tubes, shake the slurry right before pouring both tubes.
6. Centrifugation at 10,000 x *g* for 5 min at 4°C.
    a. Beckman Coulter Avanti J-E with Beckman Coulter JA-25.50 rotor.
7. Pipette two 400 µL aliquots of the supernatant into labeled, 1.5 mL centrifuge tubes (economy microtube with snap cap, nr 89000-028, VWR International)
    a. Pipette 400 µL of methanol into all of the 1.5 mL tubes before starting procedure (expedites process of getting nitrite stable in methanol).
    b. Use a new tip for each sample.
8. Vortex samples on 10 speed setting; 1-2 s each tube
    a. Let stand 10 min at 4°C
    b. Vortex again on same settings
9. Centrifuge at 13,000 x *g* for 8 min (Eppendorf centrifuge, model 5424, Brinkmann Instruments Inc.)

10. Transfer the supernatant into 1.5 mL labeled tubes
11. Store samples covered in aluminum foil, in the dark, labeled at 4°C
12. Run samples on ENO-20 NOx Analyzer within 48 hours of extraction procedure
    a. Repeat steps 8-10 as needed if samples become turbid during storage

NOx Analysis:

1. Prepare Carrier, Reactor A, and Reactor B solutions according to EiCom ENO-20 User Manual. Store at 4°C.
2. Pull Carrier solution out of refrigerated storage and allow it to come to room temperature on day of analysis.
3. Make Reactor solution by mixing 50/50 Reactor A and Reactor B. Make just enough to run samples each day (100-200 ml). Wrap bottle in aluminum foil or use an amber bottle to prevent light exposure to reactor solution, and allow to reach room temperature before using.
4. Place intake lines into each solution. Set up and turn on machine according to User Manual. Let it run 15-20 minutes, and then verify that both pumps are working by checking the flow rate into the waste bin.
5. Settings: reactor pump at 0.22 ml/min; carrier pump at 0.66 ml/min; injection settings start recording at inject and run 5 minutes after injection; hardware settings range 1 V.
6. Before running a blank, check that the absorbance line has stabilized and then press the auto zero button near the start/stop button. Make sure the absorbance line has dropped and stays at 0.
7. Run standard solutions.
    a. Using 100 µM $NaNO_2$ stock solution, dilute to 20, 40, 60, 80 µM for complete standard curve.
8. To run samples, inject 50 µl and rinse injection head with syringe of milliQ water between samples.

APPENDIX 3A: Average L. monocytogenes counts (log CFU/g) used for estimation of lag time and growth rate after initial (day 0) inoculation.

			L. monocytogenes (log CFU/g) growth after inoculation at day 0[c]											
Trt[a]	Sodium nitrite[b]	Sodium ascorbate[b]	Day 0	Day 4	Day 7	Day 11	Day 14	Day 18	Day 21	Day 25	Day 28	Day 35	Day 42	R^{2d}
1a	50	100	3.38	4.44	6.12	7.65	8.29	8.61	8.57	NT	NT	NT	NT	0.9992
2a	50	550	3.36	3.47	5.08	6.10	7.68	8.54	8.67	NT	NT	NT	NT	0.9750
3a	300	100	3.35	3.36	3.42	3.94	4.68	5.64	6.40	7.26	7.84	7.78	7.56	0.9884
4a	300	550	3.33	3.41	3.37	3.37	4.27	5.21	5.42	6.61	7.13	7.74	7.25	0.9771
5a	0	325	3.44	3.56	4.68	7.43	8.45	9.00	8.96	NT	NT	NT	NT	0.9993
6a	352	325	3.36	3.47	3.43	4.69	5.09	5.40	6.37	7.21	7.22	7.66	7.28	0.9672
7a	175	0	3.49	3.50	NT	5.37	5.86	7.16	7.79	8.29	8.29	NT	NT	0.9906
8a	175	643	3.41	3.39	3.53	4.24	5.10	6.70	7.18	8.07	7.88	NT	NT	0.9934
9a	175	325	3.46	3.83	4.29	5.16	5.95	7.38	7.83	7.48	7.68	NT	NT	0.9699
10a	175	325	3.54	3.56	3.94	4.96	6.50	7.89	8.03	8.12	8.24	NT	NT	0.9949
1b	50	100	3.38	3.70	4.70	7.13	7.43	7.67	7.79	8.06	7.95	NT	NT	0.9911
2b	50	550	3.36	3.28	3.73	6.75	7.14	7.17	7.37	7.64	7.64	NT	NT	0.9900
3b	300	100	3.42	3.32	3.32	4.12	4.48	5.11	6.07	7.57	8.03	7.84	7.91	0.9668
4b	300	550	3.44	3.26	3.41	4.13	4.17	5.18	5.83	6.34	6.77	7.07	7.11	0.9896
5b	0	325	3.40	3.64	4.45	7.19	7.47	8.13	8.35	8.81	8.68	NT	NT	0.9826
6b	352	325	3.34	3.43	3.25	4.62	5.11	6.22	6.67	6.91	7.18	6.98	7.19	0.9891
7b	175	0	3.35	3.34	3.21	4.06	4.31	5.54	6.15	7.58	7.47	7.39	7.94	0.9762
8b	175	643	3.34	3.31	3.32	4.59	5.16	6.41	6.68	7.06	7.20	7.32	7.42	0.9881
9b	175	325	3.34	3.38	3.67	4.94	5.35	6.53	7.83	7.28	7.22	7.40	7.38	0.9609
10b	175	325	3.34	3.33	3.07	4.19	3.90	4.50	5.21	6.67	7.30	7.21	7.49	0.9527

[a] Trt = treatment; a = replication 1, b = replication 2.

[b] Ingoing sodium nitrite and sodium ascorbate (ppm) on meat weight basis.

[c] Log CFU/g measured in triplicate meat samples.

[d] R^2 value of DMFit estimates of growth parameters.

APPENDIX 3B: Average *L. monocytogenes* growth (log CFU/g) used for estimation of lag time and growth rate after delayed (day 28) inoculation.

			L. monocytogenes (log CFU/g) growth after inoculation at day 28[c]										
Trt[a]	Sodium nitrite[b]	Sodium ascorbate[b]	Day 0	Day 4	Day 7	Day 11	Day 14	Day 18	Day 21	Day 25	Day 28	Day 32	R^{2d}
1a	50	100	3.36	4.04	5.89	7.81	8.58	8.74	8.45	NT	NT	NT	0.9933
2a	50	550	3.33	3.55	4.71	6.62	7.97	8.70	8.56	NT	NT	NT	0.9935
3a	300	100	3.36	3.30	3.38	4.30	5.36	6.56	7.51	8.11	8.30	NT	0.9984
4a	300	550	3.32	3.27	3.76	4.86	6.00	7.80	7.55	8.11	8.04	NT	0.9834
5a	0	325	3.38	3.83	5.47	7.16	8.22	8.91	8.75	NT	NT	NT	0.9938
6a	352	325	3.41	3.29	3.85	4.91	5.95	7.30	7.91	8.35	8.04	NT	0.9919
7a	175	0	3.35	3.39	3.65	5.68	7.55	8.34	8.36	8.51	8.38	NT	0.9980
8a	175	643	3.31	3.30	3.88	5.02	6.32	7.63	8.14	8.44	8.33	NT	0.9961
9a	175	325	3.30	3.17	3.87	4.88	6.01	7.26	8.08	8.39	8.37	NT	0.9946
10a	175	325	3.31	3.48	3.93	5.17	6.55	7.91	8.26	8.48	8.40	NT	0.9955
1b	50	100	3.27	3.46	5.55	7.01	7.31	7.78	7.62	NT	NT	NT	0.9910
2b	50	550	3.39	3.81	5.34	7.01	7.26	7.77	7.54	NT	NT	NT	0.9937
3b	300	100	3.06	3.21	4.11	4.38	5.14	6.71	6.53	7.55	7.84	NT	0.9679
4b	300	550	3.43	3.32	3.76	4.21	4.87	5.81	6.38	6.88	7.17	7.30	0.9972
5b	0	325	3.40	3.65	5.11	6.62	6.87	7.62	7.72	NT	NT	NT	0.9849
6b	352	325	3.26	3.30	3.78	4.30	4.90	5.60	6.13	6.58	6.77	7.01	0.9982
7b	175	0	3.45	3.38	3.70	4.88	5.76	7.06	7.26	8.11	7.67	NT	0.9874
8b	175	643	2.98	3.27	3.90	4.70	5.46	6.78	7.05	8.06	7.32	NT	0.9683
9b	175	325	3.37	3.31	4.03	4.81	5.33	6.65	6.96	7.94	7.31	NT	0.9673
10b	175	325	3.21	3.24	3.92	4.67	5.26	6.37	6.84	8.41	7.62	NT	0.9555

[a] Trt = treatment; a = replication 1, b = replication 2.

[b] Ingoing sodium nitrite and sodium ascorbate (ppm) on meat weight basis.

[c] Log CFU/g measured in triplicate meat samples.

[d] R^2 value of DMFit estimates of growth parameters.

APPENDIX 4: Average residual nitrite measured during storage of cooked, cured pork sausage at 7°C (RSM based treatments).

			Residual nitrite (ppm)[c]					
Trt[a]	Sodium nitrite[b]	Sodium ascorbate[b]	Day 0[d]	Day 4	Day 7	Day 14	Day 21	Day 28
1a	50	100	34	32	29	26	21	24
2a	50	550	35	22	24	18	19	14
3a	300	100	267	225	223	187	155	137
4a	300	550	273	206	171	153	135	109
5a	0	325	NT	NT	NT	NT	NT	NT
6a	352	325	318	239	226	195	167	157
7a	175	0	120	125	122	108	107	112
8a	175	643	123	112	99	90	72	66
9a	175	325	151	126	102	96	88	78
10a	175	325	151	119	111	102	86	78
1b	50	100	17	25	23	24	18	23
2b	50	550	16	23	25	25	17	16
3b	300	100	110	197	219	223	176	160
4b	300	550	125	181	174	176	149	123
5b	0	325	NT	NT	NT	NT	NT	NT
6b	352	325	188	195	227	217	185	187
7b	175	0	76	115	121	107	104	92
8b	175	643	75	100	108	112	87	79
9b	175	325	85	103	119	104	80	84
10b	175	325	97	125	113	99	79	87

[a] Trt = treatment; a = replication 1, b = replication 2.
[b]Ingoing sodium nitrite and sodium ascorbate (ppm) on meat weight basis.
[c]ppm measured in duplicate from 2 meat samples (n=4).
[d]replicate 2 day 0 samples were temperature abused before analysis. Data was not used for modeling depletion rate. Day 4 measured values were used as residual nitrite concentrations at initial inoculation.

APPENDIX 5: Kerry Ingredients Accel™ 2000 Powder product information.

Kerry Ingredients & Flavours
1 Millington Road
Beloit, WI 53511
USA
Tel: +1 (608) 363 1200
Fax: +1 (608) 363 1399

Product Data Sheet US and Canadian version

Product Name: Accel™ 2000 Powder
Product Code: I10024
Date Printed: March 15, 2012

General Description

ACCEL™ 2000 Powder is a natural flavor produced from a concentrated fermentate of celery juice. **ACCEL™ 2000 Powder** enhances flavor in all types of cured and fermented meat products, providing a mild broth, pleasant savory, vegetable note.

ACCEL™ 2000 Powder can be used to flavor and may improve color stability of cured beef, salami, summer sausage, beef sticks, beef logs, Thuringer, Cervelat, Italian salami, cured ham, bacon, turkey, hot dogs, bologna, cold cuts and other fermented and non-fermented meat products.

Use Rate
ACCEL™ 2000 Powder is recommended to be used at 0.15-0.85% based on meat green weight. Usage can be increased or decreased depending on desired requirements.

Application
ACCEL™ 2000 Powder is easily incorporated into brines or meat blends. During processing, **ACCEL™ 2000 Powder** should be added and thoroughly mixed at the stage where traditional flavor and cure would be added. No processing changes are required.

Regulatory Information

Accel 2000 is maufactured under FDA regulations. The FDA label statement for Accel 2000 is Natural Flavor.

Alternate: Cultured celery juice, maltodextrin, corn starch

Alternate: Celery Juice, lactic acid starter culture, maltodextrin, corn starch

Alternate: Cultured celery juice powder[(celery juice, lactic acid starter culture), maltodextrin, corn starch]

Customer labels approved by USDA:

Cultured celery juice powder

Celery juice powder, lactic acid starter culture

Local food regulations should always be consulted with respect to specific applications and necessary declarations. Legislation may vary from country to country.

Shelf life and Storage

Store **ACCEL™ 2000 Powder** in a cool and dry environment. The shelf life of the product in its original unopened bag is six months from the date of manufacture if stored as directed.

Packaging
ACCEL™ 2000 Powder is available in 20 pound (9.08 kg) multiwall bags stretch wrapped on pallet.

APPENDIX 6: Florida Food Products, Inc. Veg Stable™ Cherry 515 product information.

FLORIDA FOOD PRODUCTS, INC.
West CR 44, Eustis, FL USA 32726
(352) 357-4141 Fax (352) 483-3192

Product Specifications and Information

PRODUCT NAME - VEG STABLE™ CHERRY 515

FFP PRODUCT CODE - 515

INGREDIENT DECLARATION- Cherry Powder, Turbinado Sugar, and Silicon Dioxide (anti-caking).

USE- Meats, dry soups, beverages, health supplements, cosmetics and seasoning blends.

DESCRIPTION- Veg Stable™ CHERRY is a dried powder derived from fresh cherries and evaporated cane syrup. Anti-caking agents my be added.

GENERAL SPECIFICATIONS	**Veg Stable™ Cherry**
APPEARANCE	Tan free flowing powder
TOTAL DISSOLVED SOLIDS	≤ 5%
pH (5% Solution)	5.8 - 6.2
TOTAL PLATE COUNT	≤20,000 cfu/gm
YEAST AND MOLD	≤100 cfu/gm max.
TOTAL COLIFORMS	Negative
PRESERVATIVES	None

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.

Gross	47.2 lbs. (21.4 kg)
Net	44.1 lbs (20kg)

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 one year.

SUGGESTED USAGE - 0.2% 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 7: Van Hees Bombal® A-Natural MX-5100 product information.

VAN HEES
We know how!

A NATURAL

SPECIFICATION

Product: BOMBAL® A-Natural MX-5100*
Product Code: 111234726US / 111234713US
Issue Date: April 23, 2013

Description:
BOMBAL A-Natural MX-5100 is a natural flavoring for meat and poultry products.

Labeling:
Natural Flavoring (Citrus Fruit Extracts, Tropical Fruit Extracts)

Usage Rate:
0.5% based on the raw formula.

Physical / Chemical Characteristics:
Free flowing powder.

Color	Beige
Odor	Slightly Citrus
Taste	Citric
pH-value (1% sol.)	4.0 - 5.0

Nutrition Values:
Nutrient per 100 g (calculated):

Calories	≈ 257 kcal
Protein	≈ < 1.0 g
Carbohydrates	≈ 62.8 g
Sugars	≈ 7.2 g
Fat	≈ < 1.0 g
Saturated Fat	≈ < 1.0 g
Fiber	≈ < 1.0 g
Sodium	≈ 4.3 g

Storage Conditions:
Keep away from direct sunlight, in a cool and dry area, once open use product.

Shelf Life:
24 months in unopened original packaging.

Pack Unit:
TBD

VAN HEES Inc.
2500 Regency Parkway • Cary • North Carolina • 27518
Tel: 919 654-6862 • Fax: 919 654-6864 • info@vanheesinc.com • www.vanheesinc.com

APPENDIX 8: Corbion Purac Verdad N6 product information.

purac
pure by nature

Product data

4551/Rev.No.1

Verdad® Powder N6

Description

Verdad Powder N6 is the powder form of a natural product based on a label friendly ingredient: vinegar.

The white distilled vinegar is produced by fermentation using specifically selected food cultures. Verdad Powder N6 has a mild vinegar taste and is designed to apply in natural meat and poultry products.

Specification	Product	vinegar
Assay	Dry matter, loss on drying	9-15% w/w
Visual sensory characteristics	Form Appearance (visual observation)	powder off-white
Purity	Free acidity (as acetic acid) pH 10% (10.0 g product + 90 g water)	4.2-6.2% w/w 5.6-6.0
Physical chemical properties	Molecular formula Molecular weight	CH_3COOH 60
Registration/ Regulation	CAS number USA	64-19-7 GRAS
Labeling	Vinegar	
Intended Use	Food products	

APPENDIX 9: Kerry Ingredients DuraFresh 2012 product information.

KERRY

Code: 30499359
Issue date: 7/19/2013

TECHNICAL INFORMATION

Kerry Ingredients Americas Region
3400 Millington Road Beloit, WI 53511 USA
Tel: (608) 363-1200
www.kerry.com

PRODUCT SPECIFICATION

PRODUCT DETAILS

Part Number	30499359
Product Name	DURAFRESH™ 2012
Product Description	DURAFRESH™ 2012 is a white to cream colored powder derived from a controlled food grade fermentation process. The resulting spray dried product contains a specific composition of organic acids, peptides, and other naturally derived fermentation metabolites. DURAFRESH™ 2012 demonstrates efficacy against Gram-positive and Gram-negative bacteria in addition to mold strains.

INGREDIENT DECLARATION

Vinegar, Cultured Dextrose, Dried Corn Syrup

USAGE DETAILS

Use DURAFRESH™ 2012 at 0.1-1.0%. For optimal results, try DURAFRESH™ 2012 in your unique formula and process at 0.8% and adjust accordingly to achieve your specific needs.

KEY PERFORMANCE PARAMETERS

Description	Min	Max	Units
pH (10% solution)	6	7.5	PH
Moisture AOAC 927.05		5.5	%

Code: 30499359
Issue date: 7/19/2013

TECHNICAL INFORMATION

Kerry Ingredients Americas Region
3400 Millington Road Beloit, WI 53511 USA
Tel: (608) 363-1200
www.kerry.com

MICROBIOLOGICAL DATA

Coagulase Positive Staphyloccoci FDA BAM 8th Edition	NOT DETECTED
Coliforms FDA BAM 8th Edition	< 10 cfu/g
E. Coli FDA BAM 8th Edition	NOT DETECTED
Listeria monocytogenes / 25g; FDA BAM 8th Edition	NOT DETECTED
Mold FDA BAM 8th Edition	< 50 cfu/g
Salmonella / 25g; FDA BAM 8th Edition	NOT DETECTED
Yeast FDA BAM 8th Edition	< 50 cfu/g
Aerobic Plate Count FDA BAM 8th Edition	< 30000 cfu/g

SHELF-LIFE & STORAGE

365 days unopened when stored under cool and dry conditions. Keep tightly sealed after opening.

PACKAGING

Packaging Type: Multilayer Paper Bag
50 lbs net

KERRY

Code: 30499359
Issue date: 7/19/2013

TECHNICAL INFORMATION

Kerry Ingredients Americas Region
3400 Millington Road Beloit, WI 53511 USA
Tel: (608) 363-1200
www.kerry.com

NUTRITIONAL INFORMATION

Nutrient	Typical Value	Unit	Method / Source
Calories	157.1	kcal/100g	Calculated
Calories from Fat	0.5	kcal/100g	Calculated
Total Fat	0.1	g/100g	Calculated
Saturated Fat	0.0	g/100g	Calculated
Trans Fat	0.0	g/100g	Calculated
Cholesterol	0.0	mg/100g	Calculated
Total Carbohydrate	34.7	g/100g	Calculated
Sugars	9.2	g/100g	Calculated
Dietary Fiber	0.0	g/100g	Calculated
Protein	1.1	g/100g	Calculated
Calcium	9.5	mg/100g	Calculated
Iron	1.3	mg/100g	Calculated
Sodium	12,220.6	mg/100g	Calculated
Vitamin A	16.3	IU/100G	Calculated
Vitamin C	1.3	mg/100g	Calculated

Note: The information stated is given in good faith and is based upon the product formulation and data provided by raw material suppliers. While we make every effort to obtain assurances from our suppliers regarding the nutritional data supplied, manufacturers and/or suppliers do not always test their materials to obtain nutritional data and therefore will not always provide absolute values.

Code:	30499359
Issue date:	7/19/2013

TECHNICAL INFORMATION

Kerry Ingredients Americas Region
3400 Millington Road Beloit, WI 53511 USA
Tel: (608) 363-1200
www.kerry.com

RECOMMENDED ALLERGEN DATA FOR LABELING PURPOSES

Is the product FREE FROM..?	Yes = Absent, No = Present
Wheat/Wheat Derivatives	YES
Soy/Soy Derivatives	YES
Egg/Egg Derivatives	YES
Dairy/Dairy Derivatives	YES
Fish/Fish Derivatives	YES
Shellfish/Shellfish Derivatives	YES
Treenut/Treenut Derivatives	YES
Peanuts/Peanut Derivatives	YES

LEGISLATION & WARRANTY STATEMENT

The product will be manufactured in accordance with all current relevant US legislation. The information stated is given in good faith and is based upon the product formulation and the data provided by our raw materials suppliers.
All reasonable precautions that could be expected of a responsible manufacturer have been taken, however no absolute guarantees can be given that trace / carry over residues will be totally absent.

APPENDIX 10: World Technology Ingredients, Inc. LV1X product information.

WTI INC.
WORLD TECHNOLOGY INGREDIENTS | *Innovators in Marination, Further Processing & Food Safety*

LV1X

Product Specification

Description

LV1X is an all natural blend of vinegar and lemon juice concentrate designed to enhance the organoleptic properties of foods while inhibiting a broad spectrum of bacteria, yeast and molds. *LV1X* increases the water holding capacity of muscle protein systems. At low concentrations, *LV1X* does not have any flavor impact on the finished product. At higher concentrations, its slight citric taste enhances the natural flavors of meats, fish, poultry and vegetables.

Specifications

Test Parameter	Specification
Appearance	Yellowish brown opaque liquid
Odor	Slight citrus
pH (10 % solution)	5.70 ± 0.15
Viscosity (23°C)	1000 cP ± 250 cP
Viscosity (4°C)	2300 cP ± 500 cP
Density (23°C)	1.34 g/cc ± 0.30 g/cc

Nutritional Analysis (per 100 grams)

Nutrient	Value
Calories	90 calories
Total Fat	1 g
Cholesterol	0 mg
Sodium	6000 mg
Potassium	5100 mg
Total Carbohydrate	19 g
Protein	1 g

Application Information

Recommended Usage: Usage rate for shelf life enhancement is 0.25 – 2.00 % of finished product weight.
Recommended usage rate for *Listeria* inhibition is 2.3 - 2.5% of finished product weight.

Labeling: Vinegar, lemon juice concentrate

Shelf Life: 12 months. Avoid temperature extremes (< 30°F or > 85°F).
Optimum storage temperatures 34°F to 72°F

Technical Assistance: Call WTI technical services at (706) 387-5150.

281 Martin Luther King Jr. Ave, Jefferson, GA 30549-1447 USA / Office **706.387.5150** / FAX **706.387.5159** / contactus@wtiinc.com

Issued 01/04/2012

APPENDIX 11: UltraSource, LLC vacuum pouch product information.

TECHNICAL SPECIFICATIONS
3 mil, 3-sided Seal Vacuum Pouch
(PA/PE/PA/PE Structure | PE Sealant)

Properties	Unit	Typical Value	Test Method/Condition
Thickness:	μ	3 mil (75 micron)	DIN 53370 25°C / 60% RH
Gas Permeability:			
Water Vapor Transmission Rate	$g/m^2.24hr$	6 – 7.5	ASTM D3849 25°C / 90% RH
Oxygen (O^2) Transmission Rate	$cc/m^2.24hr$	50 – 70	ASTM D1249 25°C / 60% RH
Mechanical Strength:			
Tensile Strength – MD	Mpa	24-25	ASTM D882 25°C / 60% RH
Tensile Strength – TD	Mpa	22-24	ASTM D882 25°C / 60% RH
Elongation – MD	%	320 – 450	ASTM D882 25°C / 60% RH
Elongation – TD	%	320 – 500	ASTM D882 25°C / 60% RH
Recommended Heat Seal Temperature Range	°C °F	150 – 180 302 – 356	ASTM D882 25°C / 60% RH
Heat Seal Strength	N/15mm	20 – 22	FTM
Haze	%	11-15	ASTM D1003 25°C / 60% RH

Disclaimer: This data should be used as average typical properties and not as a specification. This data is offered for informational purposes only and does not represent any type of guarantee or warranty of performance. UltraSource assumes no liability for any incidents that may arise from use of this data.

Version: 06/06/13

P:\Docs\Spec Sheets (PDF)\Operational Supplies\Master\Technical Data, 3 mil, 3-sided.docx

UltraSource

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