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The physical, chemical and microbial effects of supplemental sodium nitrate on cured meat products

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The physical, chemical and microbial effects of supplemental sodium nitrate on cured meat products

by

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DEDICATION

This thesis is dedicated to my father, Fritz Usinger, and in memory of my aunt, Debra

Usinger, and my grandfather, Frederick D. Usinger III. A path in meat science would not

have been possible for me without the encouragement and love I have received from all

of you.

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ABSTRACT

The effects of supplemental nitrate from celery powder on boneless ham, cotto

salami and frankfurters were investigated. Two treatments (control and supplemental

nitrate) of each product were replicated twice to evaluate residual nitrite, residual nitrate,

rancidity, microbial growth, color, sensory properties, and proximate composition. All

analytical measurements were conducted at regular intervals for 98 days during storage at

1°C (32 − 34°F). Residual nitrite values were not significantly different ($P > 0.05$)

between treatments for cotto salami and hams, however, for frankfurters the control (15.8

ppm) and supplemental nitrate (11.9 ppm) treatments were significantly different ($P <$

0.05). Residual nitrate values were significantly different ($P < 0.05$) between the two

treatments for all three products as expected; however, the residual nitrate values for each

treatment were not significantly different ($P > 0.05$) for the effect of day. Rancidity

(TBARS) was significantly different ($P < 0.05$) for cotto salami control (0.47) and

supplemented cotto salami treatment (0.37), as well as the frankfurter control (0.38) and

the supplemented frankfurter treatment (0.49). However, no statistical difference ($P >$

0.05) in rancidity was observed between the two ham treatments. The results also showed

no statistical difference ($P > 0.05$) for microbial growth between the treatments for any of

the products. Color measurements showed that Hunter L-values for the cotto salami

control (49.65) and the supplemented cotto salami (46.94) were significantly different (P

< 0.05), and Hunter a-values for the ham control (8.59) and the supplemented ham (7.92)

were also significantly different ($P < 0.05$). The internal color measurement of Hunter a-

values for frankfurters determined that the control treatment (10.62) displayed a

significantly different ($P < 0.05$) Hunter a-value than the supplemental nitrate treatment

(10.11). None of the other physical, chemical or microbial measurements conducted were different as a result of the treatments. Sensory evaluations (15 cm line scale) supported the instrumental color results for cotto salami, frankfurters, and ham treatments. Sensory panel scores showed frankfurter (9.33) and ham (9.86) control treatments displayed a greater intensity of pink color than the frankfurter (6.93) and ham (6.56) supplemental nitrate treatments. Panelists determined the control cotto salami (6.65) treatment had a lighter visual appearance than the supplement nitrate cotto salami (9.87) treatment. Frankfurters showed no differences for sensory panel odors or flavors while the treatments for cotto salami resulted in some differences in aromas and flavors, and the greatest effect on aroma and flavor occurred with the hams. Consequently, the results showed that the overall addition of supplemental nitrate did not significantly alter physical, chemical or microbial effects on cured meat products during refrigerated storage, but some product-dependent sensory effects were observed.

CHAPTER 1. GENERAL INTRODUCTION

Sodium nitrate and sodium nitrite have been used in meat products as a cure and a preservative for centuries. The use of sodium nitrite is still common in cured meats today because it provides these products with improved quality characteristics such as color, shelf life, and flavor. The addition of sodium nitrite also contributes to meat safety by reducing the outgrowth of spore-forming bacteria in cured meat products. However, nitrate/nitrite, either as sodium nitrite in cured meat or nitrate in water sources has suffered continuous controversy due to the perception that dietary nitrate/nitrite leads to cancer in humans and causes infantile methemoglobinemia or "blue baby syndrome". The belief that nitrite in food and nitrate in water are associated with disease has resulted in numerous consumers viewing nitrate/nitrite as unhealthy and unsafe for consumption. The United States Environmental Protection Agency (EPA) regulates the amount of nitrate in water, but this regulation has suffered much debate and there is suggestion that the limit on the concentration permitted (10 ppm nitrate-N or 44 ppm nitrate) is too low and should be increased. The United States Department of Agriculture (USDA) permits the use of nitrate and nitrite for meat curing, and has concluded that cured meats are safe for consumption when cured according to current regulations. Specific regulations, created by the USDA, for both nitrate and nitrite control the concentration of these ingredients allowed to enter particular products. However, in recent developments for meat curing, celery juice powders that contain vegetable sources of nitrate and/or nitrite are now permitted as an alternative to conventional curing, but the USDA does not consider vegetable products to be curing agents when used in processed meat production, and consequently these products must be labeled "uncured". Further, these sources of

nitrate and nitrite are not limited by regulation in processed meats. It is important to note that the addition of sodium nitrate or sodium nitrite is virtually irreplaceable in cured meats due to its cumulative effect on color, flavor, antioxidant properties and antimicrobial activity.

Recent research has demonstrated that when sodium nitrite or nitrate is ingested, the body's nitric oxide will increase as a result, providing the ingested amount is sufficient (Lundberg & Weitzberg, 2010). The discovery of nitric oxide's physiological functions, along with extensive follow up research, has made it clear that sufficient nitric oxide in the human body is a significant contributor to good health (Bryan, 2006). Dietary nitrate has been shown to affect several physiological conditions, including cardiovascular health and exercise performance, by increasing nitric oxide concentrations in the blood and tissue. However, current perceptions regarding consumer health and safety will need to be overcome before the use of nitrate and nitrite in food can be viewed more positively by consumers.

The objectives of this study were to evaluate the physical, chemical and microbial effects of supplemental sodium nitrate in cured meat products, when manufactured to contain 220 mg or more of nitrate per 112 g serving of cured meat, with the target concentration accomplished by combining conventional sodium nitrate with celery powder containing additional nitrate. This concentration of dietary nitrate was developed according to a collaboration of previous reports that achieved diverse health benefits in human subjects after the consumption of dietary nitrate (Webb et al., 2008; Larsen et al., 2010; Lansley et al., 2011; Zand et al., 2011; Murphy et al., 2012; Liu et al., 2013; Gilchrist et al., 2014). It was hypothesized that this concentration of sodium nitrate would

not affect the physical, chemical or microbial properties of cured meat products. By including additional sodium nitrate in combination with USDA-regulated levels of sodium nitrate and nitrite in cured meats, it was hypothesized that cured meat products would have little or no change in color, texture, flavor or microbial growth. This thesis is organized into four chapters. Chapter one establishes a general introduction. Chapter two provides a review of the literature focusing on the topics relating to sodium nitrite and dietary nitrate. Chapter three is a complete manuscript and chapter four provides general conclusions for the entire thesis.

CHAPTER 2. LITERATURE REVIEW

Introduction

The human body can derive nitrate/nitrite/nitric oxide through two methods: endogenously by the nitric oxide synthase (NOS) pathway, and exogenously through dietary consumption. Nitrate and nitrite obtained from either source has the ability to subsequently form the free radical molecule nitric oxide. The formation of nitric oxide is so fundamentally important to human health that the 1998 Nobel Prize in Physiology or Medicine was awarded to Robert Furchgott, Louis Ignarro and Ferrid Murad for first recognizing nitric oxide as a endothelium-derived relaxing factor and for its signaling ability in the cardiovascular system. Nitric oxide is mandatory for normal function in every organ system in the body and adequate production effectively reduces the risk of developing cardiovascular diseases (Parthasarathy & Bryan, 2012).

The ability to form nitric oxide has established a new context for nitrate/nitrite in the diet. Incorporating nitrate with nitrite in cured meat systems could give consumers a significant dietary source of nitric oxide after the consumption of processed meats. Manufacturing a product that contains nitrate in combination with nitrite will allow for the essential curing reaction along with the necessary cured color, flavor and antimicrobial properties. Because nitrate is inert in cooked meat (Honikel, 2008; Sebranek, 2009), nitrate will not be depleted in the meat system during processing and storage, and can act as a source of dietary nitrate. The consumer could benefit after ingestion with the probability of increased nitric oxide levels. However, establishing this type of product will have its challenges due to the public and some scientific perceptions linking nitrate and nitrite to nitrosamine formation. Such products are unlikely to become

commonplace in the commercial market place due to the challenges of overcoming these perceptions, but demonstration of their potential for increasing physiological nitric oxide may be helpful for improving the negative perceptions of cured meat products.

Nitrate and Nitrite in the Environment

Nitrate is formed from gaseous nitrogen, which makes up 78% of the atmosphere, and it is fundamental for all life on Earth. Gaseous nitrogen is converted into nitrate and other usable forms through nitrogen fixation. Nitrate accumulates within vegetables, groundwater and some fruits. Legume plants can form nitrate by first obtaining nitrogen from the atmosphere and converting it into ammonia using bacteria (Lundberg & Weitzberg, 2010). These nitrogen-fixing bacteria within the plant, known as *Rhizobium,* have the nitrogenase enzyme that combines nitrogen with hydrogen to produce ammonia (Lundberg & Weitzberg, 2010). Bacteria first convert ammonia into nitrite ions and then into nitrate ions. Other types of plants that cannot take nitrogen from the air can absorb nitrate or ammonium ions from the soil (Lundberg & Weitzberg, 2010). Nitrate ions are essential to plants because they provide nutrients for growth, and this is why commercial fertilizers typically contain high nitrate concentrations. The amount of nitrate and nitrite within a plant is dependent on its developmental stage, but a large majority of nitrite is converted into nitrate because accumulation of nitrite can be toxic to plant life (Lundberg & Weitzberg, 2010). When animals and plants either die or excrete digestive waste, they return stored nitrogen to the soil where it is converted to nitrate and incorporated into aquifers by precipitation (Keeton, 2011). Nitrate is very water-soluble; therefore it is easily leeched into groundwater sources for the drinking water supply (Milkowski, 2011).

Sodium Nitrate and Nitrite: Preservatives and Curing Agents

Sodium or potassium nitrate or nitrite as curing agents were discovered by accident (Pearson & Gillett, 1996) in crystalline deposits as a natural contaminant of salt (sodium chloride) that has been historically used for its preservation properties and curing reactions (Parthasarathy & Bryan, 2012). Before the curing reaction was discovered, nitrate impurities in salt were observed to provide spoilage protection (Sindelar & Milkowski, 2012). During the 19th century, some salt sources containing saltpeter (potassium nitrate) were observed to be better preservers than others (Honikel, 2008). Once a better understanding of the meat curing chemistry was acquired, it was discovered that the method essential to food preservation by nitrate was achieved by the bacterial conversion of nitrate to nitrite (Binkerd & Kolari, 1975; Sebranek & Bacus, 2007). In the early 1900s the use of nitrite instead of nitrate became preferred in sausage manufacturing because nitrate is simply a precursor to nitrite, and use of nitrite allows for a faster curing time (Bryan, 2006; Sebranek & Bacus, 2007).

Today's preservation of cured, processed meats is accomplished with the incorporation of salt and nitrite (Parthasarathy & Bryan, 2012). Nitrate is currently still used, but its presence is rare except for specialty products where curing is a slower process (Sebranek, 2009). In order to act as a preservative, nitrite, cooking, and the addition of salt work collectively to protect against food poisoning caused by microorganisms (Cammack et al., 1999). Variations between factors such as meat system pH, the amount of reductants present, temperature, and time all affect the curing reactions caused by nitrite (Sebranek, 2009). Nitrite provides numerous benefits for the meat system and is, therefore, an essential ingredient in cured, processed meats. These benefits

include the delay of bacterial growth and in particular botulinum toxin production, the improvement of cured meat flavor and color, retardation of rancidity during storage, and inhibition of warmed-over flavor development (Binkerd & Kolari, 1975).

Color

The first and most prominent effect from nitrite is the development of a desirable reddish-pink cured color (Cornforth & Jayasingh, 2004; Sebranek & Bacus, 2007; Honikel, 2008; Parthasarathy & Bryan, 2012). Meat color is important because color provides the consumer with perceptions of the product's palatability and quality (Grossi et al., 2014). In order to ensure the meat product reaches optimal cured color formation, nitrate or nitrite must be added during manufacturing (Grossi et al., 2014). When nitrite is added to a meat formulation, it undergoes a series of reactions in order to combine with the pigment (Pérez-Rodríguez et al., 1996).

Nitrite is a highly reactive ion and does not, by itself, fix the pigment causing cured meat color, however, nitrosylating agents are formed and transfer nitric oxide to the pigment to stabilize the color (Sindelar & Milkowski, 2011). Nitric oxide production from nitrite is a necessary step in order to achieve cured color (Sindelar & Milkowski, 2011). Nitric oxide reacts with muscle myoglobin in the presence of endogenous or added reductants to form nitric oxide metmyoglobin, which is responsible for the brown pigment in cured meats before heating (Sebranek, 2009). Once the protein portion of myoglobin is cooked, it is then denatured and separates the mono-nitrosated heme structure from its covalent attachment to form nitrosylhemochromogen, which is

responsible for the bright pink color of cured meat (Morrissey & Tichivangana, 1985; Aberle et al., 2001).

Generally, only 40 to 50 ppm of ingoing nitrite is required to develop a cured color in most products (Sebranek & Bacus, 2007). The original amount of sodium nitrite added to meat for curing (typically 156 ppm) diminishes rapidly due to reactions during formulation and heating, leaving residual nitrite in the product at a much lower concentration, which then continues to decline during storage (Pérez-Rodríguez et al., 1996).

Flavor

The second quality aspect derived from nitrite in meat curing is the development of cured flavor. Very little specific literature is available on the chemistry of cured meat flavor (Pegg & Shahidi, 2000). Although there are several theories behind flavor development caused by nitrite, the principal mechanism is still not yet known (Sindelar & Milkowski, 2011; Villaverde et al., 2014b). It has been speculated that flavor production could be the result of nitrite's antioxidant function of inhibiting lipid oxidation (Aberle et al., 2001). Nitrosylhemochromogen formation, also responsible for cured pigment, is a reaction that stops iron porphyrins from oxidizing unsaturated fatty acids and creating volatiles, thus limiting iron's catalytic activity (Aberle et al., 2001). If these volatiles were created they would produce an unfavorable warmed-over flavor (WOF) (Aberle et al., 2001), which is a major flavor issue in cooked meat, but WOF can be largely prevented with the addition of nitrite (Sato & Hegarty, 1971).

Another explanation is that the flavor of cured meats is really the natural flavor of the meat species and nitrite inhibits the flavor overtone of carbonyls derived from lipid oxidation (Pegg & Shahidi, 2000). Sensory research has implied that aroma and flavor form from complex combinations of several compounds in collaboration with a lack of rancid flavors to establish cured flavor, instead of simply retarding lipid oxidation (Sindelar & Milkowski, 2012). A sensory study conducted by Cho and Bratzler (1970) concluded that panelists could differentiate between pork cured with nitrite and pork cured with sodium chloride. Panelists also detected a significant difference in amount of cured meat flavor between samples that were smoked and cured with nitrite and those smoked and cured without nitrite (Cho & Bratzler, 1970).

Similar to the minimum amount of nitrite needed to induce color, it appears that at least 40 to 50 ppm of ingoing nitrite is also needed to contribute cured flavor (Sebranek & Bacus, 2007). Nitrite's ability to preserve a desirable meaty flavor has been credited for the consumer demand for cured, processed meats (Aberle et al., 2001).

Antioxidant Properties

Similar mechanisms that provide cured color and flavor are also responsible for nitrite's antioxidant properties (Sebranek, 2009). Nitrite displays two antioxidant functions, which include the formation of nitrosylhemochromogen as well as the development of nitric oxide (Aberle et al., 2001). Nitrosylhemochromogen decreases catalytic activity by the immobilization of the iron complex and restricts the initiation of lipid oxidation (Aberle et al., 2001). The second antioxidant function is credited to nitric oxide, which operates as a free radical acceptor and prevents lipid oxidation by

terminating the free radical chain reaction (Aberle et al., 2001). Nitric oxide has the ability to reduce the amount of free iron released during cooking by binding and stabilizing the heme iron of meat pigment (Parthasarathy & Bryan, 2012). Preventing lipid oxidation benefits cured flavor by inhibiting the development of WOF in cooked cured products (MacDonald et al., 1980) as noted earlier, as well as providing long-term flavor stability (Sebranek & Bacus, 2007). By hindering the development of oxidative rancidity, cured meats are able to retain a higher product quality during distribution and storage (Sindelar & Milkowski, 2012).

Nitrite is considered a highly effective antioxidant when sufficient levels are used in cured meats (Sindelar & Milkowski, 2012). A study conducted by MacDonald et al. (1980) used model systems containing prooxidants such as ferrous iron and ferric iron-ethylenediaminetetraacetic acid (EDTA) to investigate lipid oxidation. This study demonstrated nitrite's antioxidant behavior by confirming that reduced levels of lipid oxidation were achieved with any of the concentrations of nitrite studied (10, 25 and 50 mg/kg) when compared to a non-nitrite treatment. Another study conducted by Sato and Hegarty (1971) investigated how different antioxidants and reducing agents effect lipid oxidation of ground beef when assessed by the 2-thiobarbituric acid (TBA) method. The authors concluded that 50 ppm sodium nitrite decreased the TBA value to less than half that of untreated ground beef, resulting in a value of 0.105 when compared to the control value of 0.297. Although 50 ppm of nitrite is needed to achieve color development, flavor and flavor stability, it has been suggested that as little as a 10 ppm nitrite can display antioxidant properties (Sebranek & Bacus, 2007).

Since nitrite displays these valuable antioxidant properties, synthetic antioxidants are prohibited by the USDA in most products already containing nitrite (Sindelar & Milkowski, 2011). However, the use of synthetic antioxidants is permitted with the incorporation of nitrate for dry cures since nitrate curing in dry cured hams or fermented sausages is a slow process (Sindelar & Milkowski, 2011) and additional fat oxidation protection (Aberle et al., 2001) is necessary.

Sodium Ascorbate and Sodium Erythorbate

A reductant, such as sodium ascorbate, sodium erythorbate, ascorbic acid or erythorbic acid is normally combined with nitrite in cured meat products (Honikel, 2008; Villaverde et al., 2014b). Most of the beneficial effects developed from curing agents are a result of the redox properties of nitrite and ascorbate (Villaverde et al., 2014a). Ascorbate acts as a free radical scavenger and possesses redox interrelationships with other antioxidants such as nitrite (Villaverde et al., 2014b). When ascorbate is combined with nitrite, the combination displays intense reducing abilities and also contributes to the prevention of microbial toxins and nitrosamine formation (Honikel, 2008).

The reductants function as cured color accelerators and can reduce the time needed for cured meat color development to hours instead of days (Aberle et al., 2001). Ascorbate and erythorbate are able to accelerate the curing reaction by encouraging a faster reduction of nitrite to nitrous acid and subsequently to nitric oxide (Redondo-Solano et al., 2013). The USDA recommends that processors use ascorbate or isoascorbate (erythorbate) at a concentration of 547 ppm to maximize curing enhancement (Redondo-Solano et al., 2013).

Ascorbate also has significant antioxidant effects, alone and in combination with nitrite (Villaverde et al., 2014b). A study of relatively high levels of sodium ascorbate concluded that lipid oxidation is decreased when assessed by TBA method (Sato & Hegarty, 1971). In this study, Sato and Hegarty (1971) added sodium ascorbate at a concentration of 5 mg/g to ground beef. After cooking and two days of storage the beef with added ascorbate had a TBA value of 0.077 when compared to the control value with no added ascorbate at 0.424.

Meat Safety: Nitrite as an Antimicrobial Agent

Along with contributing to flavor and the color of meat, nitrite hinders the growth of food spoilage bacteria, and most importantly, pathogens such as *Clostridium botulinum* (Cammack et al., 1999; Sebranek & Bacus, 2007). Nitrite application is especially important in cured meats that are vacuum packaged because of the potential for growth of anaerobic bacteria (Cassens, 1997). Even though nitrite is a more effective inhibitor of anaerobic bacteria, it can also contribute to the control of *Listeria monocytogenes* and other microorganisms (Sebranek & Bacus, 2007). Although it is not completely understood, nitrite functions as both a bacteriostatic and bacteriocidal agent (Sebranek & Bacus, 2007; Sindelar & Milkowski, 2011). It is proposed that the reactions of nitrite responsible for its antimicrobial functions are reactions likely connected with the conversion to nitric oxide (Sindelar & Milkowski, 2011). Cammack et al. (1999) found nitric oxide to have toxic effects on some bacterial cells as well as the ability to inactivate some iron-sulfur proteins such as aconitase and ferrochelatase. According to Johnston et al. (1969), possible microbiological roles of nitrite for the inhibition of *C. botulinum* include: (a) enhanced destruction of spores by heat, (b) increased spore

germination during thermal processing with subsequent destruction of the germinated spores by heat, (c) prevention of germination and outgrowth of the spores and (d) reaction with some meat component(s) to form a more inhibitory compound(s).

Botulinum Toxin

The fundamental antimicrobial utility of nitrite has been its ability to suppress the outgrowth of *Clostridium botulinum* spores (Keeton, 2011). *C. botulinum* is a spore-forming, gram-positive, anaerobic, toxin-forming bacteria that was once a serious problem with meats and sausages. Botulism toxin was commonly associated with meat products in the past, and therefore derives its name from the Latin word botulus, meaning sausage (Archer, 2002). Types A, B, C, D, E, F, and G are the seven recognized strains of neurotoxin produced by *C. botulinum.* Only types A, B, E and F are known to cause illness in humans. *C. botulinum* is dangerous because its spores are heat-resistant and can grow in the absence of oxygen, thus making canned or vacuum packaged meat products an ideal medium (Sindelar & Houser, 2009). What makes *C. botulinum* deadly to humans is that it produces one of the most lethal neurotoxins known, which causes death by respiratory paralysis (Cammack et al., 1999). A residual nitrite amount of 40-80 ppm is generally recognized as the minimum level required in meat products to inhibit outgrowth of *C. botulinum* spores (Aberle et al., 2001). Nitrate/nitrite's ability to prevent the growth of this harmful pathogen makes it a very important ingredient in processed meats.

Listeriosis

Listeriosis is an infection caused by the gram-positive bacterium *Listeria monocytogenes*. This facultative anaerobic or aerobic bacterium can be motile at

temperatures between -4°C and 50°C as well as pH values between 4.7 and 9.2

(Cammack et al., 1999), making cool and damp processing facilities an ideal

environment. Listeriosis typically affects older adults, pregnant women, newborns and

the immunocompromised to a greater extent than the rest of the population. Once ill with

listeriosis, clinical signs are diarrhea, nausea and vomiting. Listeriosis can be treated with

the use of antibiotics. The addition of sodium nitrite is able to reduce, but not inhibit the

growth of *L. monocytogenes*. Glass and Doyle (1989) concluded that 3.5% sodium

chloride in combination with 103 ppm sodium nitrite did not inhibit the growth of *L.

monocytogenes* at 32.2°C in beaker sausage and pepperoni. However, investigators

determined salami batter containing lactic starter culture showed a reduction in *L.

monocytogenes* populations during the fermentation period at 32.2°C.

USDA Regulations for Nitrite and Nitrate

Since the early 1900s the USDA has regulated nitrate and nitrite as curing salts

(Cassens, 1997; Bryan, 2006). The use of sodium or potassium nitrate and nitrite is

regulated by the Food Safety Inspection Service (FSIS) of the USDA. The Code of

Federal Regulations (2015) (Food Ingredients and Sources of Radiation, 9 CFR

424.21(c)), states the purpose, products, and amount in which a particular substance can

be added. In the United States, the permitted levels of nitrite or nitrate are dependent on

individual products that are being manufactured (Redondo-Solano et al., 2013). The

maximum ingoing sodium nitrate and sodium nitrite concentrations for immersion cured

products are 700 ppm and 200 ppm, massaged or pumped cured products are 700 ppm

and 200 ppm, and comminuted cured products are 1718 ppm and 156 ppm, respectively

(USDA, 1995). Either sodium or potassium salts of nitrate and nitrite may be used, but

the weight limitations are the same for both salts. The use of sodium or potassium nitrate and nitrite are permitted only in meat and poultry products. Specific regulations of ingoing nitrite are of importance because ingoing nitrite depletes over time, therefore the amount of ingoing nitrite rather than the residual must be standardized (Sebranek & Bacus, 2007).

USDA Regulations for Bacon

Bacon has its own set of USDA regulations for sodium nitrite or potassium nitrite due to the fact that nitrosamine formation was once a challenge to eliminate. Bacon is most commonly cooked at high temperatures and this combination of heat, nitrite and secondary amines contributes to the potential for nitrosamine formation (Pearson & Gillett, 1996). A lower level of nitrite along with sodium ascorbate or sodium erythorbate eliminates the possibility of nitrosamine formation by reducing residual nitrite concentration present at cooking. The use of sodium or potassium nitrate is no longer permitted in any curing method for bacon (USDA, 1995), in order to prevent additional residual nitrite formation. A combination of 550 ppm of ascorbate (or erythorbate) and 120 ppm of ingoing sodium nitrite (or 148 ppm potassium nitrite) is required for pumped and/or massaged bacon to diminish the problem of nitrosamines in cured, cooked bacon (USDA, 1995; Pearson & Gillett, 1996).

Risks with the Consumption of Nitrate/Nitrite

Nitrate and nitrite are not only found in processed meats, but are naturally occurring in vegetables and drinking water. Consumers hold two major concerns with the presence of nitrate and nitrite in their food and water supply, and these concerns include

the development of cancer and infant methemoglobinemia. Although there are currently

strict regulations dictating the amount of nitrate and nitrite in water sources and meat

products, such as a limit of 10 ppm nitrate-N (44 ppm nitrate) in drinking water and 156

ppm sodium nitrite in comminuted meat products, the consumer is not satisfied. This fear

has led to an increase in consumer demand for uncured meat products and has placed a

burden on farmers to control water run-off and fertilizer usage to try and reduce nitrate in

ground water. Currently, in Iowa, the Des Moines Water Works has filed a lawsuit to be

heard by a federal trial judge beginning on August 8th, 2016 against Buena Vista,

Calhoun and Sac counties over water quality. According to the Des Moines Water

Works, these three counties have been polluting the Raccoon River with nitrate-N levels

as high as 39.2 mg/L (172.5 ppm nitrate). The Des Moines Water Works hopes to be

reimbursed for continuously operating a very large nitrate removal facility for water from

the Raccoon River, which is a major source of the city's municipal water supply. This

battle is predicted to be a long and expensive one.

Association with Cancer

The use of nitrate and nitrite has been a controversial dispute for many decades

and although scientific research has established their addition to food as safe when used

as regulated, there is still belief by many consumers that consumption can cause cancer.

A report in 1970 by Lijinsky and Epstein (1970) concluded that nitrosamines are a class

of carcinogens that are related to human cancer. The authors acknowledged that large

concentrations of nitrate exist within vegetables, but claim that only limiting the exposure

to nitrite in meat and fish is necessary. These authors recommended that since cured meat

contains both nitrite and secondary amines, their consumption should be avoided. After

this conclusion, other studies began to surface about the correlation between processed

meats and cancer. A study conducted by Peters et al. (1994) stated that there is a

correlation between the consumption of hot dogs and childhood leukemia. This study and

many others are epidemiological studies and do not have valid cause-and-effect scientific

support. Even though scientific evidence is weak, large concern arose from parents and

schools with several not wanting to feed their children processed meats. This fear led

various scientific groups and associations to look into these allegations. In 1995, the

National Research Council Subcommittee on Nitrate and Nitrite in Drinking Water

reviewed previous findings and found no association between nitrate and stomach cancer

(NRC, 1995). The National Research Council (1995) also concluded that the studies in

humans were inadequate to support an association between nitrate or nitrite exposure and

reproductive or development effects. Even though various reviews confirm that there is

no harm in the consumption of processed meat with appropriate use of nitrate and/or

nitrite, organizations such as World Cancer Research Fund (2007) and the World Health

Organization (2015) continue to encourage consumers to limit consumption of processed

meats, while still encouraging consumption of large amounts of vegetables and fruits.

Alternative Curing Methods

In order to satisfy consumer demands for "healthier" and "safer" processed meat

and poultry products, a number of uncured, no-nitrate/nitrite added, meat and poultry

products have surfaced in the marketplace (Sindelar & Houser, 2009). These natural and

organic products in the meat industry that have a standard of identity, are not permitted to

incorporate the addition of sodium nitrate and/or nitrite, and therefore; must state,

"uncured" on the label of standardized cured meats if offered to consumers. Although all

of these products claim "no nitrates/nitrites added" and "uncured" on the label, there are actually two different types of uncured products that exist in the marketplace. There are those with no intent of replacing nitrate/nitrite and those that replace conventional nitrate/nitrite with a natural source of nitrate/nitrite to simulate typical curing (Sindelar & Houser, 2009). The products that replace conventional nitrate/nitrite were first developed to utilize vegetable ingredients with a high nitrate content and a nitrate-reducing starter culture (Sebranek & Bacus, 2007). Celery juice powder containing nitrate and combined with starter cultures of coagulase-negative cocci such as *Kocuria (Micrococcus) varians, Staphylococcus xylosus*, and *Staphylococcus carnosus* have been commonly used as an effective cure replacer (Sebranek & Bacus, 2007). These products require incubation time for culture activity and consequently the process is somewhat inconvenient. More recently, celery juice powder has been made available that it can be utilized in a cultured form, which has become more popular among processors since the nitrate has already been converted into nitrite, which eliminates the need for the reduction of nitrate after addition to a meat product blend. However, the products must still be labeled "uncured" because according to the USDA definitions, conventional nitrate or nitrite have not been added to the products. The use of the term "uncured" is misleading to the uneducated consumer because they are under the impression that no nitrite whatsoever is in the product when, in fact, there is, with the difference being that the nitrite present is from natural sources.

Products that use a vegetable cure and claim uncured or no-nitrates/nitrites-added are not required by the USDA to regulate the ingoing amount of nitrate/nitrite replacer because the natural source is not considered to be a curing agent by the USDA. The

USDA Processing Inspector's Calculation Handbook (USDA, 1995) suggests that in order to provide a preservative effect, at least 40 ppm of ingoing nitrite should be used. The typical ingoing nitrite level of a nitrite replacer is between 40 and 60 ppm, which is significantly less than the typical nitrite-cured product (Sebranek & Bacus, 2007). Although this amount is substantially lower than conventional cures, if at least 40 to 50 ppm of nitrite is formed from added natural nitrate sources, cured meat color, flavor and flavor stability is expected to be typical (Sebranek & Bacus, 2007).

The biggest concern is that uncured, no-nitrate/nitrite-added meat and poultry products that have no intent of replacing nitrate/nitrite can carry a higher risk for botulism than nitrate/nitrite cured products (Sindelar & Houser, 2009). Miller and others (1993) investigated whether organic acid salts (pyruvate, citrate, lactate, acetate, and propionate) would be effective in controlling *C. botulinum* in nitrite-free ground turkey in temperature-abused conditions. The samples were inoculated, cooked in water at 75°C, cooled and then incubated at 28°C for 0 to 18 days. When the salts were applied at a 6% concentration, the time to toxicity increased to 18 days for citrate (0.20 M), to less than 18 days for lactate (0.66 M), acetate (0.72 M), and propionate (0.62 M), and 7 days for pyruvate (0.54 M). Therefore, citrate was the most effective salt for delaying botulism toxin production. If any of these salts were to be combined with sodium nitrite, they have the possibility of greatly enhancing meat safety. Although the organic acid salts proved to be effective to some extent, the most alarming reminder from this study was that the citrate, lactate and pyruvate samples did not portray any off-odors or soft texture when toxic with *C. botulinum.* This result is noteworthy because without sensory indicators the consumer is not alerted of the dangers linked to a toxic product (Miller et al., 1993), a

situation very typical of *C. botulinum*. However, if a nitrite replacer such as celery juice powder is used, the reduced amount of formed nitrite may not represent a botulism concern if the product is stored and handled properly with modern technology and adequate refrigeration.

Nitrate in Drinking Water

The occurrence of nitrate in drinking water is just as large of a consumer concern as their addition in processed meats. Nitrate exists in ground water sources because of the use of nitrate fertilizers and the water solubility of nitrate (Sebranek, 2009). The Environmental Protection Agency (EPA) limits the amount of nitrate/nitrite in drinking water to prevent infant methemoglobinemia, a condition also known as the "blue baby syndrome". The EPA limit is 10 mg/L or 10 ppm of nitrate-N (44 ppm nitrate) in drinking water (U.S. EPA, 2014). The current maximum contaminant level (MCL) of 10 ppm nitrate-N was established from a survey conducted for the years $1943 - 1946$ by the American Public Heath Association committee on water supply (APHA) (Avery, 1999; L'hirondel et al., 2006; Powlson et al., 2008). The authors of the APHA survey (APHA, 1950) recommended a nitrate limit of 50 mg L^{-1} nitrate in water even though the committee conceded that they did not have detailed epidemiological and technical data to connect with the cases observed at that time. Since the survey did not find any infantile methemoglobinemia cases at concentrations less than 10 ppm, the United States, Europe and the World Health Organization declared the MCL of 10 ppm nitrate-N for nitrate in drinking water (Avery, 1999). The EPA first put regulations on nitrate in 1976 as a part of the Safe Water Drinking Act known as a National Primary Drinking Water Regulation (U.S. EPA, 2015). This regulation of 10 ppm nitrate-N still remains today.

Infant Methemoglobinemia

Infant methemoglobinemia or "blue baby syndrome" occurs in infants under six months of age, and the suggested cause is the ingestion of nitrates in drinking water. Currently, the evidence for nitrate as a source of methemoglobinemia remains controversial (Powlson et al., 2008). Methemoglobin, a form of oxidized hemoglobin that is unable to deliver oxygen, results in methemoglobinemia when it is in the blood at high enough concentrations to produce symptoms of cyanosis (Avery, 1999). Infants under six months of age have lower amounts of NADH-cytochrome b_5 methemoglobin reductase, which makes infants vulnerable to methemoglobinemia because they are unable to convert methemoglobin back to hemoglobin (Avery, 1999). Due to young infants underdeveloped methemoglobin-reducing system, overexpression of nitric oxide, separate from nitrate intake, may result in endogenous nitrite production sufficient to result in methemoglobinemia (Avery, 1999).

It has been reported that Hunter Comly (1945) first investigated methemoglobinemia in rural areas where infants were bottle-fed well water (Avery, 1999; Powlson et al., 2008). Comly's findings showed that bacteria as well as nitrate were found in the water given to infants. Comly (1945) observed that these rural wells were undesirable and reported,

> In many cases the wells were old, dug rather than drilled, had inadequate casings or none at all, and were poorly covered so that surface water, animal excreta and other objectionable material could enter freely.

Comly (1945) proposed that after infants drank the well water, nitrate ions were converted by bacterial action to nitrite ions and then were absorbed and reacted with

hemoglobin to form methemoglobin. Comly's hypothesis became accepted when further research showed a similar pattern of water with high nitrate concentrations and methemoglobinemia (Avery, 1999). Following Comly's report, Bosch et al. (1950) investigated methemoglobinemia cases in Minnesota and reported similar findings.

Over the past 20 years, re-evaluations of methemoglobinemia studies strongly suggest that fecal bacteria and not nitrate may be the cause of methemoglobinemia in infants (Avery, 1999; Powlson et al., 2008; Katan, 2009). Methemoglobinemia in infants can occur without exposure to high nitrate water or nitrates in food, and may occur as a result of diarrheal illness and gastrointestinal disturbances (Avery, 1999). Avery (1999) provided two possible explanations for the correlation between nitrate contamination of water and methemoglobinemia. The first is that nitrate is an indicator of bacterial contamination due to the description of the wells and the fact that diarrhea and vomiting symptoms are often prevalent. The water wells studied in the 1940's were found close to barnyards and manure storage facilities and since nitrogen exists in feces, it seems logical that the nitrogen found in feces is converted to nitrate, thus resulting in the association of high bacterial levels with high nitrate levels. The second explanation is that infants with existing gastrointestinal inflammation and/or infection establish an environment suited for methemoglobin through the endogenous production of nitrite from nitric oxide. Human tissues, in response to infection, produce nitric oxide and this overexpression of nitric oxide can lead to nitrite production that is sufficient enough to overwhelm an infant's undeveloped methemoglobin-reducing system. Existing gastrointestinal inflammation in combination with high concentrations of ingested nitrate can further drive chemical reactions toward nitrite accumulation.

As more evidence about the sources of methemoglobinemia accumulates there is more reason to believe that the current limits on drinking water are unnecessarily strict (Avery, 1999; L'hirondel et al., 2006; Powlson et al., 2008; Katan, 2009). Improving infant health by reducing the amount of nitrate in drinking water is not a cost effective solution (Avery, 1999). Keeping nitrate-N concentrations at 10 ppm or below is an expensive struggle for farmers in small communities (Avery, 1999; Katan, 2009). Farmers are being accused as the source of nitrate pollution in community groundwater due to their use of nitrate-rich fertilizer and the potential of fertilizer to seep into the groundwater (Katan, 2009). If the nitrate limit in drinking water was increased, many rural communities would be relieved of significant economic burden and there would be no additional increase in health risks for infants (L'hirondel et al., 2006). Potential recommendations proposed for the MCL of nitrate by investigators start at 20 ppm (L'hirondel et al., 2006) and increase up to 100 ppm (Powlson et al., 2008). Cornblath and Hartmann (1948) found that infants fed 100 mg/kg of the nitrate ion a day showed no evident signs of cyanosis, although infants who had previous been cyanotic did show apparent signs of cyanosis when fed 100 mg/kg of nitrate. Researchers suggest that further studies should be conducted to set a new standard for nitrate in drinking water and that a new recommendation should be created from science-based standards (Powlson et al., 2008).

Human Production of Nitrate/Nitrite

What the consumer does not realize is that nitrate and nitrite are not mysterious, harmful chemicals that humans solely ingest on a daily basis, but are actually also produced within the human body. Nitric oxide and other nitrogen oxides, products of

nitrite, are derived through two methods: the nitric oxide synthase (NOS) pathway and dietary consumption. The human body is able to produce its own supply of nitric oxide through the NOS pathway. Additional amounts of nitrate and nitrite are received through the ingestion of numerous dietary sources. A wide range of sources including drinking water, vegetables and some meats provide the body with either nitrate or nitrite or both. When nitrate is ingested, it is absorbed in blood plasma and subsequently secreted in saliva where bacteria reduce the nitrate to nitrite. The nitrite then re-circulates to the digestive system following secretion in saliva and is absorbed in the bloodstream.

Nitrate/Nitrite Sources in the Human Body

The predominant source of endogenous nitrate and nitrite is produced from the L-arginine-NOS pathway (Lundberg et al., 2011). Nitric oxide is produced from L-arginine as a normal function of the endothelium (Grisham & Bryan, 2010). Nitric oxide synthase enzymes catalyze an oxygen-dependent five-electron oxidation of the amino acid L-arginine to form nitric oxide and L-citrulline (Bryan & Murad, 2010). The five bound cofactors required by the NOS enzymes are flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4), calmodulin and heme iron (Bryan & Murad, 2010; Milkowski, 2011). A portion of the nitric oxide produced is then quickly oxidized by either superoxide or oxyhemoglobin to nitrate and enters the mammalian nitrate cycle (Gilchrist & Benjamin, 2011). Ingested nitrate along with nitrate derived from the NOS pathway can be taken up by the blood and excreted into the saliva and converted into nitrite (Gilchrist & Benjamin, 2011) by bacterial reduction. Mammals have three NOS isoforms, which contribute to host defense, neuronal and signaling pathways. The three NOS isoforms that can be found in mammals are inducible,

neuronal, and endothelial. Nitric oxide can be released for short periods of time in response to a receptor and physical stimulation, or can be released for long periods of time by activation of macrophages, endothelial cells, endotoxin and pro-inflammatory cytokines (Bryan & Murad, 2010).

Nitrate/Nitrite Sources in the Human Diet

Humans consume nitrate and nitrite through water, vegetables, and cured meats. Exogenous sources contribute about 95% of alimentary nitrate, with 80% of nitrate intake attributed to vegetables, and another 10 to 15% attributed to water (Archer, 2002), thus making cured meats a very small contributor. Vegetables, specifically celery, beets, spinach and collard greens contain some of the highest concentrations of naturally occurring nitrate in food (Sindelar & Milkowski, 2012). Keeton et al. (2009) investigated nitrate and nitrite concentrations (mg/kg) of vegetables and cured meat products available in retail. Investigators determined that the mean concentration of nitrate in conventional and organic celery to be 1495 ppm and 912 ppm, respectively, which was significantly greater than the amount found in conventional and organic cured cooked sausage, 32 ppm and 18 ppm, respectively. Because nitrate is reduced to nitrite by bacteria in the oral cavity, human saliva accounts for 93% of the total daily ingestion of nitrite (Sindelar & Milkowski, 2012). If nitrate was actually harmful to human health, it seems unlikely that human physiology would have evolved to purposefully concentrate nitrate by a natural biological process, and to secret nitrate in saliva, followed by bacterial reduction and chemical recycling as nitrite (McKnight et al., 1999).

Enterosalivary Circulation of Nitrate/Nitrite/Nitric Oxide

Once nitrate-containing foodstuffs are ingested, nitrate is rapidly absorbed in the

small intestine and enters the bloodstream. A maximum of 25% of circulating nitrate is

actively concentrated in saliva and secreted by the salivary glands (Lundberg &

Weitzberg, 2010). Commensal oral facultative anaerobic bacteria, primarily of the

Veillonella spp. (Doel et al., 2005), existing in the posterior crypts of the tongue, reduce

the nitrate secreted in the saliva to nitrite by the action of nitrate reductase enzymes

(Shiva & Gladwin, 2010). These oral bacteria use nitrate as an alternative electron

acceptor to gain adenosine triphosphate (ATP) in the absence of oxygen (Lundberg &

Weitzberg, 2010; Lundberg et al., 2011). Nitrate-enriched saliva appears to sustain the

survival and growth of bacteria that have the ability to respire nitrate (Doel et al., 2005).

This seems to represent a symbiotic relationship between bacteria and host (Doel et al.,

2005); the bacteria receive a substrate (nitrate), from the host, necessary for its own

respiration and in return produce nitrite, a substrate used for production of nitric oxide

(Lundberg & Weitzberg, 2010; Lundberg et al., 2011). After secretion of nitrate with the

saliva, the levels of salivary nitrate are 10- to 20- fold higher than in plasma (Lundberg &

Weitzberg, 2010). Approximately 20% of the nitrate in the saliva is reduced to nitrite

(Spiegelhalder et al., 1976), and the nitrite released raises the salivary nitrite levels to

1,000 times that of plasma in the resting state (Gilchrist & Benjamin, 2011). The salivary

nitrate and nitrite are then swallowed where they reach the acidic environment of the

stomach. When the saliva contacts the gastric juices, a majority of the nitrite is protonated

to form nitrous acid (HNO_2) (Lundberg & Weitzberg, 2010; Lundberg et al., 2011).

Nitrous acid then decomposes to nitric oxide and a variety of other nitrogen oxides

(Lundberg et al., 2011). Any residual nitrate and nitrite remaining is absorbed in the small intestine (Lundberg et al., 2008). A large portion, about 65 to 70% of the plasma nitrate, is lost to urinary excretion (Archer, 2002), but the remaining 25% of blood plasma nitrate will enter the enterosalivary cycle (Lundberg et al., 2011). While nitrate is in the blood plasma, it has a half-life of 5 to 8 hours and circulates evenly throughout the tissues (Lundberg et al., 2011).

Health Benefits of Nitrate/Nitrite Consumption

Robert Furchgott, Louis Ignarro and Ferrid Murad won the 1998 Nobel Prize in Physiology or Medicine for first recognizing nitric oxide as a signaling molecule in the cardiovascular system. It is currently understood that nitric oxide is mandatory in every organ system in the body and is responsible for regulating many different physiological effects including smooth muscle relaxation, neurotransmission, wound healing and immune response (Sindelar & Milkowski, 2011). Maintaining nitric oxide homeostasis is essential for ideal human health. The ability to produce nitric oxide reduces the risk of hypertension, atherosclerosis, peripheral artery disease, heart failure, and thrombosis leading to heart attack and stroke, the number one cause of death for all Americans (Parthasarathy & Bryan, 2012).

Cardiovascular Health

Nitric oxide provides cardiovascular health effects due to nitric oxide's role as a physiological signaling molecule. Nitrite, as a source of nitric oxide, may play several roles during ischemia or hypoxia, such as reducing to nitric oxide to dilate blood vessels, preventing irreversible oxidation, and the ability to inhibit mitochondrial respiration

(Bryan, 2006). It seems likely that nitric oxide contributes to cardioprotective effects of exercise by decreasing cardiovascular risk and injury in myocardial ischemia (Calvert, 2010). A portion of nitrite forms nitric oxide under hypoxic conditions as well as S-nitrosothiols (RSNOs) under normoxic conditions (Bryan, 2009). Nitric oxide activates soluble guanylate cyclase in smooth muscle or diffuses into the lumen of the blood vessels (Shiva & Gladwin, 2010). The activation of soluble guanylate cyclase leads to vasodilation through the production of cyclic guanosine monophosphate (cGMP) and guanosine triphosphate (GTP) (Kingwell, 2000). It is thought that nitric oxide elicits its primary cell signaling events through cGMP (Bryan, 2009). If diffused into the lumen, nitric oxide is inactivated and forms nitrate in a dioxygenase reaction with hemoglobin or forms nitrite through oxidation by the plasma protein ceruloplasmin (Shiva & Gladwin, 2010). In addition to activating soluble guanylate cyclase, nitrite may also have the potential to generate nitric oxide from the formation of iron nitrosyl complexes with other proteins to mediate signaling (Shiva & Gladwin, 2010). For example, mitochondrial respiration is regulated when nitric oxide binds $heme_{aa3}$ on cytochrome c oxidase and nitrite can alternatively mediate signaling through S-nitrosation (Shiva & Gladwin, 2010). After these reactions take place, vasodilation can be mediated and cytoprotective effects can occur in the setting of ischemia/reperfusion. Consequently, dietary sources of nitric oxide metabolites (nitrate and nitrite) could improve blood circulation and oxygen delivery thus providing a protective pathway for people at risk for cardiovascular disease (Bryan, 2006).

Improvements in cardiovascular health can be achieved by maintaining sufficient levels of nitric oxide. Studies have shown that nitric oxide has the ability to reduce blood

pressure. Webb et al. (2008) investigated the use of beetroot juice as a potential treatment of cardiovascular disease. The naturally occurring nitrate in beetroot juice was used as a source of nitric oxide to study nitric oxide benefits. This study measured the blood pressure of healthy volunteers over 24 hours after the consumption of 500 mL of beetroot juice or water. Peak differences in both systolic and diastolic blood pressure observed at 2.5 to 3 hours after ingestion of beetroot juice, and both measures of blood pressure decreased by about 10 mm Hg. The participants who consumed the beetroot juice showed a significant decrease in systolic blood pressure for up to 24 hours, while diastolic blood pressure was not significantly different from the control at 24 hours. A separate study by Liu et al. (2013) evaluated a nitrate-rich meal containing spinach for effects on blood pressure. Healthy participants consumed either a control diet or a high-nitrate diet containing 220 mg of nitrate derived from spinach. Those who consumed the high-nitrate diet had an overall lower pulse pressure ($P < 0.001$) and an overall lower systolic blood pressure ($P < 0.001$) when compared to those who consumed the control diet.

Kapil et al. (2013) explored the importance of nitrite, produced orally, on human blood pressure. The investigators used a chlorhexidine-based antiseptic mouthwash to disrupt oral nitrate reduction in healthy volunteers. Investigators measured participants' salivary nitrite concentrations, plasma nitrite concentrations, urinary nitrite concentrations, systolic blood pressure and diastolic blood pressure. During this study the volunteers were measured for a seven-day control period with no lifestyle changes and then measured for a following seven-day treatment period while using antiseptic mouthwash twice a day. The use of the antiseptic mouthwash almost eliminated oral conversion of nitrate to nitrite. Participants showed a reduction in oral nitrite production

by 90% as well as a reduction in plasma nitrite levels by 25%. After seven days' use of antiseptic mouthwash, there was an increase in volunteer's systolic and diastolic blood pressure according to the measurements acquired at the clinic. Systolic blood pressure was increased by 3.5 ± 1.0 mm Hg, and diastolic blood pressure was increased by 2.2 ± 1.0 mm Hg. A Prospective Studies Collaboration (2002) of 61 studies claimed that a 2 mm Hg decrease in systolic blood pressure would result in 10% lower stroke mortality. Therefore, this 3.5 ± 1.0 mm Hg increase might increase mortality. This study demonstrates that oral nitrate-reducing bacteria play a role in blood pressure regulation.

Athletic Performance

The production of nitric oxide has the potential to increase athletic performance. Matching tissue oxygen and substrate supply to demand in the event of physical activity is regulated by both blood delivery and the capacity of cells to extract these substrates (Kingwell, 2000). Nitric oxide is involved in both of these processes. Vascular shear stress is a stimulus for the release of nitric oxide from endothelial cells, which induces vasodilation of arteries (Kingwell, 2000). Nitric oxide is then able to diffuse to vascular smooth muscle where its production of cGMP and GTP by the activation of guanylate cyclase leads to further vasodilation (Kingwell, 2000).

A study conducted by Murphy et al. (2012) researched the running velocity of healthy individuals after the consumption of whole beetroot. Volunteers consumed 200 g portions of beetroot containing 500 mg or more nitrate. In order to measure running velocity, participants ran a 5 km distance 75 minutes after beetroot or placebo ingestion. The investigators determined that the running velocity was marginally faster after

beetroot consumption when compared to the placebo. Volunteers obtained a 5% faster running velocity during the last mile of the run with the prior consumption of beetroot. Lansley et al. (2011) saw similar results when examining dietary nitrate supplementation on trained cyclists. Cyclists consumed a half-liter of beetroot juice containing 6.2 mmol of nitrate two and a half hours before cycling. Investigators concluded that the beetroot juice improved a 4 km race performance by 2.8%.

Nitric Oxide Homeostasis

As the human body ages, nitric oxide production and homeostasis becomes a challenge. Young and healthy adults have the ability to efficiently produce nitric oxide from the amino acid L-arginine; however, aging adults experience a reduced ability to synthesize endothelial-derived nitric oxide (Bryan & Loscalzo, 2011). Depletion of L-arginine combined with a diet low in nitrate/nitrite creates a condition where nitric oxide synthase is insufficient and the body is unable to maintain homeostasis and cardiovascular disease ensues (Bryan, 2006). Egashira and others (1993) conducted a study that investigated the effects of age on endothelium-dependent vasodilation of resistance coronary arteries. Eighteen healthy patients ($23 - 70$ years old) were used to observe coronary blood flow response to acetylcholine (an endothelium-dependent vasodilator) and papaverine (an endothelium-independent vasodilator). The findings of this study showed that there was a modest change in blood flow response to papaverine with increasing age, whereas the blood flow response to acetylcholine decreased significantly with aging. Since papaverine decrease was not significantly associated with aging, the concept that age is an independent factor causing the weakening of endothelium-dependent vasodilation can be supported. Observations from this study and

others provided the conclusion that the reduced availability of endothelium-derived nitric oxide occurs with aging and may lead to increased risks of cardiovascular disease in the older population (Bryan & Loscalzo, 2011).

Since research has provided knowledge of the importance of maintaining nitric oxide homeostasis, perhaps this offers the ability to prevent or at least reduce diseases that occur with aging (Bryan & Loscalzo, 2011). Regular exercise and a diet high in nitrate/nitrite can enhance nitric oxide levels. Although exercise has been shown to enhance endothelial production of L-arginine, nothing will help increase nitric oxide more than dietary choices (Bryan & Loscalzo, 2011). Dietary nitrate/nitrite can account for half of steady state nitric oxide concentrations (Hord et al., 2009). The US National Institutes of Health developed the Dietary Approaches Stop Hypertension (DASH) diet to lower blood pressure without the use of medication (Bryan & Loscalzo, 2011). This diet is composed of fruits, vegetables, and low-fat dairy foods. Hord and others (2009) found that two hypothetical vegetable and fruit consumption patterns based on the DASH diet would exceed the World Health Organization's Acceptable Daily Intake for nitrate by 550% for a 60-kg adult. It is ironic to consider that a diet, which exceeds recommended nitrate consumption by more than 500%, is considered beneficial to overall health, but yet processed meats or water containing more than 10 ppm nitrate-N is considered detrimental to human health.

Nitrate in Today's Human Physiology and Medicine

Aside from dietary consumption, there are currently several products on today's market that are directly linked to nitric oxide: nitroglycerin, inhaled nitric oxide therapy,

sildenafil (Bryan & Loscalzo, 2011), and Neogenis Laboratory Inc.'s line of nitric oxide

supplements (Neogenis Laboratories, 2015). The use of nitroglycerin started well before

the discovery of nitric oxide and has been used to treat patients with cardiovascular

diseases such as acute angina (Bloch et al., 2011). Inhaled nitric oxide is used for the

treatment of newborns with persistent pulmonary hypertension and bronchopulmonary

dysplasia (Bloch et al., 2011). Sildenafil (Viagra®) is a phosphodiesterase inhibitor

(Bryan & Loscalzo, 2011) that is used to treat erectile dysfunction. Lastly, Neogenis

Laboratory's line of supplements include Neo40® daily, SuperBeets® and BeetElite

NeoShot®. All three products claim to enhance the body's daily nitric oxide levels and

improve overall health and/or athletic performance.

Potential for Health Benefits of Nitrate Addition in Processed Meats

The current literature suggests that supplementing cured meat with additional

nitrate could contribute to nutritional sources of nitric oxide. This proposes the possibility

that someday a processed meat product could be perceived to have health effects similar

to spinach. Incorporating nitrate with nitrite in the meat system could provide consumers

with a dietary source of nitric oxide. The addition of nitrate has potential to benefit the

consumer with increased nitric oxide levels after ingestion.

Nitrate, considered essentially inert in cooked meat, has been shown to act as a

dietary substrate for systemic nitric oxide formation (Lundberg & Weitzberg, 2010). This

is seemingly contradictory to reports of what occurs in meat curing where cured meat

products produce relatively little nitrite from nitrate, and thus, the production of nitric

oxide from nitrate is unlikely in cured meat (Honikel, 2008). Honikel's report, however

does not consider human physiology where nitrate can be considered bioactive when absorbed, secreted in saliva and reduced to nitrite by oral bacteria (Lundberg & Weitzberg, 2010). A study conducted by Wierbicki and Heiligman (1973) demonstrated the effects of added nitrate and nitrite levels on residual amounts of these curing agents in hams that were either irradiated or non-irradiated. This study was originally conducted to investigate if irradiation with a reduced amount of sodium nitrite, in combination with sodium nitrate, could prevent nitrosamine formation and still provide bacterial safety. The results of this study showed that non-irradiated ham cured with 25 ppm sodium nitrite in combination with 100 ppm sodium nitrate had a residual nitrate level of 145 ppm after 10 days of storage, and a level of 169 ppm after 90 days of storage. These observations indicate that residual nitrate concentrations were unchanged and remained high or slightly increased when compared to the ingoing nitrate concentrations. It is also noteworthy that residual nitrite concentrations remained relatively low at 1.1 ppm and 2.3 ppm after 10 and 90 days of storage, respectively. Because nitrate remains relatively unchanged in cooked meat products, it is an excellent potential source of dietary nitrate and a reservoir for nitric oxide. Additional studies have also confirmed constant residual nitrate levels when compared to ingoing nitrate levels (Shults et al., 1997; Honikel, 2008). It is nitrate's enterosalivary conversion to nitrite and subsequently nitric oxide, which associates nitrate with possible health benefits. In order for nitrate in meat to act as a supplement for nitric oxide, the individual must possess the oral commensal bacteria to effectively reduce nitrate to nitrite and subsequently produce nitric oxide.

Kapil et al. (2014) suggested that inorganic and dietary nitrate is more advantageous than nitrite as a source of nitric oxide. Nitrate is a more stable anion and

has a 5 to 8 hour half-life in the blood plasma (Lundberg et al., 2011), which is far greater

than nitrite's half-life of 110 seconds (Bryan & Lancaster, 2011). Therefore, Kapil et al.

(2014) speculated that nitrate could be given as a once-daily dose regime. After nitrate

ingestion, the rise in plasma nitrite levels is slow and sustained for 6 hours and then

remains slightly elevated for 24 hours after one dose (Kapil et al., 2014). This viable dose

strategy could be effective in hypertensive patients, although further research of this idea

may be needed for improved compliance in the clinical setting (Kapil et al., 2014). Bryan

(2010) suggested that nitrate/nitrite could be viewed as a vitamin, which is in support of

Kapil et al. (2014) proposing nitrate as a daily dose regime. Nitrate/nitrite falls under the

definition of a vitamin because small amounts are produced in normal metabolism by L-

arginine oxidation and minute amounts are found in food sources, and if one does not

consume sufficient amounts of nitrite/nitrate-rich foods, specific health disorders such as

cardiovascular disease are likely to occur (Bryan, 2010). If processed meats were to

provide additional dietary nitrate, it is possible that cured meat could supply the public

with an increased dietary nitrate dose and help to protect the body from the development

of cardiovascular disease. While unlikely to become a widely adopted practice,

information about the potential effects of supplementary nitrate in cured meat may

facilitate improvement in the perceptions consumers currently hold relative to cured meat

in the diet.

Summary

Sodium nitrite is a one-of-a-kind ingredient that provides numerous contributions

to cured meat systems such as cured color, flavor, antioxidant, and antimicrobial

properties. Incorporating nitrate, in combination with nitrite, in a cooked, cured meat

product can be expected to result in all the typical characteristics of a product that was cured with nitrite alone since nitrate in cooked meat is typically inert. However, the addition of nitrate to cured meat products would provide an additional dietary source of nitrate, which could ultimately lead to increased nitric oxide production *in vivo* and reduced risk of cardiovascular diseases, such as heart attack and stroke. While nitrate in cooked meat is considered largely inert, it is not clear how supplemental nitrate at concentrations above normal amounts might affect product characteristics. Therefore, the objective of this study was to investigate the physical, chemical and microbial effects of supplemental nitrate in cured meat products, when manufactured to contain 220 mg or more nitrate per 112 g serving of cured meat. It was hypothesized that addition of supplemental nitrate to cured meat will not alter cured meat quality or microbiological properties, and thus demonstrate that cured meat could serve as a viable dietary source of nitrate and subsequently, nitric oxide.

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CHAPTER 3. THE PHYSICAL, CHEMICAL AND MICRIOBIAL EFFECTS OF SUPPLEMENTAL SODIUM NITRATE ON CURED MEAT PRODUCTS

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Abstract

The effects of supplemental nitrate from celery juice powder on residual nitrite, residual nitrate, rancidity, microbial growth, color, sensory properties, and proximate composition of frankfurters, cotto salami and boneless ham during storage ($32 - 34°F$) were studied. The products were assigned one of two treatments, which were replicated twice: control (156 ppm sodium nitrite) or supplemental nitrate (156 ppm sodium nitrite, 1718 ppm sodium nitrate, in combination with 2% VegStable 502). Sensory parameters and proximate composition were measured once for each replication. All other analytical measurements were conducted at regular intervals for 98 days. The supplemental nitrate had no significant increase ($P > 0.05$) on residual nitrite. No changes ($P > 0.05$) were observed in residual nitrate concentrations during storage for any of the products. The results showed that addition of supplemental nitrate did not significantly alter most physical, chemical or microbial properties of cured meat products during refrigerated storage, but some product-dependent sensory effects were observed.

Keywords: sodium nitrate, sodium nitrite, cured meat, residual nitrite, residual nitrate

Introduction

Sodium nitrate and sodium nitrite have been used in meat products as curing agents and preservatives for centuries. Sodium nitrite is used in cured meats because it provides these products with improved quality characteristics such as color, shelf life, and flavor. The addition of sodium nitrite also contributes to meat safety by reducing the potential for outgrowth of several microbial pathogens including *C. botulinum* and *L. monocytogenes* in cured meat products (Cammack et al., 1999; Sebranek & Bacus, 2007). Sodium nitrite provides these benefits to cured meat by undergoing reactions within the meat system to form nitric oxide. Nitric oxide production from nitrite is the necessary step to achieve cured characteristics (Sebranek, 2009).

While cured meats contribute a very small portion of human dietary intake of nitrate and nitrite, the human body derives nitrate and nitrite through two methods: endogenously through the nitric oxide synthase (NOS) pathway and exogenously through dietary consumption. In the diet, nitrate and nitrite can be found in vegetables, water and some meats (Archer, 2002). The ingestion of nitrate from food leads to the conversion of nitrate to nitrite through bacteria in the mouth and subsequently to nitric oxide. Consequently, once a product containing nitrite or nitrate is ingested, the body's nitric oxide levels have been shown to increase as a result, provided the ingested amount is sufficient (Lundberg & Weitzberg, 2010). The discovery of nitric oxide, along with follow-up research, has made it clear that nitric oxide is one of the most important signaling molecules in the human body for regulation of physiological functions such as blood flow to the tissues and organs (Bryan, 2009).

The ability to form nitric oxide and the important physiological role of this molecule, has established a new context for nitrate and nitrite. Incorporating supplemental nitrate with nitrite in the meat system has potential to provide consumers with a meat product that could have a physiological impact similar to leafy green vegetables. Manufacturing a product that contains nitrate in combination with nitrite will allow a typical curing reaction by nitrite to provide the necessary cured meat characteristics. However, because nitrate is inert in cooked meat (Honikel, 2008; Sebranek, 2009), it is not typically depleted in a cooked meat system during storage and distribution and, thus, can act as a source of dietary nitrate. This dietary source of nitrate could ultimately lead to increased nitric oxide production *in vivo* and reduce the risk of cardiovascular diseases, such as heart attack and stroke (Bryan, 2006). However, current perceptions of nitrate and nitrite regarding consumer health and safety will need to be overcome before the use of supplemental nitrate in food can fully be accepted. This study was initiated to test the hypothesis that the addition of supplemental nitrate to cured meat, utilizing celery powder to achieve a nitrate concentration that could potentially impact nitric oxide concentrations in consumers, will introduce no significant changes in meat product quality or microbial characteristics.

Materials and Methods

Experimental Design

The experimental design consisted of two treatments of three different products, each of which were replicated twice. A control and a supplemental nitrate treatment were manufactured using boneless ham, cotto salami and frankfurter products. All products were manufactured in the Iowa State Meat Laboratory under USDA inspection. A.C.

Legg (A.C. Legg, Inc., Calera, AL, U.S.A) provided spices, and celery powder (VegStable 502) was provided by Florida Food Products, Inc. (Florida Food Products, Inc., Eustis, FL, U.S.A.) to be used as a supplemental nitrate source. The supplemental treatment products were formulated to achieve a target of 220 mg or more nitrate per 112 g serving (1964 ppm) of cured meat. This concentration was achieved by including sodium nitrate at 1718 ppm as permitted by the USDA as well as including 2% celery powder containing 30,000 ppm nitrate according to the supplier. The use of sodium nitrate and nitrite are permitted to be used together in a single curing method and each one is permitted to be used up to the maximum individual limits (USDA, 1995). However, the combination must not result in more than 200 ppm of nitrite, calculated as sodium nitrite, in the finished product (USDA, 1995). For the supplemental nitrate products, potassium chloride was substituted for 8% of the sodium chloride used in the control treatment to compensate for the additional sodium content of the sodium nitrate and to keep the sodium content of the two treatments similar. A dietary nitrate concentration of 220 mg or more has been reported by several authors to achieve a reduction of blood pressure in human subjects (Bryan, 2009; Liu et al., 2013). Two percent celery powder is a higher concentration than typically used in processed meats, but was chosen to achieve the desired amount of added nitrate.

Product Manufacturing

The ingredients and the processing steps for the products are as follows:

Table 1. Control Ham Treatment

Ingredients per batch	% of Meat Block	Weight (lbs)
Boneless ham	100	50
Water/Ice	20	10
Salt	2.2	1.1
Phosphate	0.28	0.14
Sugar	1.3	0.66
Modern Cure (6.25% nitrite)	0.25 (156 ppm sodium nitrite)	0.125
Sodium Erythorbate	0.05 (547 ppm)	0.0275

Table 2. Supplemental Nitrate Ham Treatment

Ingredients per batch	% of Meat Block	Weight (lbs)
Boneless ham	100	50
Water/Ice	20	10
Salt	2.0	1.01
Phosphate	0.28	0.14
Sugar	1.3	0.66
Modern Cure (6.25% nitrite)	0.25 (156 ppm sodium nitrite)	0.125
Sodium Erythorbate	0.05 (547 ppm)	0.0275
VegStable 502	2.0 (600 ppm nitrate)	1
Sodium Nitrate	0.17 (1718 ppm sodium nitrate)	0.088
Potassium Chloride	0.17	0.088

Boneless ham was obtained from and processed in the Iowa State Meat Laboratory. Control and supplemental nitrate ham treatments and both replications were processed separately, but in the same manner. Boneless ham was ground through a Biro® grinder (Model 7.5 424852, The Biro® Manufacturing Co., Marblehead, OH, U.S.A) fitted with a 3/8" plate. The ground ham was mixed with water/ice and spice ingredients in a Higashimoto Kikai paddle mixer (Model 90.3.3, Nava, Japan) for five minutes. The mixture was ground a second time in the Biro® grinder with a ¼" grinder plate. The

product was loaded into a vacuum filler (RS 1040C, Risco U.S.A. Corp., South Eaton, MA, U.S.A) and stuffed into 9x26" clear, fibrous casings (Kalle, Gurnee, IL, U.S.A). The stuffed ham was hung on a smoke truck and placed into a Maurer (Maurer AG, Reichenau, Germany) oven with a natural smoke generator (Raucherzeuger Goliath 11, Reichenau, Germany), for thermal processing (Table 3). After cooking was completed, the products were cooled and stored at 1°C (32 − 34°F) overnight. Ham was sliced the following day using a Bizerba slicer (Model No. 10191442, Piscataway, NJ, U.S.A) and vacuum packaged into half-pound packages using high barrier bags (Cryovac Sealed Air Corporation, 6x12, Duncan, SC, U.S.A) with an oxygen transmission rate of 3 − 6 cc at 73°F (m^2, 24 hrs atm @ 73°F, 0 RH) and a water vapor transmission rate of 0.5 − 0.6 g at 100°F (100% RH, 100 in^2, 24 hr), with a Ultravac Model UV 2100 packaging machine (Koch, Kansas City, MO, U.S.A). All ham treatments were stored at 1°C (32 − 34°F).

Table 3. Thermal Processing for Control and Supplemental Nitrate Ham Treatments

Step	Step Time	Dry Bulb Temperature (°F)	Wet Bulb Temperature (°F)	% Relative Humidity	Internal Temperature (°F)	Main Blower	Exhaust Damper
Cook	0:40	165	0	0		8	Auto
Cook	0:30	170	0	0		8	Auto
Smoke Cook	0:45	175	0	0		6	Closed
Smoke Cook	1:00	175	161	71		8	Closed
Cook	0:01	180	160	62	140	10	Auto
Steam Cook	0:01	185	185	100	160	10	Closed
Cold Shower	0:10	50	50	0		0	Auto

Table 4. Control Cotto Salami Treatment

Ingredients per batch	% of Meat Block	Weight (lbs)
Beef 90s	60	30
Pork 50s	40	20
Water/Ice	30	15
Caseinate	3.5	1.75
Salt	2.7	1.37
Garlic	0.09	0.047
Black Pepper	0.25	0.125
Cracked Black Pepper	0.19	0.095
Cardamom	0.12	0.06
Modern Cure (6.25% nitrite)	0.25 (156 ppm sodium nitrite)	0.125
Sodium Erythorbate	0.05 (547 ppm)	0.0275

Table 5. Supplemental Nitrate Cotto Salami Treatment

Ingredients per batch	% of Meat Block	Weight (lbs)
Beef 90s	60	30
Pork 50s	40	20
Water /Ice	30	15
Caseinate	3.5	1.75
Salt	2.5	1.26
Garlic	0.09	0.047
Black Pepper	0.25	0.125
Cracked Black Pepper	0.19	0.095
Cardamom	0.12	0.06
Sodium Erythorbate	0.05 (547 ppm)	0.0275
Modern Cure (6.25% nitrite)	0.25 (156 ppm sodium nitrite)	0.125
VegStable 502	2.0 (600 ppm nitrate)	1
Sodium Nitrate	0.17 (1718 ppm sodium nitrate)	0.088
Potassium Chloride	0.22	0.1096

Beef and pork were obtained from and processed in the Iowa State Meat

Laboratory. Control and supplemental nitrate treatments for both replications of the cotto

salami were processed separately, similar to the hams. Beef and pork were ground

separately through a Biro® grinder (Model 7.5 424852, The Biro® Manufacturing Co.,

Marblehead, OH, U.S.A) fitted with a 3/8" plate. The ground beef was mixed with the

salt and half of the water/ice in a Higashimoto Kikai paddle mixer (Model 90.3.3, Nava,

Japan) for five minutes. The pork and the rest of the water and spice ingredients were

added to the mixer and mixed for five minutes. The mixture was then ground a second

time in the Biro® grinder with a ¼" grinder plate, loaded into a vacuum filler (RS 1040C,

Risco U.S.A Corp., South Eaton, MA, U.S.A) and stuffed into 9x26" clear, fibrous

casings (Kalle, Gurnee, IL, U.S.A). The stuffed salami was hung on a smoke truck and

placed into a Maurer (Maurer AG, Reichenau, Germany) oven with a natural smoke

generator (Raucherzeuger Goliath 11, Reichenau, Germany) for thermal processing

(Table 6). After cooking was completed, the products were chilled in a 1°C (32 − 34°F)

cooler overnight. Salami was then sliced using a Bizerba slicer (Model No. 10191442,

Piscataway, NJ, U.S.A) and vacuum packaged into half-pound packages using the same

pouches and machines as for the hams. Salami treatments were stored at 1°C (32 − 34°F)

for the remainder of the study.

Table 6. Thermal Processing for Control and Supplemental Nitrate Cotto Salami
Treatments

Step	Step Time	Dry Bulb Temperature (°F)	Wet Bulb Temperature (°F)	% Relative Humidity	Internal Temperature (°F)	Main Blower	Exhaust Damper
Cook	1:00	100	89	65		8	Auto
Cook	0:45	130	104	42		8	Closed
Cook	0:45	150	115	34		8	Closed
Smoke Cook	1:00	176	150	52		6	Auto
Cook	0:01	176	158	64	140	10	Auto
Cook	0:01	185	176	81	164	10	Closed
Cold Shower	0:20	50	0	0		0	Auto

Table 7. Control Frankfurter Treatment

Ingredients per batch	% of Meat Block	Weight (lbs)
Beef 90s	50	25
Pork 50s	50	25
Water/ice	30	15
Salt	2.0	1
Modern Cure (6.25% nitrite)	0.25 (156 ppm sodium nitrite)	0.125
Phosphate	0.5	0.25
White Pepper	0.21	0.105
Onion Powder	0.28	0.14
Garlic Powder	0.14	0.07
Frankfurter Seasoning (Contains 547 ppm Sodium Erythorbate)	3.0	1.5

Table 8. Supplemental Nitrate Frankfurter Treatment

Ingredients per batch	% of Meat Block	Weight (lbs)
Beef 90s	50	25
Pork 50s	50	25
Water/ice	30	15
Salt	1.8	0.92
Modern Cure (6.25% nitrite)	0.25 (156 ppm sodium nitrite)	0.125
Phosphate	0.5	0.25
White Pepper	0.21	0.105
Onion Powder	0.28	0.14
Garlic Powder	0.14	0.07
Frankfurter Seasoning (Contains 547 ppm Sodium Erythorbate)	3.0	1.5
Potassium Chloride	0.16	0.08
VegStable 502	2.0 (600 ppm nitrate)	1
Sodium Nitrate	0.17 (1718 ppm sodium nitrate)	0.088

Beef and pork were obtained from and processed in the Iowa State Meat

Laboratory. Both replications of the control and supplemental nitrate frankfurter

treatments were processed separately, similar to the hams and cotto salami. Beef and pork

were first ground separately through a Biro® grinder (Model 7.5 424852, The Biro®

Manufacturing Co., Marblehead, OH,U.S.A) fitted with a 3/8" plate. The ground beef

was then added with the salt, cure and half of the water/ice to a vacuum bowl chopper

(Model VSM65 Kramer, and Grebe GmbH and Co., KG, Biendenkopf-Wallau,

Germany). The beef was chopped with the salt, cure and water/ice until a homogenous

mixture was formed and a temperature of 43°F was reached. The pork and the rest of the

water/ice and spice ingredients were then added to the bowl chopper and chopped until

the emulsion reached 54°F. The product was then loaded into a vacuum filler (Model

4003-165, Risco U.S.A Corp.) and stuffed into size 27 mm diameter peelable cellulose

casings (Viscofan, Danville, IL, U.S.A). Each frankfurter was stuffed to a weight of 70

grams. The frankfurters were hung on a smoke truck and placed into a Maurer oven

(Maurer AG, Reichenau, Germany) with a natural smoke generator (Raucherzeuger

Goliath 11, Reichenau, Germany) for thermal processing (Table 9). After cooking was

completed, the products were cooled and stored at 1°C (32 − 34°F) overnight. Casings

were removed from the frankfurters before packaging using an automated peeler (Model

2600, Townsend Engineering, Des Moines, IA). Frankfurters were vacuum-packaged,

four links per package, using the same pouches and machine as for the hams and cotto

salami, and subsequently stored at 1°C (32 − 34°F).

Table 9. Thermal Processing for Control and Supplemental Nitrate Frankfurter Treatments

Step	Step Time	Dry Bulb Temperature (°F)	Wet Bulb Temperature (°F)	% Relative Humidity	Internal Temperature (°F)	Main Blower	Exhaust Damper
Cook	00:10	110	100	70	0	Low	Auto
Cook	00:15	145	0	0	0	High	Auto
Smoke Cook	00:30	145	138	82	0	High	Closed
Cook	00:20	155	0	0	0	High	Auto
Cook	00:30	175	136	36	0	High	Auto
Steam Cook	00:01	180	180	100	160	High	Closed
Cold Shower	00:15	50	0	0	0	Off	Auto

Residual Nitrite Analysis

Residual nitrite analysis was conducted using AOAC method 973.31 (AOAC, 2005e). Approximately 5 g of sample were stirred with hot distilled water, placed into a 500 mL volumetric flask and heated in a hot water bath at 100°C for 2 hours. Flasks were swirled every half hour while in the hot water bath. Flasks were removed from the hot water bath and cooled to room temperature. Contents of the flasks were filtered into 50 mL volumetric flasks. Sulfanilamide solution and N-(-1-Naphthyl) ethylenediamine dihydrochloride (NED) reagent were added to the flasks. Sample absorbance was read using a spectrophotometer at 540 nm (Model 4320940, DU 640, Beckman, Fullerton, CA, USA). Duplicate measurements were conducted for each sample on days 1, 6, 13, 27, 41, 55, 69, 83, and 97 after packaging.

Residual Nitrate Analysis

Hormel Laboratories (Hormel Foods, LLC, Austin, MN, U.S.A.) conducted residual nitrate testing using AOAC method 993.30 (AOAC, 2005c). Because residual nitrite also is measured as part of this method, both nitrate and nitrite concentrations were recorded. Samples were frozen at Iowa State University on days 1, 6, 13, 27, 41, 55, 69, 83, and 97 after packaging, and shipped overnight to Hormel for analysis. Once received by Hormel the samples were held at 0°F until 18 hours before evaluation and then were stored at 38°F. Testing was typically conducted within 1 to 2 weeks of sample arrival. Nitrate and nitrite were extracted from the meat samples with hot water and then analyzed using an ion chromatography system (Thermo Scientific Dionex ICS-1100, Thermo Fisher Scientific Inc., Sunnyvale, CA, U.S.A). Data was collected using Chromeleon 7 (Thermo Scientific Chromeleon 7, Thermo Fisher Scientific Inc., Sunnyvale, CA, U.S.A).

TBA Analysis

The 2-thiobarbituric procedure of Tarladgis et al. (1960) modified by Zipser and Watts (1962) was used to measure oxidative rancidity. Approximately 10 g of sample were weighed into a round bottom flask and attached to a distillation apparatus. Samples were boiled, in combination with 97.5 mL of distilled water, hydrogen chloride solution and sulfanilamide solution, until 50 mL of distillate was collected. Five mL of TBA reagent was added to 5 mL of sample distillate and placed into a boiling water bath for 35 minutes. After the samples cooled, measurements were read using a spectrophotometer at 532 nm and multiplied by a factor of 7.8 to achieve mg malonaldehyde per 1,000 g of

meat (Model 4320940, DU 640, Beckman, Fullerton, CA, USA). Duplicate

measurements of each sample were conducted on days 1, 14, 28, 42, 56, 70, 84, and 98

days after packaging.

Color Analysis

Color measurements (L, a, b) were conducted on the surface of the ham, cotto

salami and frankfurters using a Hunterlab LabScan instrument (Model LS 1500, Hunter

Associated Laboratories Inc., Reston, VA, U.S.A.) using illuminant D65 (daylight @

6500K) and 10° observer angle. In order to imitate retail packaging, instrument

calibrations were done by placing Saran wrap over the calibration plate. Ham and cotto

salami surface measurements were taken on the surface of sliced samples using a 1.00 cm

port insert. Frankfurters included an additional color measurement to include both

external surface and internal surface color. Frankfurters were cut in half length-wise and

wrapped with Saran wrap. External and internal frankfurter measurements were then

taken using a 0.25 cm port insert. A total of three random surface measurements were

collected for each product sample and averaged. Samples, designated for color analyses

(15 samples for each control and treatment), were constantly stored under fluorescent

lighting until day of analysis. Color measurements were conducted on days 1, 6, 13, 20,

27, 34, 41, 48, 55, 62, 69, 76, 83, 90, and 97 after packaging.

Total Plate Counts

Total plate counts were measured by first blending 10 g of sample in combination

with 90 mL peptone water (Hardy Diagnostics, Cat no. D290, Santa Maria, CA, U.S.A.)

in a stomacher bag (Whirl Pak, Jackson, WI, U.S.A.) using a lab blender (EasyMix, AES

Laboratories, France) for sixty seconds. One milliliter of each sample was plated on to

3M petrifilm (3M Health Care, St. Paul, MN, U.S.A.) containing peptone diluent

(Becton, Dickinson and Company, Sparks, MD, U.S.A.). The petrifilms were incubated

for 72 hours at 23°C and then counted (USDA, 2013). Total plate counts were conducted

on day 0, 7, 14, 30, 60, and 90 days after packaging. Day 0 samples were immediately

frozen following packaging then thawed and plated on day 8.

Lactic Acid Bacteria

Lactic acid bacteria were enumerated by first blending 10 g of sample in

combination with 90 mL peptone water (Hardy Diagnostics, Cat no. D290, Santa Maria,

CA, U.S.A.) in a stomacher bag (Whirl Pak, Jackson, WI, U.S.A.) using a lab blender

(EasyMix, AES Laboratories, France) for sixty seconds. One tenth of a milliliter of

sample containing peptone diluent (Becton, Dickinson and Company, Sparks, MD.,

U.S.A.) was plated on to 100 mm x 15 mm petri plates (Fisherbrand, Fisher Scientific,

Chicago, IL, U.S.A.) containing MRS (deMan, Rogosa and Sharpe) agar (Becton,

Dickinson and Company, Sparks, MD, U.S.A.). MRS agar was modified by adjusting the

pH to 5.5 with the incorporation of acetic acid and was prepared according to the

Compendium of Methods for the Microbiological Examination of Foods (APHA, 1992).

The petri plates were incubated for 72 hours at 23°C and then counted (USDA, 2013). If

no growth was observed, plates were incubated for an additional 72 hours at 23°C and

then counted. Lactic acid bacterial counts were conducted on day 0, 7, 14, 30, 60, and 90

days after packaging. Day 0 samples were immediately frozen following packaging, then

thawed and plated on day 8.

Sensory Analysis

Sensory evaluation of the products was conducted using a nine-member, trained panel. The panel was comprised of students, staff and faculty at Iowa State University. Three separate training sessions were held before evaluation of the first replication of each product and a brief re-training was conducted before panelists evaluated the second replication. Two sessions for evaluation of each replication were conducted to obtain sensory data. A three-digit code was randomly assigned to each sample of the products evaluated in each session. Panelists evaluated samples using a 15-cm line scale. Data was collected using Compusense five Release 5.6 sensory evaluation software. Panelists evaluated cured aroma, "other aroma," texture, cured flavor, "other flavor," saltiness and intensity of pink color for ham products (Figures 1 and 2). For cotto salami, panelists evaluated the same properties as for hams (cured aroma, "other aroma," texture, cured flavor, "other flavor," saltiness, and intensity of pink color), but also included spice intensity and intensity of light to dark color (Figures 3 and 4). For frankfurters, panelists again evaluated the same properties as for hams and cotto salami (cured aroma, "other aroma," texture, cured flavor, "other flavor," saltiness, and intensity of internal pink color), but in this case also included pepper intensity, intensity of brown surface color, and intensity of internal light to dark color (Figures 5 and 6). Panelists were asked to determine "other aroma" and "other flavor" as attributes that would be unusual from that typical product identity.

The ham and cotto salami slices were cut into one-inch square pieces for evaluation. The pieces were mixed in a large mixing bowl to ensure panelists would receive a random sampling of the small pieces from each product. Four to five pieces

were placed in a cup and covered with a lid. Ham and cotto salami treatments were held at room temperature for 5 minutes prior to being served. Frankfurters were heated in boiling water prior to evaluation. Frankfurters were placed in water, and the water was brought to a boil, after which the pot was removed from the heat and the frankfurters remained in the water for seven minutes. Frankfurters were then cut into one-inch pieces, and four to five pieces, each from a different frankfurter link from a given treatment or control, were placed into a cup and covered with a lid. In addition to the samples used for tasting, one whole slice of ham and cotto salami and one frankfurter link, were used for appearance and color evaluations by the panel. The frankfurter was cut lengthwise, one side was left intact and the other side was cut vertically into two pieces so panelists could view both external and internal color.

SENSORY EVALUATION OF HAM

Date_____　　　　**Sample _____**　　　　**Panelist_____**

Evaluate the sample and indicate the intensity of each attribute.

Cured Ham Aroma

_____
None　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　Intense

"Other Aroma" (If present)

_____
None　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　Intense

Describe the "Other Aroma" (If present)_____

Texture

_____
Soft　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　Firm

Cured Ham Flavor

_____
None　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　Intense

"Other Flavor" (If present)

_____
None　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　Intense

Describe the "Other Flavor" (If present)_____

Saltiness

_____
None　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　Intense

Figure 1. Sensory evaluation form for attributes of ham treatments

SENSORY EVALUATION OF HAM - APPEARANCE

Date_____   **Participant ID number:** _____

Code number of the <u>first</u> sample: _____

Evaluate the intensity of the SURFACE CURED HAM COLOR – (PINKNESS)

_____

None   Intense

Code number of the <u>second</u> sample: _____

Evaluate the intensity of the SURFACE CURED HAM COLOR – (PINKNESS)

_____

None   Intense

Figure 2. Sensory evaluation form for appearance characteristics of ham treatments

SENSORY EVALUATION OF SALAMI

Date_____   **Panelist_____**

Salami Aroma

_____
None   Intense

"Other Aroma" (If present)

_____
None   Intense

Describe the "Other Aroma" (If present)_____

Texture

_____
Soft   Firm

Salami Flavor

_____
None   Intense

"Other Flavor" (If present)

_____
None   Intense

Describe the "Other Flavor" (If present)_____

Spice Intensity

_____
None   Intense

Saltiness

_____
None   Intense

Figure 3. Sensory evaluation form for attributes of cotto salami treatments

SENSORY EVALUATION OF SALAMI - APPEARANCE

Date_____   **Participant ID number:** _____

Code number of the <u>first</u> sample: _____

Evaluate the intensity of the SALAMI COLOR

_____

No Pink   Intense Pink

_____

Light   Dark

Code number of the <u>second</u> sample: _____

Evaluate the intensity of the SALAMI COLOR

_____

No Pink   Intense Pink

_____

Light   Dark

Figure 4. Sensory evaluation form for appearance characteristics of cotto salami treatments

SENSORY EVALUATION OF FRANKFURTERS

Date_____   **Panelist**_____

Cured Frank Aroma

_____
None   Intense

"Other Aroma" (If present)

_____
None   Intense

Describe the "Other Aroma" (If present)_____

Texture

_____
Soft   Firm

Cured Frank Flavor

_____
None   Intense

"Other Flavor" (If present)

_____
None   Intense

Describe the "Other Flavor" (If present)_____

Pepper Intensity

_____
None   Intense

Saltiness

_____
None   Intense

Figure 5. Sensory evaluation form for attributes of frankfurter treatments

SENSORY EVALUATION OF FRANKFURTERS - COLOR

Date_____   **Participant ID number:** _____

Code number of the <u>first</u> sample: _____

Evaluate the intensity of the OUTSIDE SURFACE CURED FRANK COLOR – (BROWN)

_____

None   Intense

Evaluate the intensity of the INSIDE CURED FRANK COLOR

_____

No Pink   Intense Pink

_____

Light   Dark

Code number of the <u>second</u> sample: _____

Evaluate the intensity of the OUTSIDE SURFACE CURED FRANK COLOR – (BROWN)

_____

None   Intense

Evaluate the intensity of the INSIDE CURED FRANK COLOR

_____

No Pink   Intense Pink

_____

Light   Dark

Figure 6. Sensory evaluation form for appearance characteristics of frankfurter treatments

Salt Content

Sodium chloride content was determined using AOAC method 971.19 (AOAC, 2005f). Approximately 10 g of ground sample was stirred for 30 seconds into 90 mL of boiling water. Quantab titrator strips (27513 − 40, high range 300 − 6000 ppm Cl⁻, Hach, Loveland, CO, USA), used to indicate salt content, were placed into folded filter paper and administered into each sample solution once cooled to room temperature. Duplicate measurements were conducted for each sample. Salt content was measured once on day 6 after packaging.

Protein Content

Protein content was analyzed according to AOAC method 992.18 (AOAC, 2005a) to determine the percentage of nitrogen. One gram of ground sample of product was analyzed using a TruMac N (Leco Corporation, St. Joseph, MI, U.S.A). Duplicate measurements were conducted for each sample. Percent protein was calculated by multiplying the nitrogen percentage by 6.25. Protein content was measured once on day 6 after packaging to confirm product composition.

Moisture Content

Moisture content was analyzed according to AOAC method 950.46 (AOAC, 2005d). Approximately 5 g of ham and 4 g of cotto salami and frankfurter samples were weighed into cotton thimbles. Thimbles were placed in the drying oven for 18 hours at 100 − 102°C. Measurements were conducted with a VWR drying oven (Model 1370GM, Sheldon Manufacturing Inc., Cornelius, OR, U.S.A). After drying, thimbles were placed in a desiccator to cool and then weights were recorded. Duplicate measurements were

conducted for each sample. As for protein, moisture content was measured on day 6 after

packaging. Percent moisture was calculated as follows:

% Moisture = ([sample weight – dried weight] /sample weight))* 100

Fat Content

Fat content was analyzed according to AOAC method 960.39 (AOAC, 2005b).

Approximately 5 g of ham and 4 g of cotto salami and frankfurter samples were weighed

into cotton thimbles. Thimbles were first dried for 18 hours at $100 - 102°C$ in a VWR

drying oven (Model 1370GM., Sheldon Manufacturing Inc., Cornelius, OR, U.S.A) and

moisture content was determined as described above. After drying, thimbles were placed

in a desiccator to cool and then extracted with hexane for 7 hours using a Soxhlet multi-

unit extraction heater (Lab-Line Instruments, Inc., Melrose Park, IL, U.S.A). Duplicate

measurements were conducted for each sample. In conjunction with protein and moisture,

fat content was measured once on day 6 after packaging to confirm product composition.

Percent fat was calculated as follows:

% Fat = ([dried weight – extracted weight]/ sample weight))*100

Statistical Analysis

The experiment was replicated twice with separate production days for each of the

replications and all of the products were analyzed over a five-month period. Data was

statistically analyzed using PROC MIXED by the Statistical Analysis System (SAS,

v9.4). A p-value of 0.05 was used to establish least squares means. Total plate counts

were analyzed using WINKS SDA Software (Texasoft, Cedar Hill, TX). Log

transformation was used to report for exponential growth rates of bacteria. A p-value of 0.05 was used to establish least squares means.

Results and Discussion

Residual nitrite measurements for frankfurters, cotto salami and ham are presented in Figures 7 − 9, respectively. Frankfurter results for residual nitrite showed a significant difference ($P < 0.05$) between the control treatment and the supplemental nitrate treatment. Although the results were statistically different between the two treatments, the least squares means show a 3.95 ppm difference between the two treatments, which is equivalent to an average difference of approximately 4 ppm. This difference in the frankfurter treatments was consistent for both replications and may simply reflect a formulation effect. More importantly, the supplemental nitrate frankfurter treatment had a lower least squares means value than the control treatment indicating slightly less residual nitrite than the control. This was expected because nitrate is inert in cooked meat (Honikel, 2008), and therefore should not result in any additional residual nitrite levels compared with a similar product cured only with nitrite. The results showed no significant difference ($P > 0.05$) between the control treatment and the supplemental nitrate treatment when analyzing cotto salami and ham, independently. Further, the residual nitrite concentrations observed are very typical and similar to those reported by others (Sindelar et al., 2007; Redfield & Sullivan, 2015). Thus, again nitrate in combination with nitrite did not produce higher residual nitrite values than a similar product only containing nitrite. The results from both treatments within all of the three products showed a significant decline ($P < 0.05$) in residual nitrite during the storage time. This result was expected because as time in storage increases, residual nitrite is

typically depleted. The relationship amongst the depletion of residual nitrite and increased storage time is an observation reported by numerous investigators (Hustad et al., 1973; Sindelar et al., 2007; Redfield & Sullivan, 2015). An interaction assessment conducted between day and treatment showed no significant difference ($P > 0.05$) between the two treatments as storage time increased for frankfurters, cotto salami or ham. This indicates that the residual nitrite in both treatments depleted within each product at a similar rate.

Hormel Laboratories also conducted residual nitrite analyses in conjunction with nitrate analyses. However, these results showed a wide variation among storage times, probably due to the freeze-thaw treatments necessary for shipping the products to the Hormel Laboratory and the highly reactive, temperature sensitive nature of nitrite in meat. However, the Hormel results also showed no significant difference ($P > 0.05$) between treatments, and no significant difference ($P > 0.05$) for the interaction between day and treatment for all three products. Because of a wide variation in the residual nitrite results during storage, the Hormel data for frankfurters showed no significant decline ($P > 0.05$) in residual nitrite as storage time increased, whereas cotto salami and ham both showed a significant decline ($P < 0.05$) in residual nitrite as storage time increased. The variation in the residual nitrite results from Hormel is most likely due to the freezing of samples for shipping followed by thawing prior to analysis coupled with the affinity of nitrite for reactions with meat components. Consequently, we have focused on the residual nitrite results obtained in our laboratory for our discussion of the results of this study.

Residual nitrate measurements, conducted by Hormel Laboratories, for frankfurters, cotto salami and ham are shown in Figures $10 - 12$, respectively and were consistent and unchanged over time, again confirming the inert nature of nitrate in a cooked meat mixture. Results also clearly show a significant difference ($P < 0.0001$) between the control treatment and the supplemental nitrate treatment for all three products. The difference between treatments was expected due to the addition of nitrate in the supplemental nitrate treatment and clearly shows that nitrate is not converted to nitrite in a cooked meat product. The presence of nitrate, approximately $37 - 75$ ppm, was observed in the control treatments to which only nitrite was added. Pérez-Rodríguez et al. (1996), who reported similar findings while investigating sodium nitrite and potassium nitrate in frankfurters, found that approximately 50% of added nitrite could be detected as residual nitrite and $10 - 15\%$ was detected as nitrate. Sindelar et al. (2007) suggested that secondary oxidation involving nitrous acid during curing could be the explanation for the conversion of some nitrite to nitrate. Honikel (2008) also agreed that oxidation of nitrite to nitrate is why nitrate can be observed in products to which only nitrite was added. No significant difference ($P > 0.05$) was observed for an interaction between treatment and day for either treatment within the three products. These results show that the amount of nitrate added in the supplemented treatment remained constant as storage time of the products increased. This confirms the hypothesis that residual nitrate will remain constant even with the relatively high nitrate concentrations used in this study. Several researchers have reported constant residual nitrate levels when compared to conventional (not supplemented) ingoing levels of nitrate (Wierbicki & Heiligman, 1973; Shults et al., 1977; Sindelar et al., 2007; Honikel, 2008). No significant

differences ($P > 0.05$) were observed independently with storage time, therefore confirming that no changes in residual nitrate levels occurred in these products during storage.

The TBA (2-thiobarbituric acid) measurements are presented in Table 10. Frankfurters and cotto salami displayed a significant difference ($P < 0.05$) between the two treatments. The control frankfurter treatment showed an overall TBA value of 0.38 compared to the supplemental nitrate treatment with a TBA value of 0.49, while the cotto salami control treatment showed an overall TBA value of 0.47 compared to the cotto salami supplemental nitrate treatment with a TBA value of 0.37. All of these TBA values are very low and because the control is lower for frankfurters, but higher for cotto salami this suggests that there is no practical effect of the added nitrate. Possible explanations for the statistical differences may be that different meat sources were acquired for each product replication and the meat ingredients may have had different initial TBA values. Frankfurter and cotto salami products both have relatively high fat contents, thus some variation between TBA values for raw materials for the two treatments is likely. Further, no significant difference ($P > 0.05$) was observed for control and the supplemental nitrate treatments in ham where fat content was considerably less. TBA values remained relatively low for all products over storage time, which was expected due to nitrite's ability to function as an antioxidant (Sebranek, 2009). No significant differences ($P > 0.05$) were shown for the effect of day for the treatments within the three products. The interaction between treatment and day also showed no significant differences ($P > 0.05$) for the treatments within the three products. These results indicated that as storage time increased, there was no increase in TBA values for either the control or the supplemental

nitrate treatments. Shults et al. (1977) also reported low TBA values that showed no increase over a 4-week storage period for nonirradiated corned beef brisket manufactured with 600 ppm sodium nitrate in combination with 150 ppm sodium nitrite.

Color measurements were conducted using Hunterlab L (lightness), a (redness), and b (yellowness). These characteristics were measured on three random locations on the sliced surface of each sample. Frankfurters included an additional measurement on the external surface as well as the interior after splitting the sample vertically. The results, shown in Table 11, indicate that the frankfurters (both internal and external) ($P > 0.05$) and ham, while nearly significant ($P = 0.05$) did not differ in L-values. However, the supplemental nitrate treatment of cotto salami had a significantly lower L-value ($P < 0.05$) than the control cotto salami treatment. Redfield and Sullivan (2015) observed that turkey products cured with sodium nitrite had a lighter appearance than those cured with celery juice powder as a source of nitrite. Celery juice powder has a yellow-green pigment and this natural coloring may contribute to a darker meat color. None of the products showed an effect ($P > 0.05$) of day or for the interaction between day and treatment. Therefore, no changes in lightness were observed over the storage period.

The a-value results are shown in Table 12. The supplemental nitrate treatment and the control were not different for the external color of the frankfurters ($P > 0.05$) or the cotto salami ($P = 0.07$). However, the a-value for hams and the internal frankfurter measurements were significantly different ($P < 0.05$) between the two treatments. The ham and the internal frankfurter measurements showed the control treatment with a higher a-value than the supplemental nitrate treatment. This means that the internal surface of the control treatment for hams and the frankfurters had a redder appearance.

All three products showed no significant difference ($P > 0.05$) for the effect of day or for the interaction between day and treatment. Therefore, no changes in a-color development were observed during storage.

Product b-color value results are displayed in Table 13. The supplemental nitrate treatment and the control were again not different for the external frankfurter color ($P > 0.05$) or for the cotto salami ($P = 0.07$). And again, as with the a-values, the b-values for ham and for the internal frankfurter measurements were different ($P < 0.05$). The ham and the internal frankfurter measurements resulted in the supplemental nitrate treatment having a higher b-value than the control treatment, meaning a more yellow appearance. A greater b-value in products with the added nitrate was not unexpected because of the yellow-green color of the celery juice powder that was added to increase nitrate content. All three products showed no significant effect ($P > 0.05$) of day or for the interaction between day and treatment.

Overall, there was no significant effect ($P > 0.05$) of day or for the interaction between day and treatment for any of the color measurements in this study. Redfield and Sullivan (2015) also concluded that time did not have an impact ($P > 0.05$) on any CIE L*, a* and b* color measurements when evaluating turkey products cured with sodium nitrite or with celery juice powder as a source of nitrite, which supports this study's findings in that no changes in color development occurred over storage time. However, Sindelar et al. (2007) observed that the main effect of day had a significant effect ($P < 0.05$) on CIE L*, a* and b* results when evaluating ham cured with different concentrations of celery juice powder as a source of nitrite. These investigators also reported a decrease in cured meat color over storage time, a change not observed in this

current study. Such difference between similar studies might be due to differences in storage temperatures and/or packaging methods and materials.

Total aerobic plate count analyses were conducted for frankfurters, cotto salami and ham and are shown in Figures 13 − 15, respectively. Plate counts were conducted to determine if any microbial differences might develop between the control and the supplemental nitrate treatments for the frankfurters, cotto salami and ham, independently. Microbial data was transformed into logs to interpret the exponential growth rates of bacteria. A value of 5 CFU/g was used as the detection limit when plotting the results. The results showed no statistical significance ($P > 0.05$) for the counts between the control and the supplemental nitrate treatments in regard to all three products. Frankfurters were the only product that showed a statistical significance ($P < 0.05$) for the effect of day with a small decrease in counts over time. Although the effect of day was significantly different over time, frankfurters showed no interaction ($P > 0.05$) between day and treatment. Likewise there was no interaction between day and treatment for cotto salami and ham ($P > 0.05$). No significant microbial growth was expected in either treatment due to sodium nitrite's ability to perform as a bacteriostatic and bacteriocidal agent (Sebranek & Bacus, 2007) especially in cooked, vacuum-packed products stored at 32 − 34°F.

Lactic acid bacteria were also enumerated for frankfurters, cotto salami and ham, and results are shown in Figures 16 − 18, respectively. Lactic acid bacteria were counted to determine if differences in growth of these common spoilage organisms in vacuum-packaged, cooked, cured meat products were apparent between the control and supplemental nitrate treatments for the frankfurters, cotto salami and ham. Microbial data

was transformed into logs to interpret the exponential growth rates of bacteria. A value of 50 CFU/g was used as the detection limit when plotting the results. Samples from days 7 and 14 were plated and counted, but were later eliminated from the lactic acid bacteria analysis due to erratic counts that were later determined to be due to airborne contamination in the laboratory. The results for days 0, 30, 60, and 90 showed no statistical differences ($P > 0.05$) between the two treatments for lactic acid bacteria counts, for the effect of day, or for the interaction between day and treatment for all three products.

Salt, protein, fat and moisture content, seen in Figures 19 − 22, were measured to determine if any proximate compositional differences occurred between the control and supplemental nitrate treatment for frankfurters, cotto salami and ham. No statistically significant differences ($P > 0.05$) in regards to salt, protein, fat or moisture were found between the control and supplemental nitrate treatments for frankfurters, cotto salami and ham. These results indicate that the addition of sodium nitrate and VegStable 502 did not change the proximate composition of the products when compared to the control treatment. The control and supplemental nitrate treatment were identical in formulation except for the additional sodium nitrate, celery juice powder and the substitution of a portion of the sodium chloride with potassium chloride. Potassium chloride was substituted for 8% of sodium chloride in the supplemental nitrate products to keep sodium content of the two treatments similar because the additional sodium content from the addition of sodium nitrate would be likely to impact the sensory evaluations of these products.

The sensory results for frankfurters are shown in Table 14. The cured aroma, "other aroma", texture, cured flavor, "other flavor", pepper intensity, saltiness, intensity of brown surface color and intensity of internal light to dark color were not significantly different ($P > 0.05$) between the control and the supplemental nitrate treatment. Frankfurter treatments were manufactured with a greater concentration of spices than the cotto salami or ham and received a greater amount of smoke deposition per unit weight given the greater surface area of frankfurters. Consequently, this is probably why panelists did not observe any differences in aroma or flavor for the frankfurters, despite the addition of 2% celery juice powder. However, panelists determined that the intensity of frankfurter internal pink color was significantly greater ($P < 0.05$) for the control treatment than the supplemented treatment.

The sensory results for cotto salami are displayed in Table 15. Cured aroma evaluations determined that the control treatment had a significantly greater ($P < 0.05$) cured aroma than the supplemental nitrate treatment. Panelists also determined there was a significant difference ($P < 0.05$) in the "other aroma" score between the control and supplemental nitrate treatments with the supplemental nitrate treatment having a greater "other aroma" than the control cotto salami. When evaluating texture, cured flavor, spice intensity, and saltiness no statistical differences ($P > 0.05$) were found between the control and the supplemental nitrate treatment. However, panelists found a significant difference ($P < 0.05$) in "other flavor" between the two treatments. The supplemental nitrate treatment possesses a greater "other flavor" than the control treatment. It is possible that the "other aroma" panelists observed could have affected their expectation of the supplemental nitrate treatment and therefore, concluded it to have a greater "other

flavor" as well. The intensity of pink color was not different ($P = 0.07$) between the control and supplemental nitrate treatments for cotto salami, but the supplemental nitrate treatment was significantly darker ($P < 0.05$) in color comparison to the control treatment.

Sensory results for the hams are shown in Table 16. The control and supplemental nitrate treatments showed no statistical difference ($P > 0.05$) for the attributes of saltiness, texture and "other flavor", but there was a significantly increased ($P < 0.05$) cured aroma and cured flavor, as well as a more intense pink color in the control hams. Results for "other aroma" were not significant ($P = 0.07$), but the trend was suggestive. The differences in cured aroma and flavor that are most likely due to the inclusion of celery powder were not unexpected due to the mild flavor profile of ham. The ham treatments were manufactured with no spices or flavoring agents other than the curing ingredients, whereas the frankfurters and cotto salami each included typical spice blends for those products. Thus, the ability to detect unexpected flavors is typically greater for hams than cotto salami or frankfurters.

Shults et al. (1977) investigated sensory characteristics on irradiated corned beef with different levels of ingoing sodium nitrite, sodium nitrate or a combination of both. Investigators concluded that there was no significant changes ($P > 0.05$) in color, odor, flavor and texture between corned beef cured with 150 ppm sodium nitrite and corned beef cured with 150 ppm sodium nitrite in combination with 600 ppm sodium nitrate. Hustad et al. (1973) also researched sensory effects on frankfurters at different levels of added sodium nitrite as well as a combination of added sodium nitrite with sodium nitrate. Hustad et al. (1973) determined that the nitrite and nitrate used in combination at

all concentrations tested did not have a significant effect ($P > 0.05$) on flavor quality

when compared to any of the ingoing sodium nitrate levels tested. Thus, the addition of

supplemental nitrate alone should not impact sensory properties of cured meat products.

The findings of Shults et al. (1977) and Hustad et al. (1973) correlate with what was

observed in this study for the frankfurter treatments, where the only difference observed

was for internal pink color. For the cotto salami in this study, differences in odor and

"other flavor" were observed, but cured flavor was not affected by nitrate

supplementation. Both of these products included spices and smoke for a relatively

intense flavor profile, thus making sensory changes due to the treatments, particularly the

celery powder less noticeable. In the case of the hams, cured flavor as well as aroma was

affected by the supplemental nitrate treatment and are most likely attributed to the

characteristics of the celery juice powder. In order to manufacture meat products to

contain 220 mg or more nitrate per 112 g serving of cured meat, the addition of 2%

VegStable 502 in combination with 1718 ppm sodium nitrate was necessary and is likely

to have greater impact on sensory characteristics of the products with a low-intensity

flavor profile. Although celery juice powder is likely to affect color and sensory

attributes, depending on the concentration used, celery juice powder is preferred for meat

applications over other natural nitrate sources because of advantages it has over other

vegetables containing high nitrate concentrations. Celery is known to have mild flavors

and a light pigment, thus its use is well accepted in meat processing (Sebranek & Bacus,

2007). Typical use, however, is 0.4%, considerably less than the 2% used in this study.

The Hunter color results are supported by the evaluations collected from the

trained panel. Panelists observed that the control treatments for ham and frankfurters

displayed a greater intensity of pink color than the supplemental nitrate treatments. Hunter results also showed that ham and internal color of frankfurters had a higher a-value, indicating that these products were redder in color. Color scores by the panelists and Hunter results both showed the cotto salami supplemental nitrate treatment to be darker in color than the control, but also found no difference for lightness in the frankfurters. Although the color variations identified by panelists and Hunter showed statistical differences between the control and supplemental nitrate treatments, these differences are relatively small and will most likely go undetected by the consumer.

Conclusions

The results of this study demonstrate that supplemental nitrate at the USDA-regulated level of 1718 ppm sodium nitrate in combination with nitrate from 2% VegStable 502 is not converted to nitrite during storage time of up to 97 days at 32 − 34°F. Because no conversion to nitrite took place, the amount of added supplemental nitrate remained consistent over refrigerated storage, and thus is available as a source of dietary nitrate when the product is consumed. The results also confirmed that nitrate does not impact microbial growth even at the relatively high concentration used in this study. The combination treatment tends to produce a less intense red color than products manufactured with nitrite alone, probably due to the effect of the 2% added celery powder, which contributes no redness to a meat mixture. The impact of the addition of the treatment with celery powder on sensory properties was very product-dependent with limited impact in the presence of more intense flavor profiles and greater impact on mild flavor profiles. Flavor and odor were unaffected, for example, for frankfurters, while "other" aroma and flavor was different for cotto salami, and cured aroma and flavor were

affected for hams. The "other" aroma and flavor difference suggests that panelists sense an unidentifiable difference in cotto salami. In the case of hams, the detected difference in cured flavor and aroma suggests a more clearly identifiable difference in these properties. These findings suggest that the amount of supplemental nitrate from celery juice powder added should be determined at some point of 2% or less of the meat block depending on the product in order to maintain sensory characteristics similar to a conventional product. With a proper spice formulation, smoke and similar flavor profile contributors, an acceptable level of supplemental nitrate using celery powder offers potential to provide a significant dietary source of nitrate. While such products are not likely to become commonplace due to the challenges of overcoming the current perceptions of nitrite and nitrate, the demonstration of potential effects of supplementary nitrate in cured meat for increasing physiological nitric oxide may help to improve the current perceptions of cured meat. While this holds promise, the next step needed to achieve this objective is a human feeding study, with nitrate-supplemented cured meat to demonstrate the expected impact of the dietary nitrate on human physiological parameters, such as nitrate, nitrite, and nitric oxide concentrations in blood plasma and associated effects on blood pressure and other physiological parameters.

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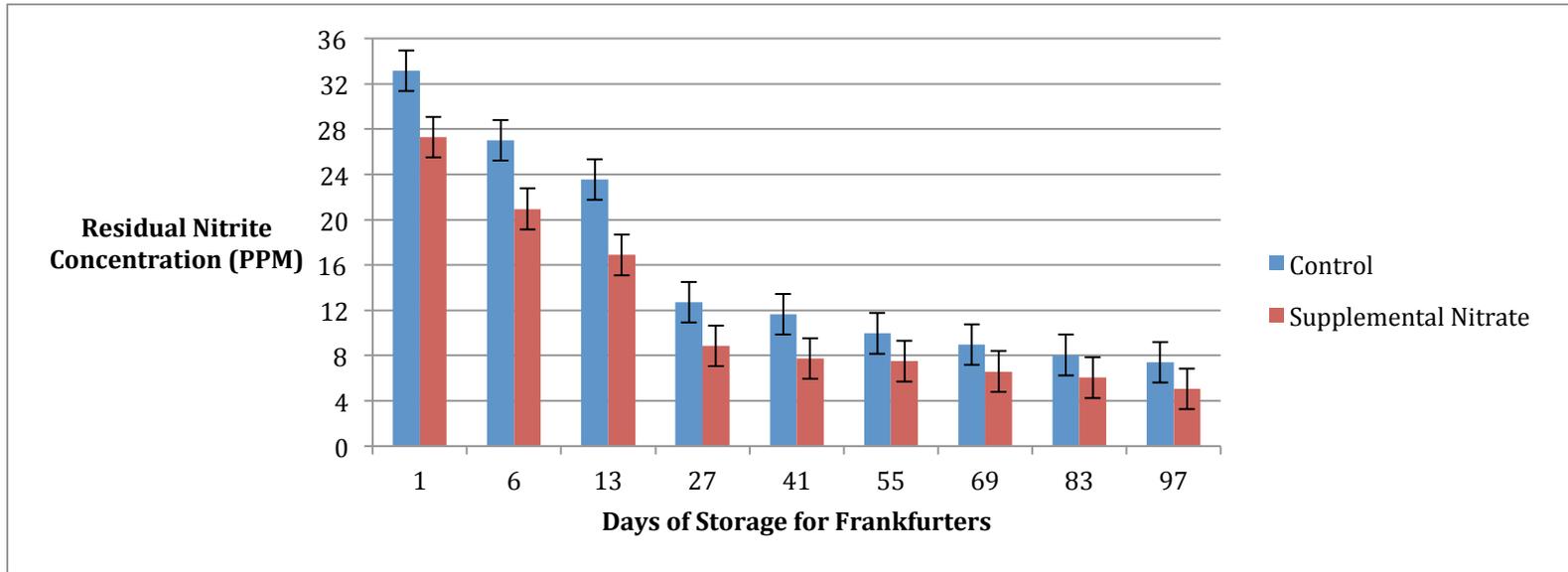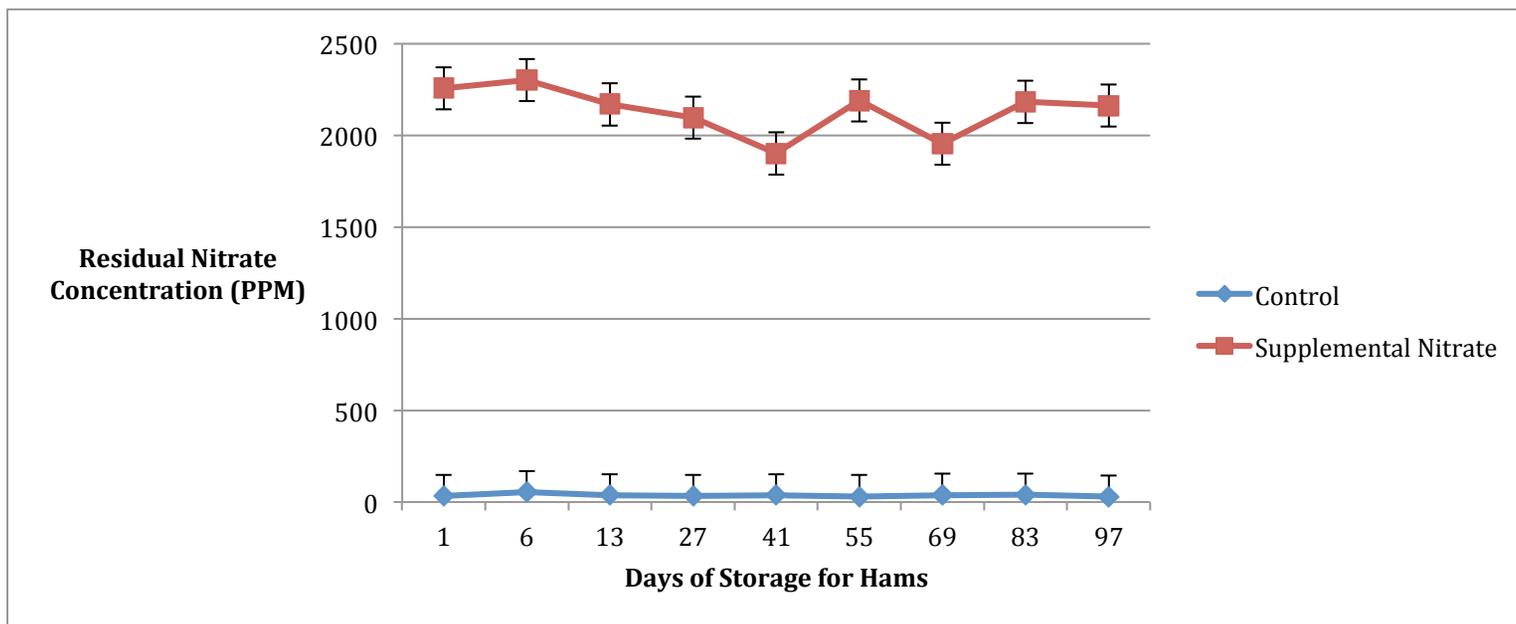
Figure 7. Least squares means for residual nitrite values of control and supplemental nitrate treatments of frankfurters during storage. (S.E. = 1.8).
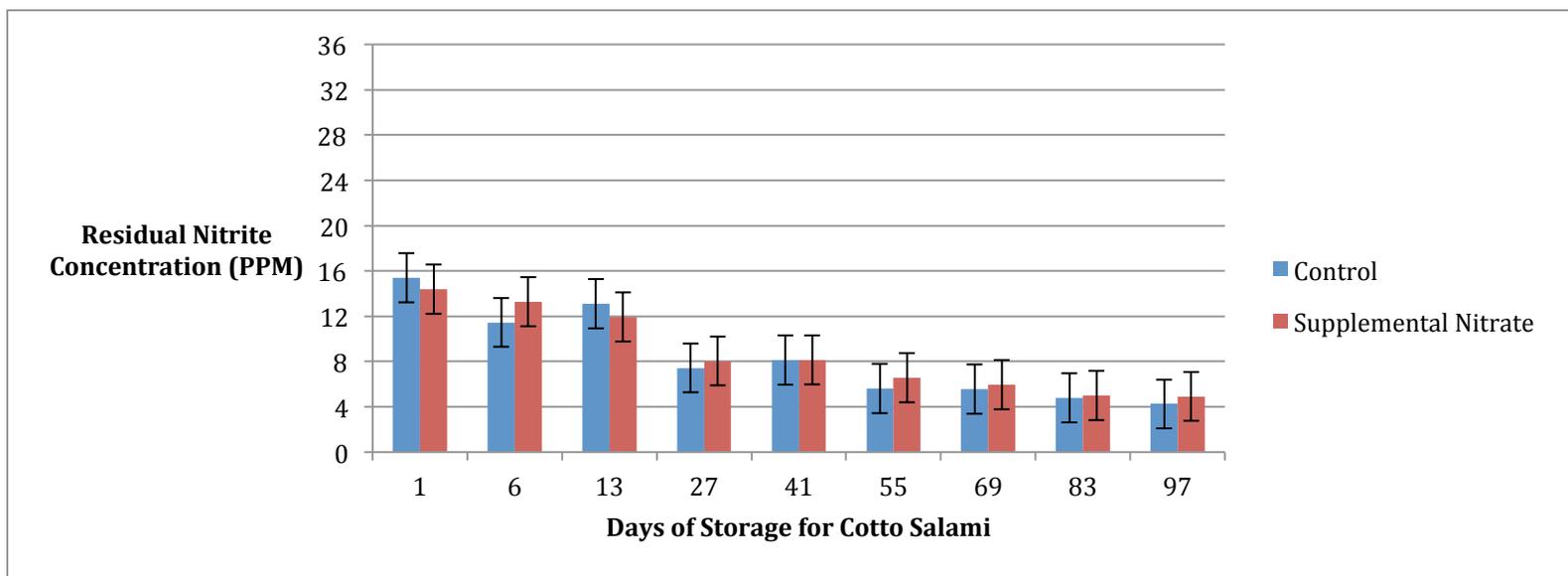
Figure 8. Least squares means for residual nitrite values of control and supplemental nitrate treatments of cotto salami during storage. (S.E. = 2.2).
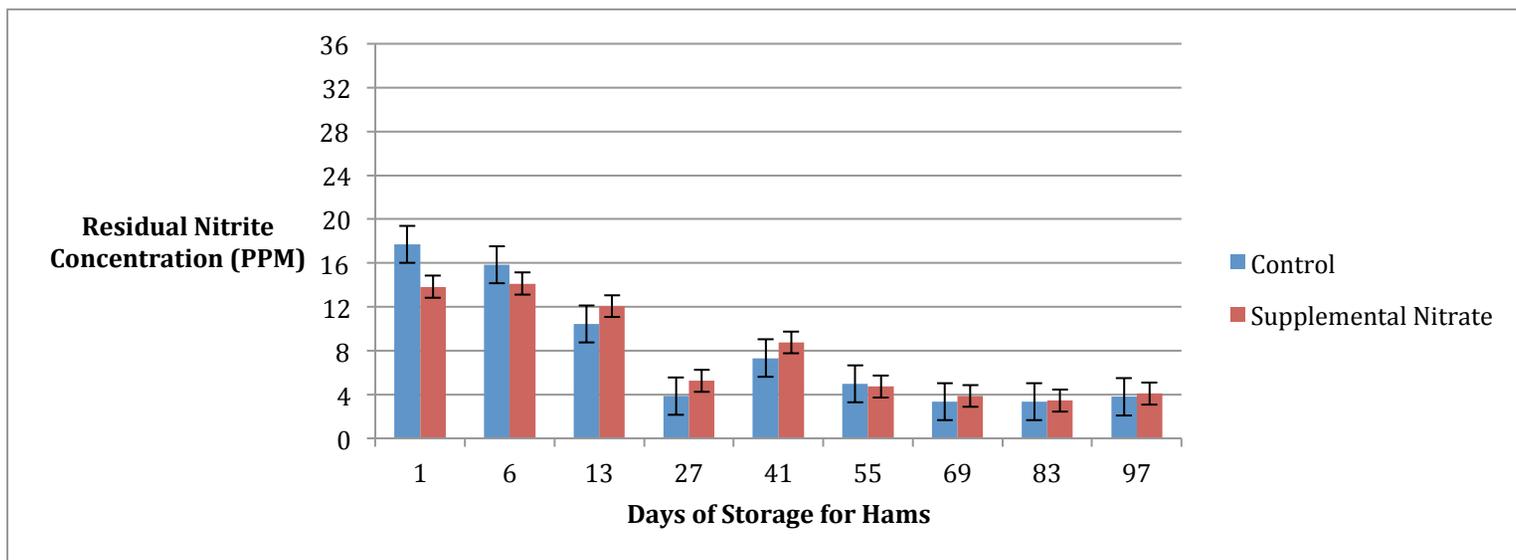
Figure 9. Least squares means for residual nitrite values of control and supplemental nitrate treatments for hams during storage. (S.E. = 1.7).
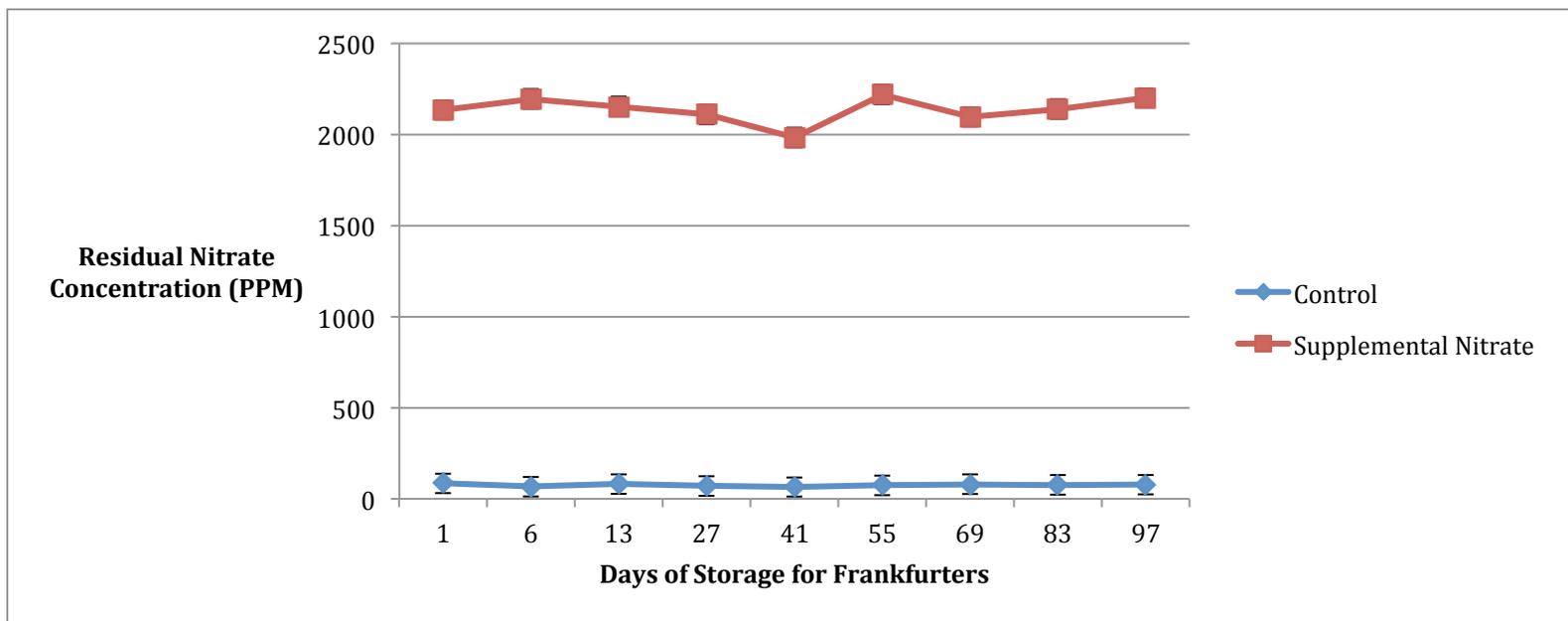
Figure 10. Least squares means for residual nitrate values of control and supplemental nitrate treatments for frankfurters during storage. (S.E. = 53.5).
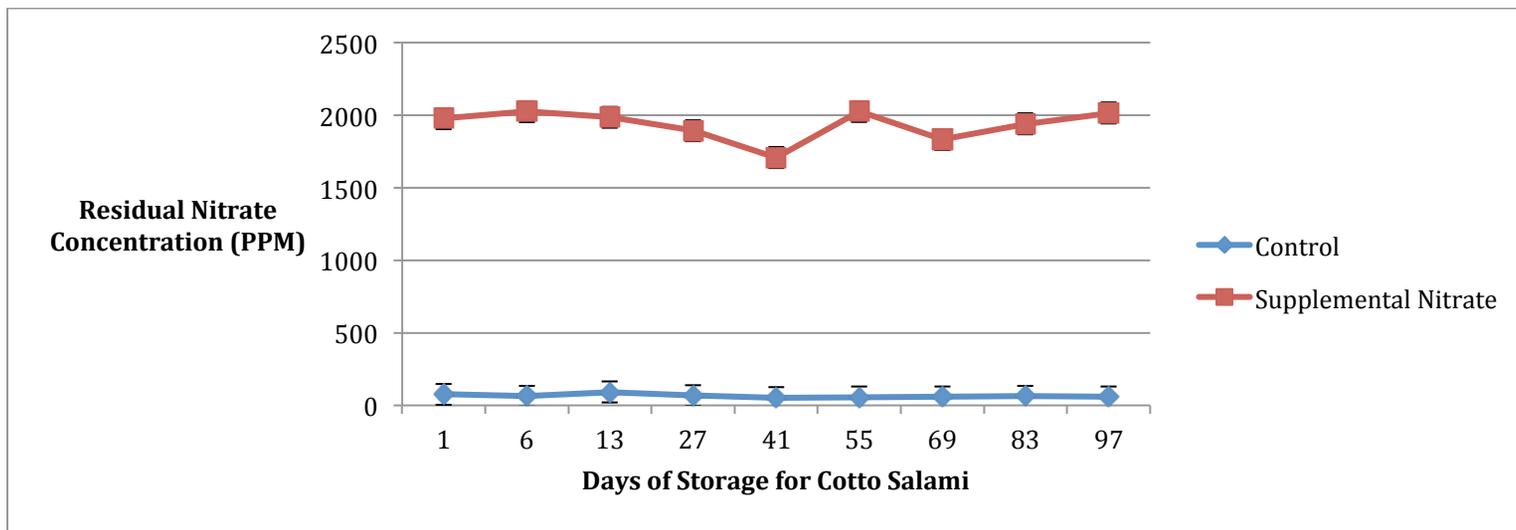
Figure 11. Least squares means for residual nitrate values of control and supplemental nitrate treatments for cotto salami during storage. (S.E. = 72.3).

Figure 12. Least squares means for residual nitrate values of control and supplemental nitrate treatments for hams during storage. (S.E. = 115.1).

Table 10. The effect of treatment on the least squares means of TBA values for frankfurters, cotto salami and hams.

Product Treatment	Frankfurters	S.E.[a]	Cotto Salami	S.E.[a]	Hams	S.E.[a]
			TBA Values			
Control	0.38^c	0.02	0.47^b	0.02	0.21^b	0.02
Supplemental Nitrate	0.49^b	0.02	0.37^c	0.02	0.16^b	0.02

[a]Standard error of means
[b-c]Means within same column with different letters are significantly different at $P < 0.05$

Table 11. The effect of treatment on the least squares means of color L-values for color of frankfurters, cotto salami and hams.

Product Treatment	Frankfurters	S.E.[a]	Frankfurters Internal	S.E.[a]	Cotto Salami	S.E.[a]	Hams	S.E.[a]
				Color L-Values				
Control	43.1^b	0.56	59.46^b	0.64	49.65^b	0.47	60.44^b	0.35
Supplemental Nitrate	43.34^b	0.56	59.61^b	0.64	46.94^c	0.47	59.43^b	0.35

[a]Standard error of means
[b-c]Means within the same column with different letters are significantly different at $P < 0.05$

Table 12. The effect of treatment on the least squares means of color a-values for color of frankfurters, cotto salami and hams.

	Color a-Values							
			Frankfurters					
Product Treatment	Frankfurters	S.E.[a]	Internal	S.E.[a]	Cotto Salami	S.E.[a]	Hams	S.E.[a]
Control	15.73[b]	0.15	10.62[b]	0.15	9.85[b]	0.17	8.59[b]	0.18
Supplemental Nitrate	15.54[b]	0.15	10.11[c]	0.15	10.29[b]	0.17	7.92[c]	0.18

[a]Standard error of means
[b-c]Means within the same column with different letters are significantly different at $P < 0.05$

Table 13. The effect of treatment on the least squares means of color b-values for color of frankfurters, cotto salami and hams.

	Color b-Values							
			Frankfurters					
Product Treatment	Frankfurters	S.E.[a]	Internal	S.E.[a]	Cotto Salami	S.E.[a]	Hams	S.E.[a]
Control	18.49[b]	0.23	10.39[c]	0.12	9.42[b]	0.11	8.02[c]	0.08
Supplemental Nitrate	18.6[b]	0.23	10.96[b]	0.12	9.71[b]	0.11	9.23[b]	0.08

[a]Standard error of means
[b-c]Means within the same column with different letters are significantly different at $P < 0.05$
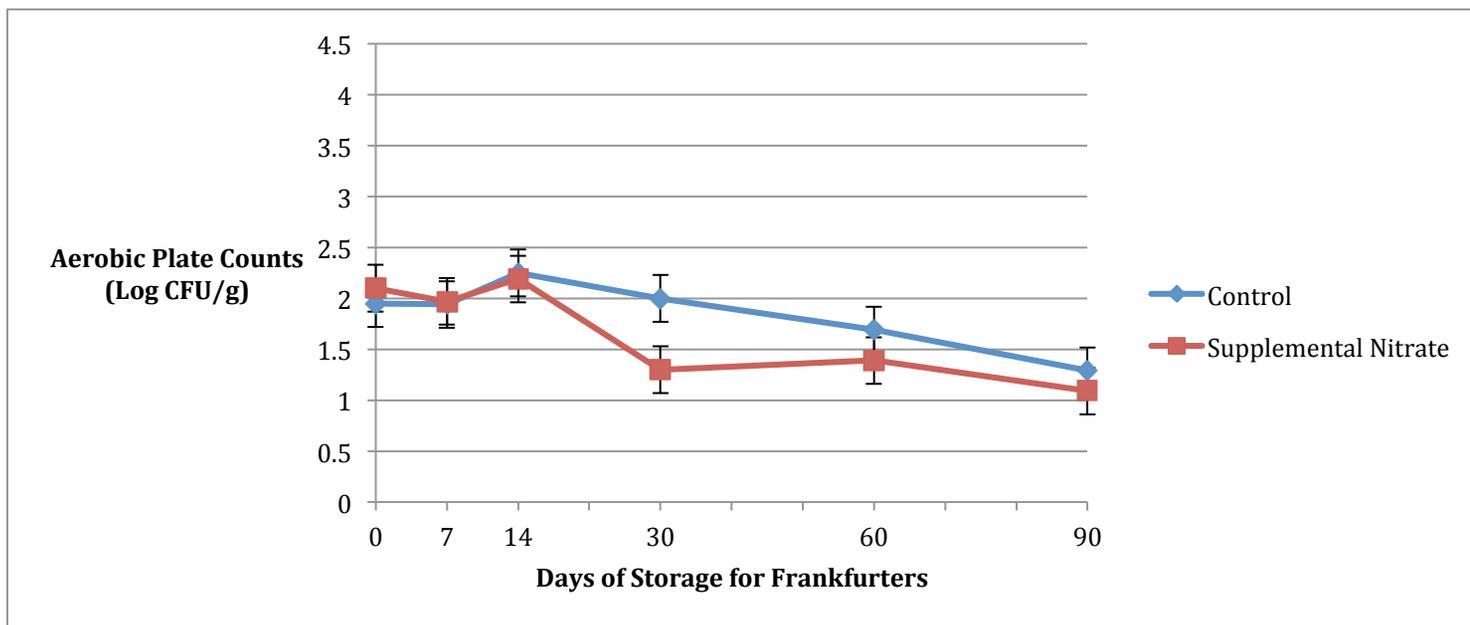
Figure 13. Aerobic plate count values (Log_{10} CFU/g) on frankfurters during storage. (S.E. = 0.23).
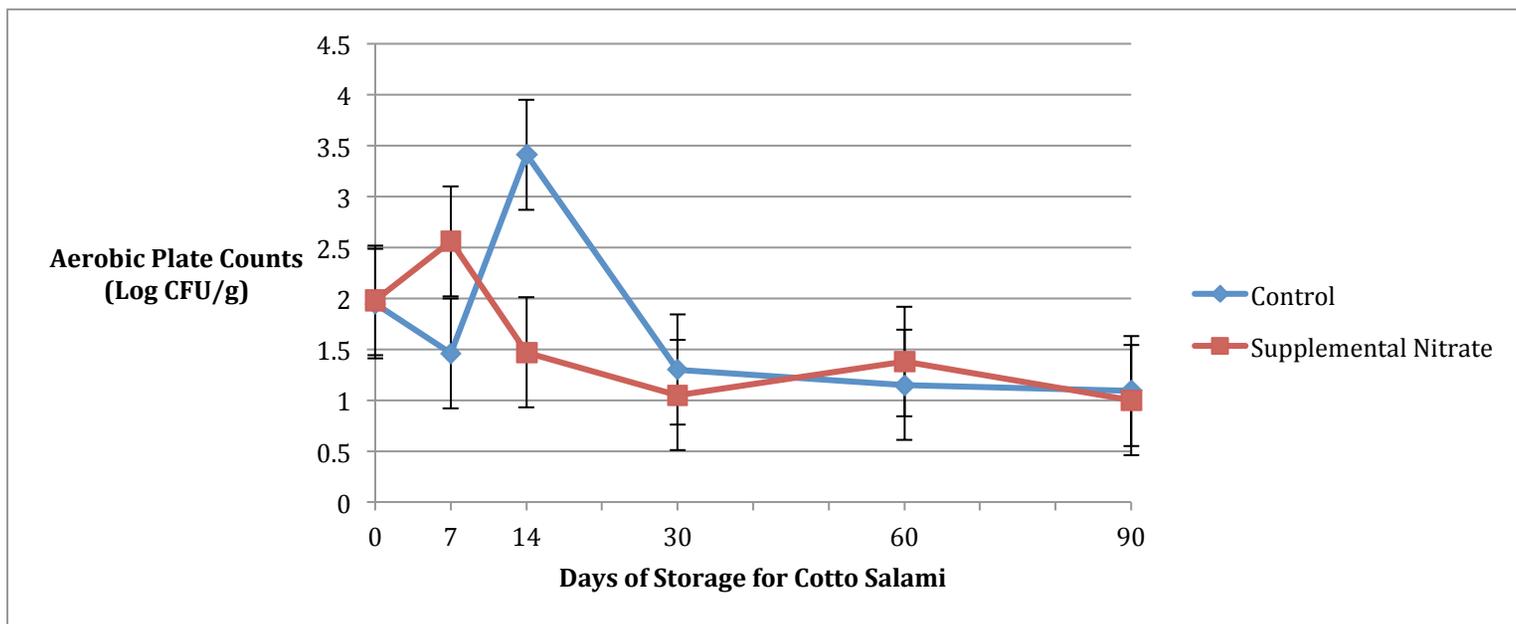
Figure 14. Aerobic plate count values (Log_{10} CFU/g) on cotto salami during storage. (S.E. = 0.54).
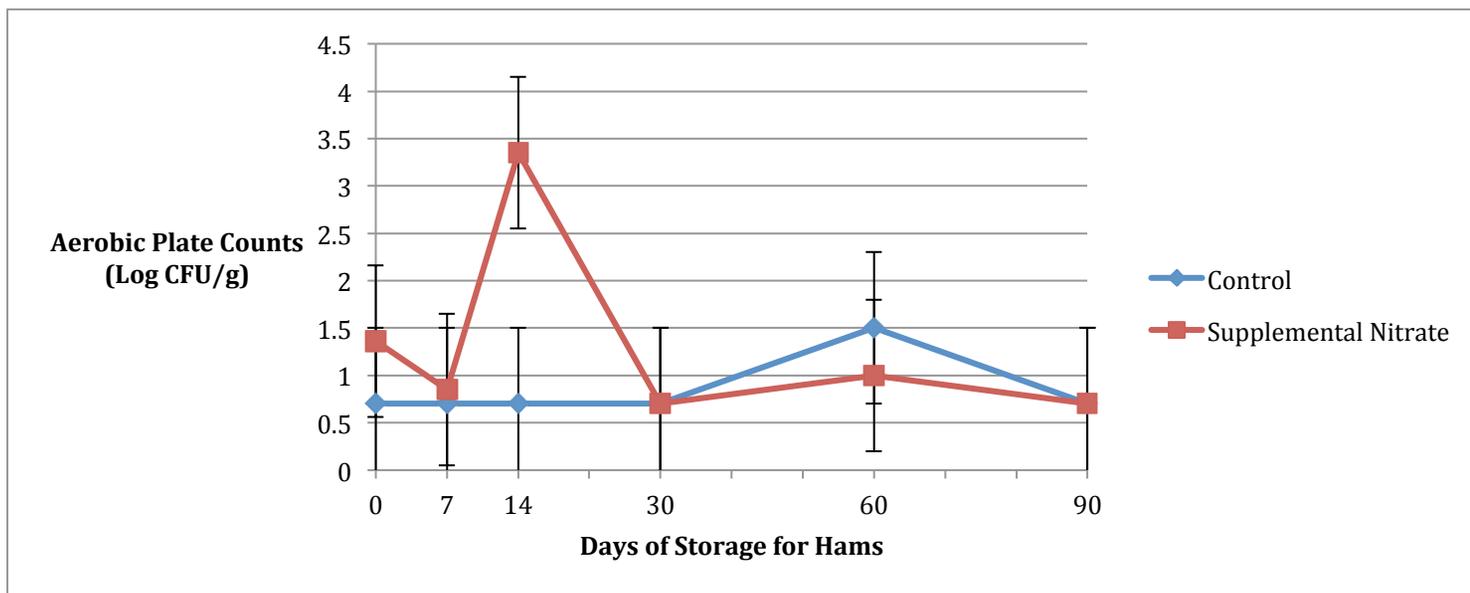
Figure 15. Aerobic plate count values (Log_{10} CFU/g) on hams during storage. (S.E. = 0.80).
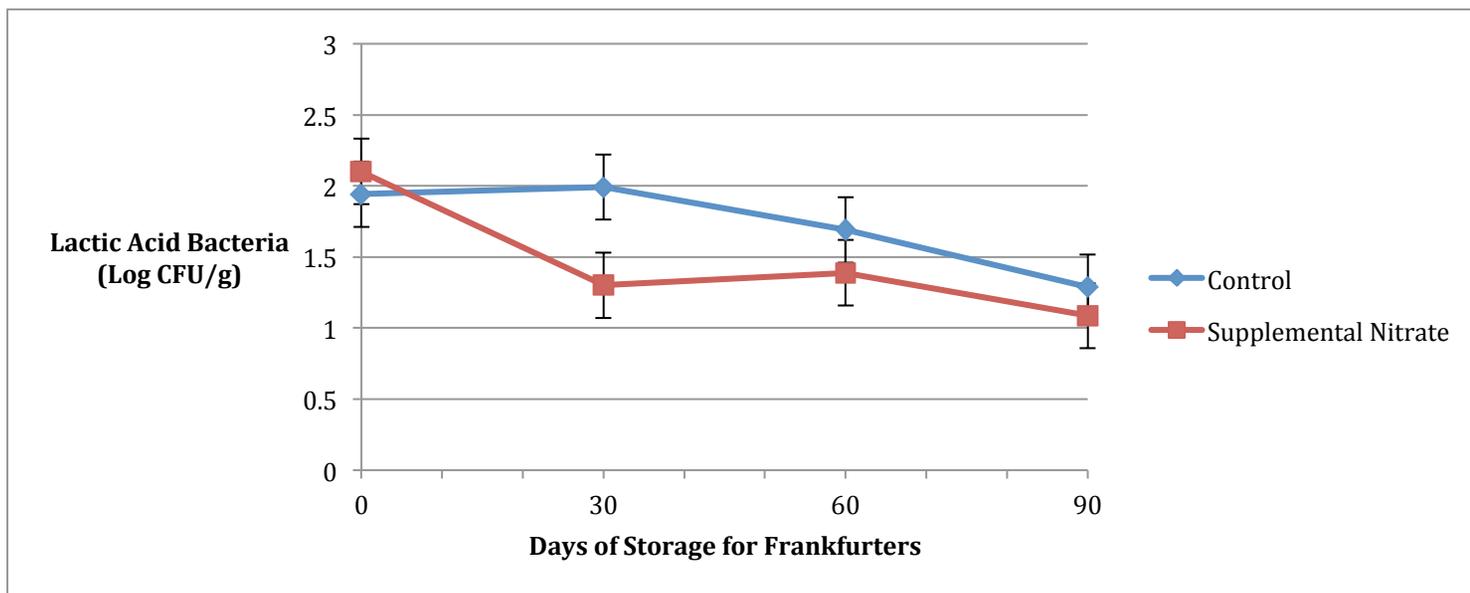
Figure 16. Lactic acid bacteria plate count values (Log_{10} CFU/g) on frankfurters during storage. (S.E. = 0.23).
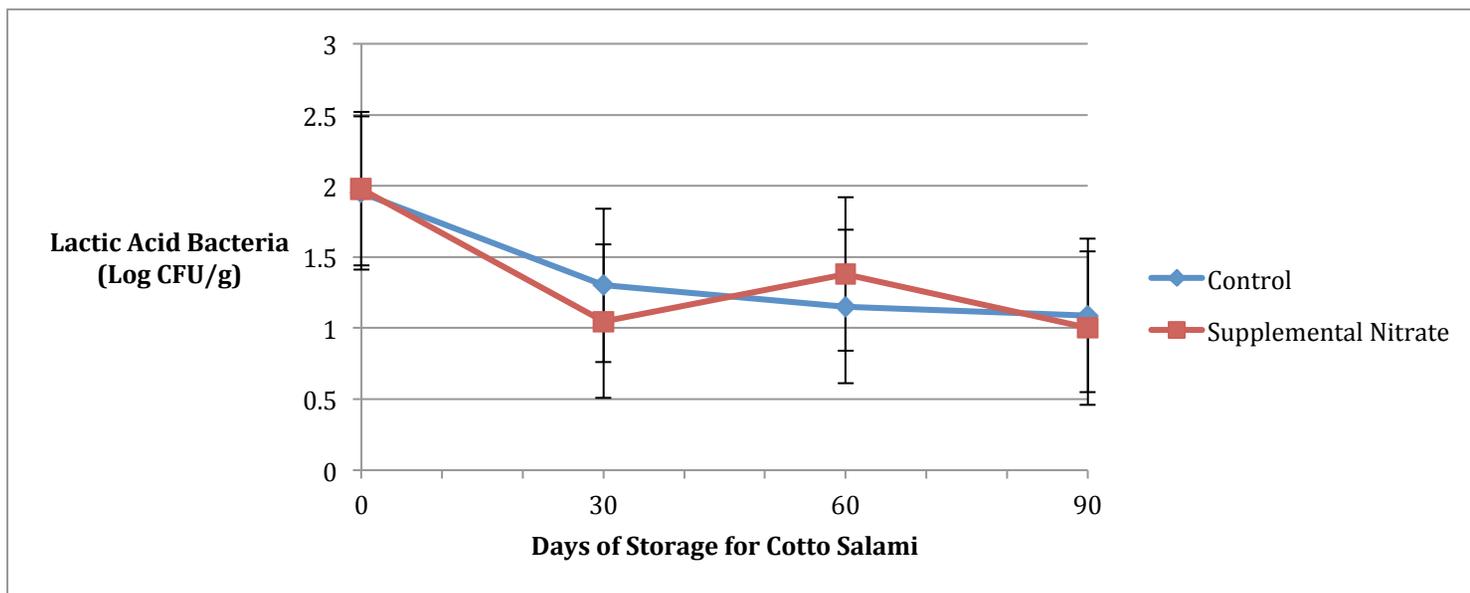
Figure 17. Lactic acid bacteria plate count values (Log_{10} CFU/g) on cotto salami during storage. (S.E. = 0.54).
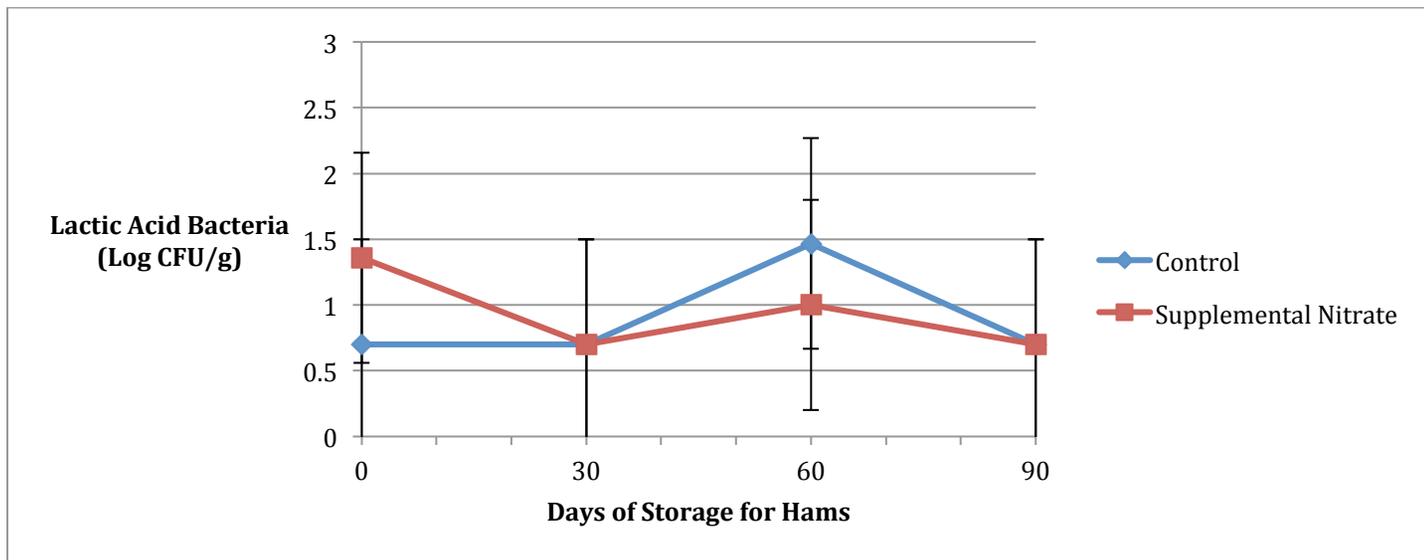
Figure 18. Lactic acid bacteria plate count values (Log_{10} CFU/g) on hams during storage. (S.E. = 0.80).
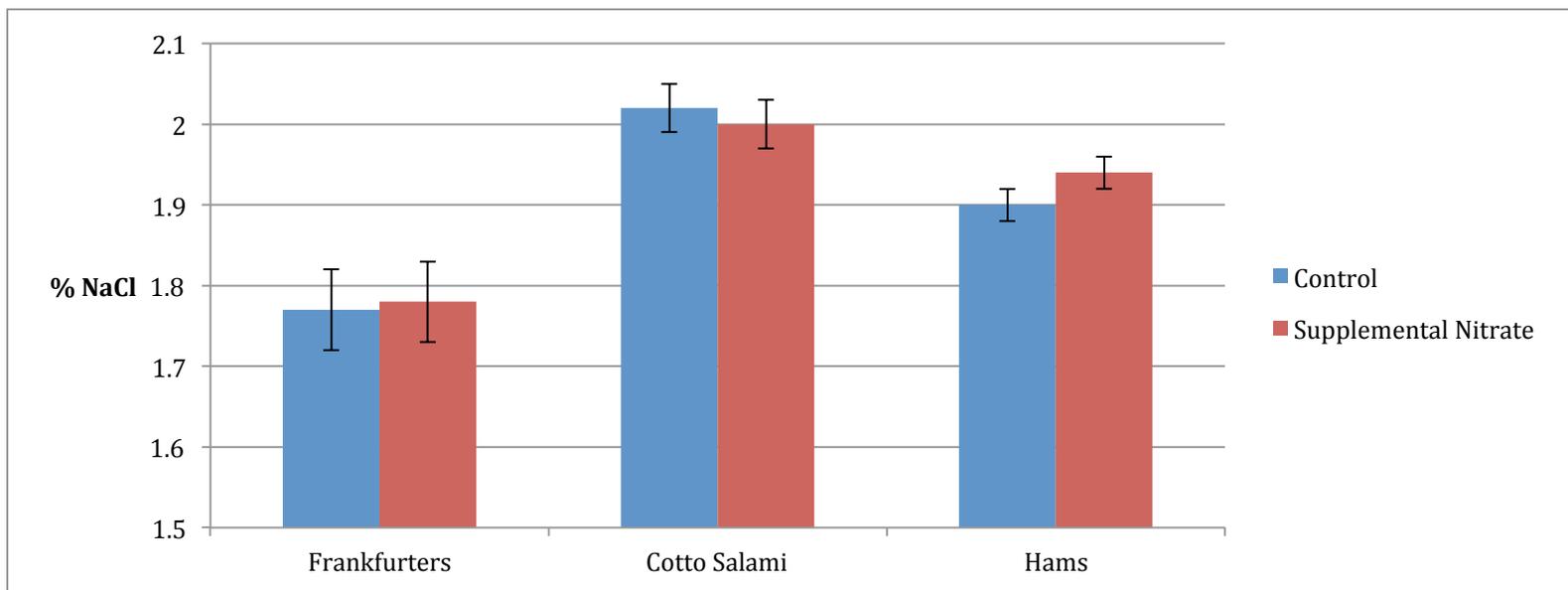
Figure 19. Least squares means for salt content values of control and supplemental nitrate treatments for frankfurters, cotto salami and hams. (Frankfurters S.E. = 0.05) (Cotto salami S.E. = 0.03) (Hams S.E. = 0.02).
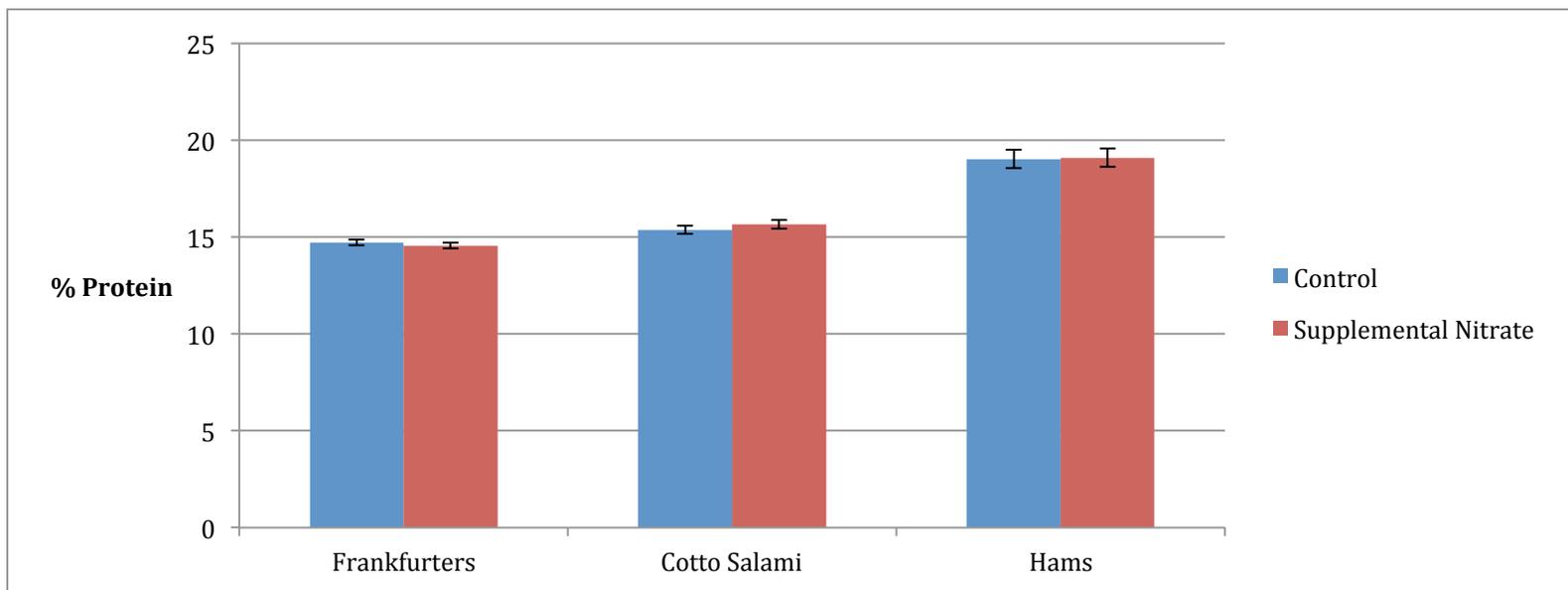
Figure 20. Least squares means for protein content of control and supplement nitrate treatments for frankfurters, cotto salami and hams. (Frankfurters S.E. = 0.14) (Cotto salami S.E. = 0.22) (Hams S.E. = 0.47).
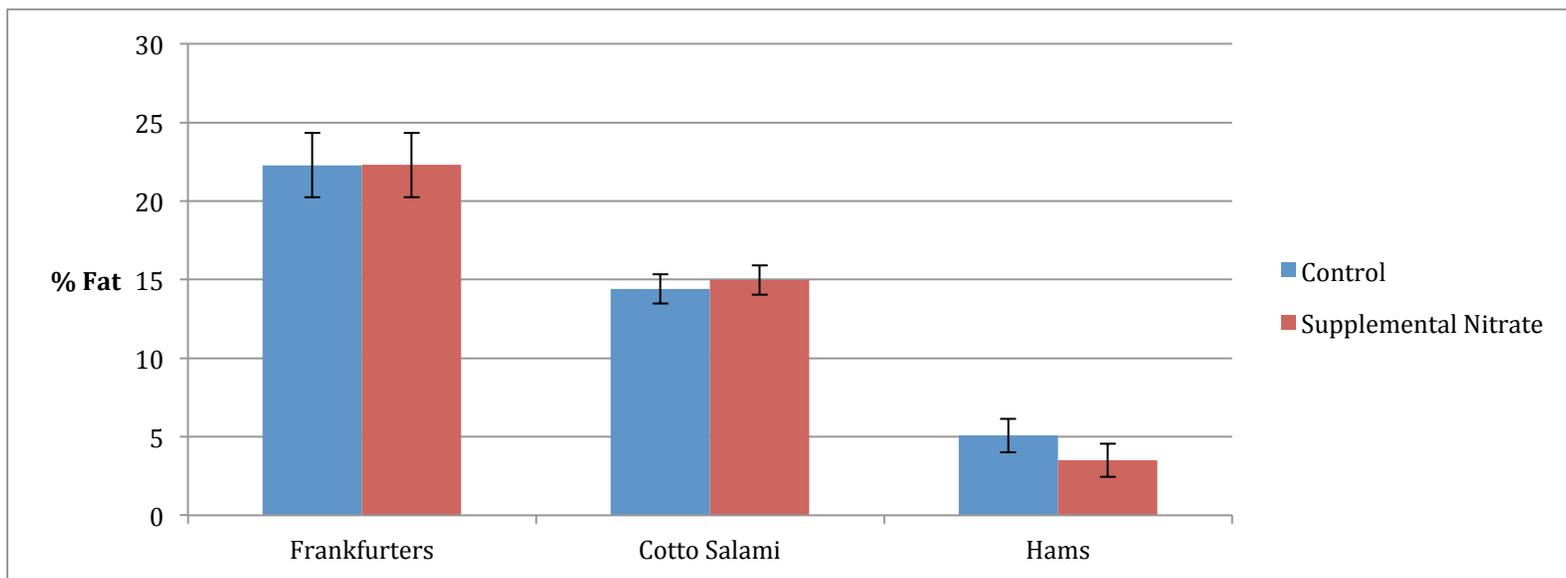
Figure 21. Least squares means for fat content of control and supplemental nitrate treatments for frankfurters, cotto salami and hams. (Frankfurters S.E. = 2.05) (Cotto Salami S.E. = 0.93) (Hams S.E. = 1.06).
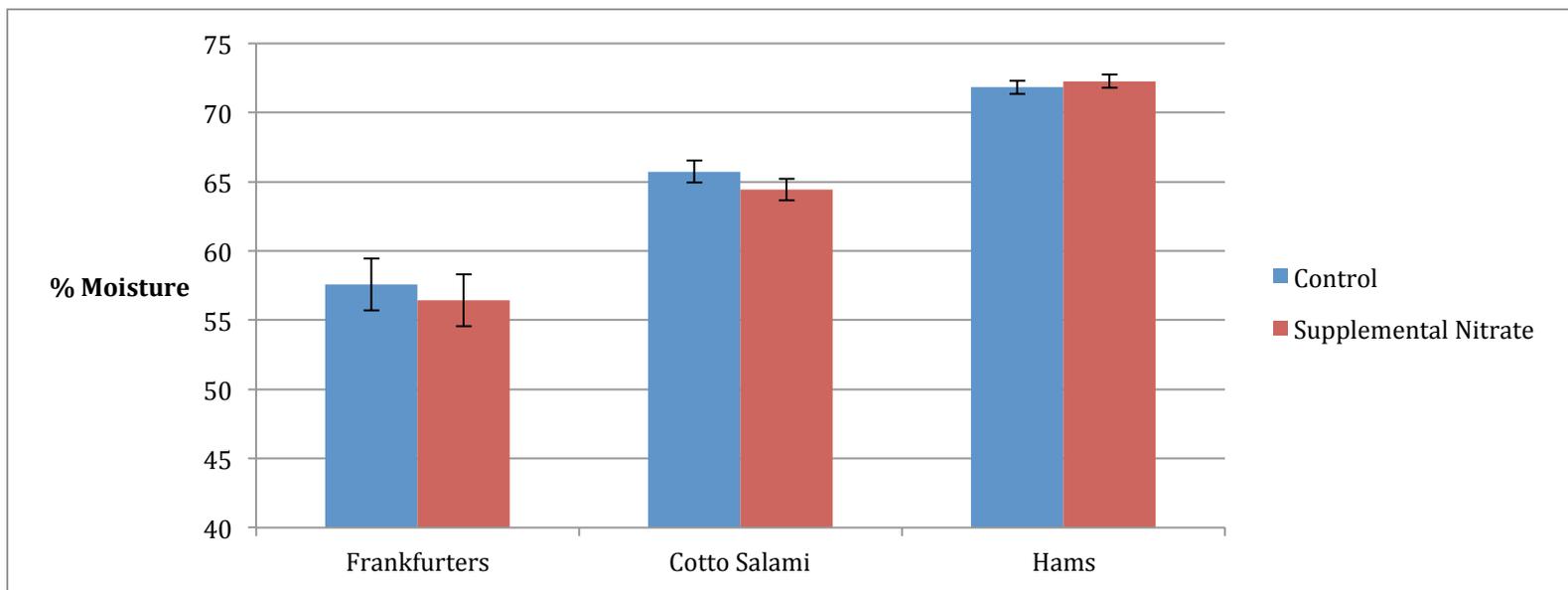
Figure 22. Least squares means for moisture content of control and supplemental nitrate treatments for frankfurters, cotto salami and hams. (Frankfurters S.E. = 1.89) (Cotto salami S.E. = 0.79) (Hams S.E. = 0.48).

Table 14. The effect of treatment on the least squares means for sensory characteristics (15 cm line scale) of frankfurters.

Product Treatments	Sensory Characteristics									
	Cured Aroma	Other Aroma	Texture	Cured Flavor	Other Flavor	Pepper Intensity	Saltiness	Brown Intensity	Pink Intensity	Light to Dark
Control	9.69[b]	0.05[b]	9.55[b]	9.55[b]	0[b]	7.29[b]	5.73[b]	9.03[b]	9.33[b]	7.69[b]
Supplemental Nitrate	8.93[b]	0.13[b]	8.75[b]	8.94[b]	0.08[b]	7.70[b]	5.80[b]	8.52[b]	6.93[c]	7.66[b]
S.E.[a]	0.3	0.04	0.98	0.59	0.06	0.48	0.27	1.91	0.23	1.88

[a]Standard error of means
[b-c]Means within the same column with different letters are significantly different at $P < 0.05$

Table 15. The effect of treatment on the least squares means for sensory characteristics (15 cm line scale) of cotto salami.

Product Treatments	Sensory Characteristics								
	Cured Aroma	Other Aroma	Texture	Cured Flavor	Other Flavor	Saltiness	Pink Intensity	Light to Dark	Spice Intensity
Control	8.89[b]	0.14[b]	7.78[b]	9.38[b]	0[b]	6.14[b]	9.43[b]	6.65[b]	7.82[b]
Supplemental Nitrate	7.79[c]	1.33[c]	8.12[b]	8.52[b]	0.05[c]	6.51[b]	7.01[b]	9.87[c]	7.75[b]
S.E.[a]	0.01	0.12	0.43	0.35	0.004	0.17	0.49	0.48	0.42

[a]Standard error of means
[b-c]Means within the same column with different letters are significantly different at $P < 0.05$

Table 16. The effect of treatment on least squares means for sensory characteristics (15 cm line scale) of hams.

				Sensory Characteristics			
Product Treatments	Cured Aroma	Other Aroma	Texture	Cured Flavor	Other Flavor	Saltiness	Pink Intensity
Control	8.65[b]	0.40[b]	8.81[b]	9.17[b]	0.17[b]	7.23[b]	9.86[b]
Supplemental Nitrate	6.85[c]	2.78[b]	9.50[b]	7.37[c]	1.80[b]	7.23[b]	6.56[c]
S.E.[a]	0.23	0.46	0.37	0.27	0.48	0.76	0.1

[a]Standard error of means
[b-c]Means within the same column with different letters are significantly different at $P < 0.05$

CHAPTER 4. GENERAL CONCLUSIONS

Sodium nitrate and sodium nitrite have been used in meat products for their curing properties for centuries, but today consumers associate these ingredients with negative health effects. This consumer perception has led to products cured with sodium nitrate and/or nitrite being viewed as undesirable and unhealthy. However, years of extensive research and numerous publications have proven the use of nitrate and nitrite to be safe for human consumption when used according to USDA regulations. In addition to being safe for consumption, it has been discovered that dietary nitrate and nitrite have the potential to increase nitric oxide levels *in vivo* with potential human health benefits. Since cured meat products are typically cured with sodium nitrite, this study investigated the effects of sodium nitrite in combination with supplemental nitrate, utilizing both conventional sodium nitrate and nitrate from celery juice powders.

The results of the current study determined that the addition of supplemental nitrate to a cured meat product has the potential to provide a source of dietary nitrate without major effects on product quality. Nitrate, in combination with nitrite, in the cured meat products studied did not convert into nitrite during the storage period. Therefore, over the course of the storage period the concentration of ingoing nitrate remained stable and no additional residual nitrite was produced. The addition of supplemental nitrate did not affect most physical, chemical or microbial properties of the cured meat products studied, but product-dependent sensory effects were observed. Consequently, the appropriate use of celery juice powder as a source of supplementary nitrate will have to be determined on a product-dependent basis in consideration of potential sensory impacts.

This study has the potential to establish a new context for nitrate and nitrite. The addition of supplemental nitrate has the ability to remain as dietary nitrate and potentially form nitric oxide after consumption to potentially give consumers a physiological impact similar to leafy green vegetables. Therefore, cured meat products could serve as a delivery vehicle for increasing dietary nitrate. Previous publications have shown positive health effects due to increased nitric oxide levels after the consumption of dietary nitrate from vegetable sources. Further research should focus on metabolic effects seen after the consumption of a processed meat product manufactured with supplemental nitrate. Metabolic research is needed to demonstrate the expected impact of dietary nitrate on human physiological parameters.

APPENDIX: RESIDUAL NITRITE LEVELS COLLECTED BY HORMEL LABORITORIES
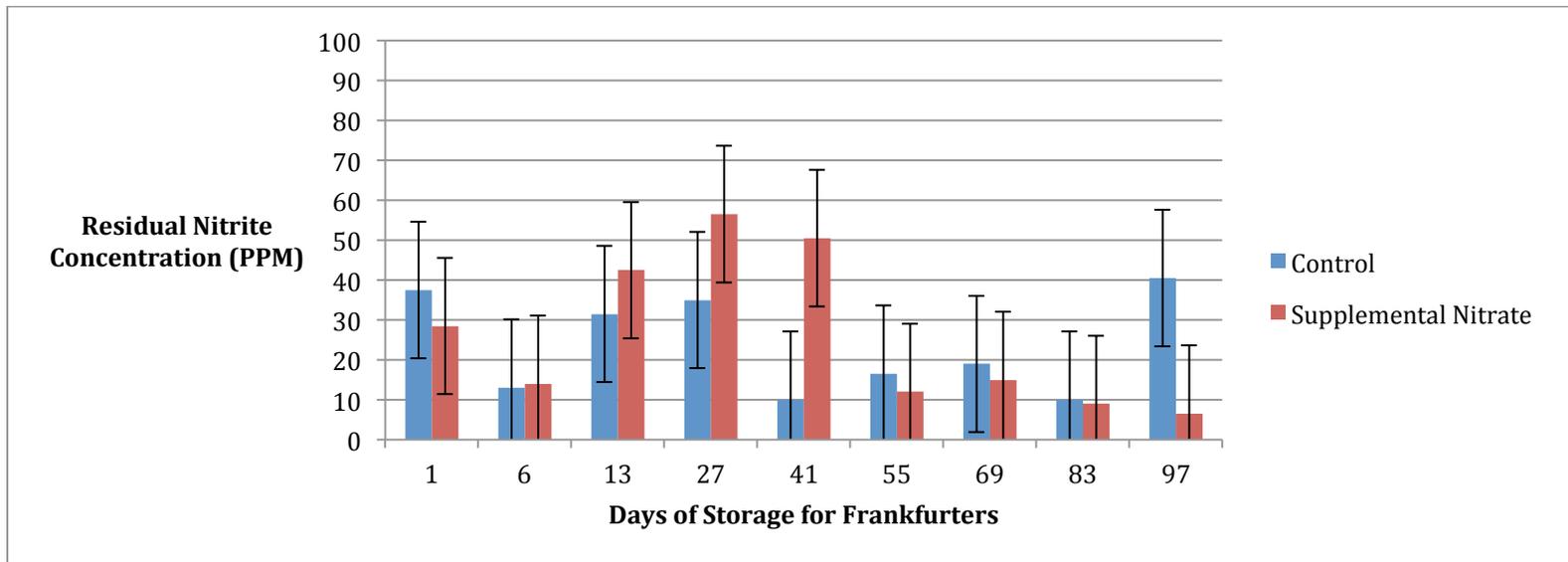
Figure 23. Least squares means for residual nitrite values of control and supplemental nitrate treatment of frankfurters during storage. (S.E. = 17.1).
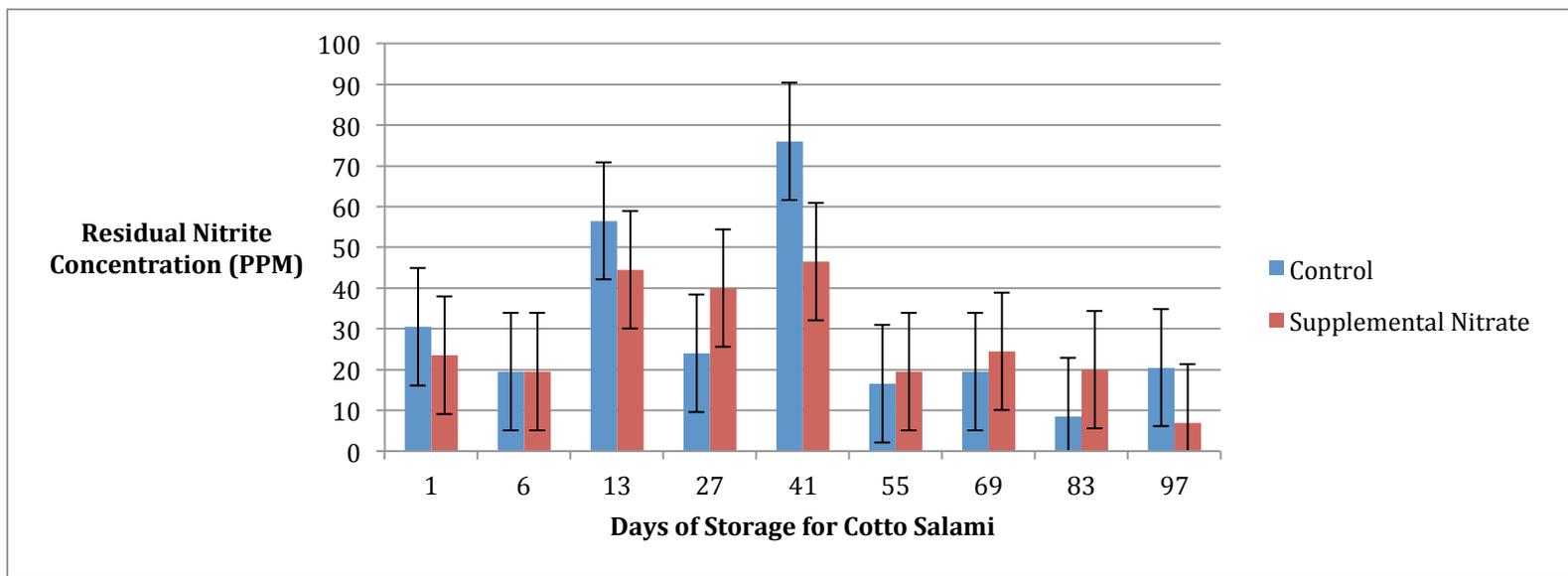
Figure 24. Least squares means for residual nitrite values of control and supplemental nitrate treatments for cotto salami during storage. (S.E. = 14.4).
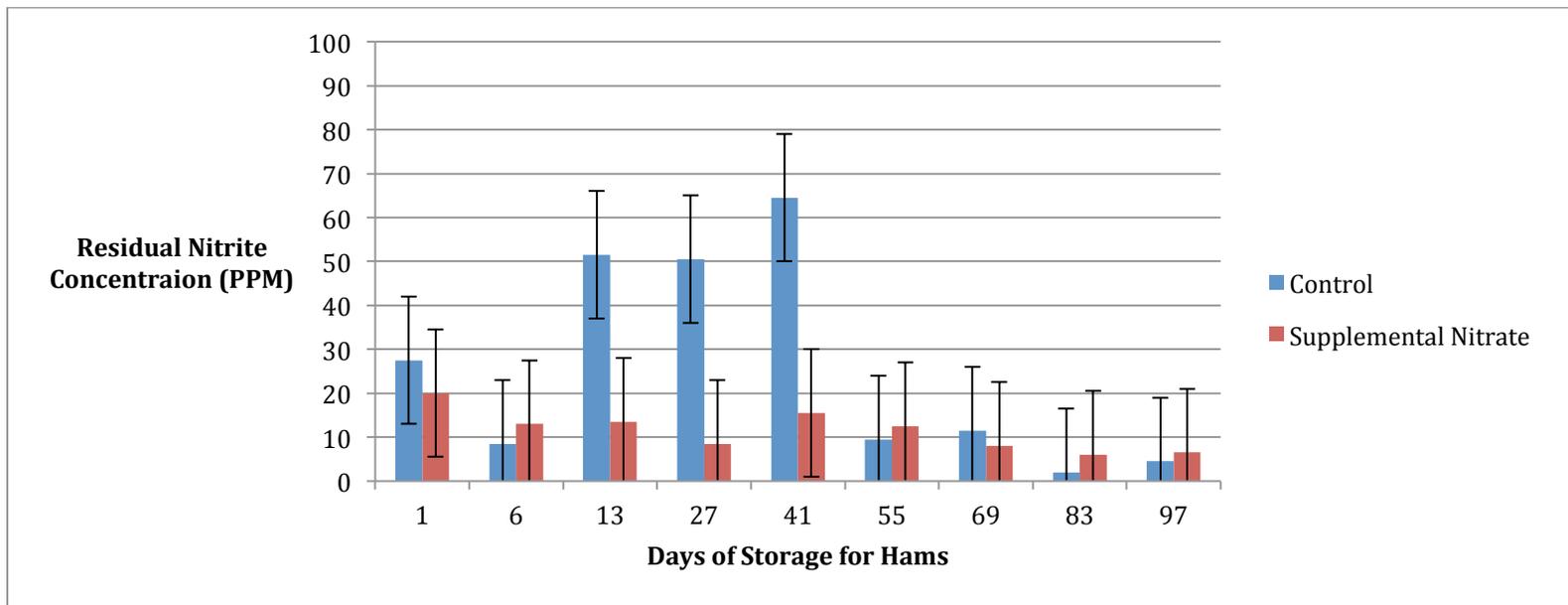
Figure 25. Least squares means for residual nitrite values of control and supplemental nitrate treatment of hams during storage. (S.E. = 14.5).

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