VEGETABLE NITRATE: ISOLATION OF NITRATE REDUCING BACTERIA FOR FERMENTATION OF NITRATE TO NITRITE AND USE OF VEGETABLE-DERIVED NITRITE TO INHIBIT GERMINATION OF *CLOSTRIDIUM* SPORES IN RTE MEATS

By

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VEGETABLE NITRATE: ISOLATION OF NITRATE REDUCING BACTERIA FOR FERMENTATION OF NITRATE TO NITRITE AND USE OF VEGETABLE-DERIVED NITRITE TO INHIBIT GERMINATION OF *CLOSTRIDIUM* SPORES IN RTE MEATS

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Abstract:

Nitrate reducing bacteria (NRB) are used to produce vegetable-derived nitrite allowing clean label status as per USDA-FSIS definition of 'natural nitrite' which can be used in place of sodium nitrite. Nitrite in processed meats is also responsible for cured meat color, microbial inhibition of spores, and contributes to flavor. One objective was to isolate NRB's that could possibly be used to generate nitrite from vegetable nitrate for use in processed meats. An 'on-agar' colony-screening assay was developed using the principle of the liquid nitrate reduction assay to detect the conversion of nitrate to nitrite on agar plates using M17 agar base plates. Samples that might have NRBs were spread-plated onto this base agar, and overlaid with sterile M17-Nitrate agar as the source of nitrate. Another objective was to use *Clostridium sporogenes* as a 'surrogate organism' for evaluating nitrite suppression of spore germination and identify permissive conditions to facilitate validation and confirmation of spore inhibition during the comparison of sodium and vegetable (celery) nitrite in cooked meat products. A three-strain spore crop from *Clostridium sporogenes* (ATCC 3584, ATCC 19404, and ATCC BAA-2695) was applied during ingredient formulation of low and high fat hotdogs that were divided into 3 batches (control without nitrite, hotdogs with sodium nitrite, hotdogs with celery nitrite). In both processes, effects with celery nitrite was compared to comparable levels of sodium nitrite. Manufactured hotdogs followed standard preparation procedures and cook processes (nitrite was used at 156 ppm). In assays, we examined spore germination at 5^{o}C, 15^{o}C, 35^{o}C so that we could best understand the contribution of temperature on the inhibition of spore germination by nitrite under various conditions, compared to controls processed without nitrite. Celery nitrite was as good or better than sodium nitrite in both low and high fat hotdogs and spore outgrowth was only observed at 35^{o}C abuse temperature conditions and not at 5^{o}C or 15^{o}C even after extended shelf life periods. HPLC analysis was used to quantify nitrite in culture fermentations and during hotdog manufacture and shelf life. The nitrite spore validation assay described herein allows easy determination of nitrite levels and whether nitrite can prevent spore germination under the most permissive conditions to help keep processed meat safe.

TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

II. REVIEW OF LITERATURE 3
Clostridium botulinium 3
Clostridium perfringens 4
Surrogate use of *Clostridium sporogenes* 5
The History of Nitrites and Nitrates 5
Nitrites and Nitrates 6
Regulations: United States Department of Agriculture (USDA-FSIS) 6
Advantages of Using Nitrates / Nitrites 7
Microbiological Advantages of Nitrate/Nitrite Addition 7
Qualitative Aspects of Nitrate/Nitrite Addition 9
Meat Curing 9
The Curing Reaction 10
Curing Accelerators 11
Factors affecting Cured Meat Color 12
Nitrates/ Nitrites and Humans 12
Concerns of Using Nitrates/Nitrites 13
Nitrate/Nitrite Poisoning 14
Nitrosamine and Cancer Connection 15
Alternatives to Nitrates/ Nitrites 16
Chemical Alternatives 16
Natural Source of Alternatives to Nitrates/Nitrites 17
Clean Label or Green Label Concept 19
Nitrate Reducing Bacteria 20
Vegetable Nitrate and Nitrate Reducing Bacteria's 21
Quantification of Nitrate/Nitrite content Using HPLC 23
Residual Nitrate Analysis 24
Application of Vegetable Based Nitrite in Hotdogs 24

Chapter Page

III. ISOLATION, CHARACTERIZATION, AND HPLC QUANTIFICATION: NITRATE REDUCING BACTERIA AND THE FERMENTATION OF NITRATE TO "NATURAL VEGETABLE NITRITE"
Abstract 26
Introduction 27
Materials and Methods 29
Bacterial Cultures and Media 29
The Nitrate Reduction Assay (in Broth) 29
The Nitrate Reduction Assay (on Agar) 30
Extraction of bacterial DNA 31
Isolation and Identification of Nitrate Reducing Bacteria 31
Vegetable Juice Extraction and Nitrate Detection 32
Fermentation Using Nitrate-Reducing Bacterial Isolates 32
Quantitation of Nitrate and Nitrite using High Performance Liquid Chromatography (HPLC) 33
HPLC Sample preparation 33
Statistical Analysis 34
Results and Discussion 34
Screening of Cultures Using the Nitrate Reduction Assay (Liquid) with Modified-Nitrate Broth 34
Screening of Bacterial Samples from Foods and Animals using Nitrate Assay Modified for on Agar 41
HPLC Analyis of Nitrate and Nitrite in Nitrate Broth, and After Bacterial Fermentation 42
Conclusion 49

IV. COMPARISION OF SODIUM NITRITE AND CELERY NITRITE ON THE INHIBITION OF SPORE GERMINATION OF *CLOSTRIDIUM SPOROGENES* IN LOW AND HIGH FAT All BEEF FRANKFURTERS

Abstract 50
Introduction 51
Materials and Methods 53
Bacterial Cultures and Media 53
Antibiotics, Disc Assay, and Media Validation of Resistance of Strains of *Clostridium sporogenes* 53

Chapter Page

Inducing Sporulation of Vegetative cells and Harvesting Spore Crops for Use as a Challenge Inoculum 55
Electron Microscopy of *Clostridium sporogenes* and Spore Crops 58
Screening of bacteriocin producing strains against *Clostridium sporogenes* (ATCC 19404, ATCC BAA-2695, ATCC 3584) 58
Meat formulations and Hotdog Manufacture 59
Statistical Analysis 60
Results and Discussion 62
Antibiotic Resistance of Inoculum Strains of *Clostridium sporogenes* and Use in Developing Selective Medium 62
Electron Microscopy of *Clostridium sporogenes* and Spore Crops 66
Manufacturing of All Beef Low Fat Hotdogs Challenged with Spores from *Clostridium sporogenes* 68
Manufacturing of All Beef High Fat Hotdogs Challenged with Spores from *Clostridium sporogenes* 73
Manufacturing of Low and High Fat All Beef Hotdogs Containing Bacteriocins and Challenged with Spores from *Clostridium sporogenes* 76
HPLC Analysis of Residual Nitrite/Nitrate 78
HPLC Analysis of Residual Erythorbate/Ascorbic Acid 80
Conclusions 83
REFRENCES 84

LIST OF TABLES

Table Page

Chapter III
Screened culture collection....37
Nitrate Reducing Bacteria Isolates....43
Time Course Conversion of Nitrate (Nitrate broth) to Nitrite 47

Chapter IV

1. Hotdog Ingredient Formulation for Low and High Fat hotdogs 61
2. Antibiotic Disc Assay Using B-D Sensi-Discs on Lawn of Various Strains of *Cl. sporogenes* to Determine Susceptibility or Resistance 63
3. Screening of Bacteriocin Producing Strains Against *Cl. sporogenes*....77
4. Low Fat Hotdogs, Residual Nitrite and Nitrate Analysis 82
5. High Fat Hotdogs, Residual Nitrite and Nitrate Analysis....82
6. Ascorbic Acid Analysis on Low and High Fat Hotdogs....82
7. Erythorbate Analysis on Low and High Fat Hotdogs....83

LIST OF FIGURES

Figure Page

CHAPTER III

1. Method for Nitrate Reduction Test in Broth 35
2. Nitrate Reduction Test on Isolates 36
3. Agar based Method for Isolation of Nitrate Reducing Bacteria 42
4. Extraction of Vegetable Juices 45
5. HPLC Analysis of Nitrate and Nitrite 46

CHAPTER IV

1. Determination of Antibiotic Resistance for Use in Selective Media 54
2. Schematic Diagram for Spore Crop Assay 57
3. Enumeration of Individual Strains of *Clostridium sporogenes* on Reinforced *Clostridium* Medium (RCM) Agar with Antibiotics 64
4. Enumeration of Individual Strains of *Clostridium sporogenes* on Reinforced *Clostridium* Medium (RCM) Agar with Antibiotics (2) 65
5. SEM Images of *Clostridium sporogenes* from agar 67
6. Manufacture of All Beef Hotdogs in the Meat Pilot Plant 69
7. All Beef Low Fat Hotdogs Shelf Life Study ($5^{o}C$, $15^{o}C$) 71
8. All Beef Low Fat Hotdogs Shelf Life study ($35^{o}C$) 72
9. All Beef High Fat Hotdogs Shelf Life Study ($5^{o}C$, $15^{o}C$, $35^{o}C$) 74
10. All Beef Low Fat and High Fat dogs with Bacteriocin 75
11. HPLC Analysis of Nitrate and Nitrite using C_8-RP Ion-pairing Chromatography 81

CHAPTER I

INTRODUCTION

Nitrite is widely used as a curing agent in the meat industry because of its multiple beneficial characteristics. Nitrite provides meat its characteristic flavor, color, and most importantly acts as an antimicrobial agent against *Clostridium botulinum* spores. Despite several advantages, it raises concern due to its role in potential carcinogen formation (i.e., nitrosamines). In recent years, consumer demand for more natural and organic food products has increased. Approximately, 41% of consumers in the United States seek clean label food products. This increasing demand for 'clean label' and 'all-natural food products' has the meat industry looking for an alternative to chemical nitrite. Vegetables are known to have natural nitrate in excesses, such as celery (3,151 ppm), turnip greens (9,040 ppm), beets (3,288 ppm), spinach (2,470 ppm) and melon (4,932). Food ingredient companies have taken advantage of this and have used nitrate-reducing bacteria to produce vegetable-derived nitrite from nitrate-containing vegetable extracts. Because the source of vegetable-derived nitrite is natural, USDA-FSIS has referred to this as 'natural nitrite'. The use of vegetable nitrite instead of sodium nitrite has allowed processors to claim green/clean label status for products in which this is used. Natural alternatives sources of ingredients such as celery juice concentrate, celery juice powder (CJP), sea salt, and evaporated cane juice are substituting synthetic ingredients for natural ones.

Nitrate reducing bacteria play a significant role in the conversion of vegetable nitrate into 'natural nitrite'. *Staphylococcus carnosus* is a commonly used nitrate-reducing bacterium for this conversion. Additional bacteria and strains could be useful for this purpose but this has not been fully explored or exploited. Considering the number of vegetable extracts that are high in nitrate, the industry lacks the variety of nitrate-reducing bacteria, some of which may be better suited for specific vegetable extracts, that can ferment vegetable nitrate efficiently and directly into 'natural nitrite'. The objective of our research is to screen and characterize nitrate-reducing bacteria and their ability to reduce nitrate to nitrite. The quantification of nitrite produced will be quantified using High-Performance Liquid Chromatography (HPLC). Further, as a testament to the application of natural nitrite in RTE meats, we are examining the ability to prevent germination of spores of *Clostridium sporogenes* in hotdogs as a surrogate organism to pathogenic *Clostridium* spore formers.

CHAPTER II

REVIEW OF LITERTAURE

Clostridium botulinum

Clostridium botulinum is a Gram-positive, rod-shaped, endospore-forming, heat resistant, strictly anaerobic, a ubiquitous pathogen, especially in soil. The spores of this organism are reverently heat resistant and can lie dormant for long periods (Houschild & Dodds, 1993). Clostridia's prevalence on animals and produce alike allows for multiple contamination points along the spectrum of food products from meats to vegetables. However, food product contamination does not indicate definite illness. *Clostridium botulinum's* ability to cause illness is through its exotoxin. This exotoxin is a neurotoxin produced during its germination phase in its life cycle. Germination is brought about in temperature as low as 3°C but only in anaerobic conditions (Elliott & Schaffner, 2001). There are seven types of botulinum neurotoxins differentiated by their serological reactions, denoted type A-G. *Clostridium botulinum* group's I-IV have the ability to produce these toxins. Of these seven types, four have the ability to infect humans by different disease manifestations: Foodborne botulism, intestinal botulism, infant botulism, and wound botulism (Peck, 1997) . Our interest is in foodborne botulism. Groups I and II are responsible for foodborne botulism. Botulism is a paralysis causing disease that occurs after ingesting the botulinum neurotoxin. Though this toxin is very dangerous, it is heat labile. (Woodburn et al., 1979).

Clostridium perfringens

Clostridium perfringens is a Gram-positive spore former that can be isolated throughout the environment. *C. perfringens'* affinity to *C. botulinum* is also in its ability to also cause illness. Unlike *C. botulinum*, however, the foodborne illness caused by *C. perfringens* is typically limited to gastrointestinal distress. Symptoms are typically being diarrhea and abdominal pain from damaged intestinal epithelial cells (Wahl et al., 2013). *C. perfringens* strains can be divided into five types denoted as A through E. The major toxin produced is what differentiates the specification of each strain. The alpha (α) toxin is found within all types of *C. perfringens*, while beta (β), epsilon (ε), and iota (ι) toxins identify the differences between types. Type A strains make up the majority of illness present in humans (Ohtani & Shimizu, 2016). *C. perfringens* produces toxin only during sporulation (Acheson et al., 2016) . Upon ingestion of foods contaminated with vegetative cells, an incubation period ranging from 5 to 24 hours is used for the organism to propagate and colonize the gut. The enterotoxaemia occurs shortly thereafter as *C. perfringens* lacks many genes for amino acid synthesis, relying on the degradation of the epithelial intestinal cell to produce sugars through an anaerobic glycolysis pathway (Ohtani & Shimizu, 2016). Thermal inactivation studies show that within 5 minutes of exposure to temperatures at 60°C, the biological activity of the *Clostridium perfringens* enterotoxin ceased (Naik & Duncan, 1978). This data, while revealing how sensitive the toxin is, does little to impede the illness rate as C. perfringens toxin production occurs in vivo (Bennett et al., 2013).

Surrogate use of *Clostridium sporogenes*

Surrogate organisms are used to minimize the danger associated with the study of pathogens (Sinclair et al., 2012). For *Clostridium botulinum* and *Clostridium perfringens*, *Clostridium sporogenes* is a preferable choice. There has been much research in the efficacy of using *Clostridium sporogenes* as a surrogate. *C. sporogenes* is a Gram-positive, endospore-forming, obligate anaerobic organism. As it is non-pathogenic and there being no significant

difference between the $D_{121.1\,°C}$ and z-values of *C. sporogenes* spores and *Clostridium botulinum* spores, it is a great candidate for surrogacy (Diao et al., 2014). It has also been shown that with pH 4.6 or less, both *C. botulinum* and *C. sporogenes*, have their germination and outgrowth inhibited due to the concentration of hydrogen ions (Brown et al., 2012). (Cui et al., 2010)When *C. sporogenes* was tested alongside *C. botulinum*, it showed similar concentrations of plant extract sensitivities (Cui et al. ,2010). In researching exosporium proteins of *Clostridium sporogenes*, (Janganan et al., 2016) determined that there was 75 – 100% amino acid match to many groups 1 strain of *C. botulinum*. In an attempt to characterize non-toxigenic *Clostridium spp.* for surrogacy, it was concluded a 2-strain cocktail consisting of strains 3676 and 3678 was sufficient for chilled food challenge testing. Their study, however, was not tested upon food items, but was solely tested in vitro (culture tubes) (Parker et al., 2015).

The History of Nitrates/Nitrites

Meat curing using nitrates and nitrites for food preservation evolved centuries ago. The history of meat curing reveals that it was discovered by accident. Earlier, salts were used to control food spoilage and extend their longevity for future use in times of scarcity. Meat that was processed with rock salt which was contaminated with saltpeter (potassium nitrite) resulted in a stable red color in the meat leading to the modern-day practice of purposeful use of nitrite (Faustman & Cassens, 1990). Recent scientific studies have proven that it is nitrite which gives meat it's characteristic red color. Currently, meats are also processed with color stabilizers, sweetening agents, seasonings, smoke, and other aids to improve quality and safety.

Nitrates and Nitrites

The USDA definition of cured meats includes the addition of sodium nitrite, sodium nitrate, potassium nitrite, or potassium nitrate. The traditional process for the manufacture of cured meat is to incorporate salt, nitrite, a reducing agent (such as ascorbate or erythorbate), and other adjuncts

such as seasonings. Curing of meats with nitrites is marked by its characteristic red color, distinct flavor, and texture properties, and most crucial is its microbiological preserving effect against *Clostridium botulinum* spores (Cassens, 1997).

Many scientific research findings have shown that nitrates are reduced into nitrites and consequently reduced further to nitric oxide and is attributed to the biochemical functions of microorganisms. The reduction of nitrate to nitrite is responsible for cured meat color, microbial inhibition, and flavor. The author stated that nitrate is converted to nitrites and further reduced to nitric oxide by microbial enzymatic action which reacts with myoglobin present in meat to produce a red color (Cerveny, 1980). Saltpeter (potassium nitrate) or sodium nitrate is reduced nitrite due to reducing action of bacteria (Gray et al., 1981).

Regulations: United States Department of Agriculture (USDA-FSIS)

The existing regulations on the use of nitrite and nitrate in the United States differ as per the manner of curing and the product being cured. For comminuted products, the maximum allowed concentration of sodium or potassium nitrite based on the weight of the fresh meat (green weight) is 156 ppm or 7g/45.4kg (USDA-FSIS, 1995). While for immersion cured or injected products, sodium or potassium nitrite is limited to 200 ppm, again based on the green weight of the meat block. In dry-cured products, the amount of ingoing sodium or potassium nitrite is restricted to 625 ppm. The USDA regulation allows a minimum of 120 ppm ingoing sodium nitrite for all cured "Keep refrigerated" products unless "… safety is assured by some other preservation process …" (USDAFSIS, 1995).

For comminuted, immersion cured and massaged products, maximum ingoing nitrate concentration is 1,718 ppm and 700 ppm, respectively, based on the green weight of the meat block. While for dry-cured meat products 2,187 ppm of ingoing nitrate is allowed. In the application of

both nitrite and nitrate, the final analytical concentration of nitrite must not exceed more than 200 ppm in the finished product (Sebranek & Bacus, 2007)

The U.S. Department of Agriculture (USDA-FSIS) regulations have prohibited the use of artificial flavoring, color, chemical preservatives, or synthetic ingredients for producing and labeling natural foods. Sodium nitrite and nitrate are not acceptable to use in labelled 'natural' products. In such products deemed 'natural', the meats are processed without the addition of sodium nitrite and replaced with natural nitrate or nitrite sources while maintaining characteristics similar to conventionally cured products. But the USDA regulations necessitate these products to be labeled as "uncured" and "no nitrate or nitrite added except those naturally occurring in added ingredients"(Sullivan et al., 2012a).

Advantages of Using Nitrates / Nitrites:

Microbiological Advantage of Using Nitrates/Nitrites

The use of nitrates and nitrites in the meat and poultry industry is beneficial for the production of products with enhanced food safety and prolonged shelf-life with an excellent storage ability. The role of nitrite as an antimicrobial agent in meat and its action against germination of spores produced by *Clostridium botulinum* is significant to prevent the introduction of botulinum toxin in meat products that are produced during spore germination.

Clostridium botulinum is a Gram-positive, anaerobic spore-forming rod. *C. botulinum* is known to be ubiquitous in soil and aquatic deposits. *C. botulinum* produces 7 immunologically diverse toxins, labeled by the letters A-to-G. *Clostridium botulinum* forms spores under stressful conditions that can endure standard cooking and food-processing measures. Specific conditions may allow spore germination: anaerobic atmosphere (i.e., vacuum packaging), nonacidic pH, low salt and sugar content, and temperature of 40^{o}C-121^{o}C. The consumption of food contaminated with botulinum toxin causes the illness, foodborne botulism. From 1990 to 2000, 160 cases of foodborne botulism were recorded affecting 263 people in the United States (Sobel, 2005).

The effect of nitrite on *C. botulinum* is significant as it is highly effective against *C. botulinum* spores. Earlier, it was thought nitrite only has a role in color development of meat but gradual progress in meat processing and research has proven the anti-botulinum property of nitrite. Only 5 ppm is needed for color development while 20 ppm of nitrite is needed for color stability in meat products. It was found that as nitrite levels increase so does the control of *C. botulinum* germination and reduction of toxin production. Nitrite also works synergistically with salt and other accelerators (ascorbic acid) which aids its anti-botulinum action (Ramarathnam & Rubin, 1994) (Pearson & Tauber, 1984).

Nitrite inhibitory action on bacterial spores is also pH-dependent, i.e. more effective at acidic pH values. Roberts (1975) has stated that there is a significant interaction between heat, salt, and nitrite, and experimentally validated that nitrite is more effective with this combination of ingredients than when present alone. Several additional factors may come into play such as competitive flora, iron availability in the product, and other factors (i.e., ascorbate, erythorbate, phosphate). Whether the product is cooked and/or cured, involvement of packaging, and storage temperatures are additional crucial factors (Roberts & Gibson, 1986). *Clostridium botulinum* has two different stages in its life cycle, the vegetative cell and spore states. The nitrite anti-botulinal effects in thermally processed meat products depend on these stages. The primary role of nitrite in control of *C. botulinum* is by inhibiting the germination of spores to develop into vegetative cells, and secondly, by preventing the multiplication of vegetative cells if any develop from spores (Pierson et al., 1983).

Qualitative aspects of Nitrate/Nitrite Addition

Nitrite also plays a substantial role in qualitative aspects of processed meat products and thereby is recognized as a multifunctional food additive. Alongside its antimicrobial property against *C. botulinum*, nitrite is responsible for typical cured meat color and flavor, and is also reported to have antioxidant properties (Roberts & Gibson, 1986).

The most evident effect of adding nitrite in meat products is the development of color i.e. pink/red color in cured meats. The color results due to the reaction of nitrite with heme pigments present muscle. The color formation is a two-step process: the first nitrite is reduced to nitric oxide and iron in the heme pigment (myoglobin) is reduced to the ferrous state resulting in unstable nitric oxide and myoglobin compound. Secondly, during thermal processing unstable compound is converted into more stable compound i.e. nitrosohemochrome (Pegg & Shahidi, 2008).

Nitrite is known to provide characteristic fresh meat flavor which is an additional role to color development and antimicrobial property. Though, studies have been carried out to explain nitrite's relationship to the development of the typical cured flavor in the meat they fail to show any substantial results (Pierson et al., 1983).

Meat Curing

Meat curing is defined as the addition of salt, sugar, spices, and either nitrate or nitrite for aiding in flavor and preservation which often alters the chemical, physical, and microbiological properties of meat products. Meat curing began historically to preserve meat. Today, meat curing is required to address consumer demands for flavor, color, and taste. The sensory aspect of meat products also plays a vital role as it depends on the process of meat curing (Sindelar, 2006).

The curing process is multifaceted as different chemical reactions, use of curing agents, meat pigment changes, as well as sensory effects the final product. This multifaceted process has altered either use of varying raw materials, formulations, and processing that leads to different kinds of products that provides alternatives to various consumer preferences (Cassens et al., 1979).

The Curing Reaction

Sodium chloride and sodium nitrite are two distinctive ingredients in cured meat and are responsible for the color, texture, flavor, safety, and storage stability, giving processed meat its unique characteristics. Exclusion of either salt or nitrite alters the meat characteristics for which it

is no longer considered 'cured'. It can be concluded that salt and nitrite are both vital ingredients of typical cured meat products (Pegg & Shahidi, 2008; Pegg et al., 1997).

Nitrite is a generic term used to denote the anion NO_2-, and the neutral nitrous acid HNO_2. Sebrank and Fox (1985) have explained that nitrous acid HNO_2 forms nitrosating compounds (N-nitroso producing) which are the compounds involved in nitrosamine formation. The reaction also depends upon the pH of the meat and pKa value of HNO_2 (Sebranek & Fox Jr, 1985).

Nitric oxide (NO) is another important modified form of nitrite. Nitric oxide binds with heme pigments in meat forming complexes like meat nitrosylmyoglobin, nitrosylhemoglobin, and dinitrosylhemochrome which gives the pink and red colors in cured meats. The total nitrous oxide produced during curing is dependent on pH, temperature, and time.

As nitrite is supplemented to meat pieces, a browning effect is observed due to nitrite acting as a strong heme oxidant. Myoglobin and oxymyoglobin are oxidized to metmyoglobin by nitrite. An unstable complex nitrosylmetmyoglobin is formed as an intermediate complex but as meat is thermally processed it forms stable compounds like nitrosylmyochromogen or nitrosylhemochrome(Cassens et al., 1979). Nitrate is used as a curing agent but it requires an additional step to reduce nitrate into nitrite. The reduction of nitrate to nitrite is achieved by nitrate-reducing bacteria. Bacterial reduction of nitrate to nitrite is attained either by the natural flora of meat or intentional use of starter cultures composing nitrate-reducing bacteria (Sanz et al., 1997). Several authors have suggested that some nitrite added to meat during the curing process is converted back to nitrate. This theory is credited with information from Dethmers and Rock (1975) that nitrous acid might yield nitric oxide and nitrate from the oxidation of nitric acid by oxygen to yield nitrite which later reacts with water to form nitrite and nitrate. They suggest that sodium ascorbate, used as cure accelerators, might also play a role in the conversion of nitrite to nitrate (Dethmers et al., 1975). The nitrite is converted to nitrate due to the inert properties of nitrite in a

cooked cure meat system (Price & Schweigert, 1987). Residual nitrite and nitrate analysis of processed meat might help in establishing the significance of nitrite conversion to nitrate.

Curing accelerators

The reduction of nitrite to nitric oxide is a key step for cured meat color development and as well for other cured meat properties. The use of reducing compounds and acidulants catalyzes the reduction of nitrite to nitric oxide. Ascorbic acid, sodium ascorbate, erythorbic acid, and sodium erythorbate are reducing compounds that are widely used for cured meat processing. The role of reductants is beneficial as they allow for high volume, rapid processes, and have enabled the development of high-speed, continuous processing lines because less time is required for nitric oxide production before cooking and color fixation. That's why these reducing compounds are called curing accelerators. Ascorbic acid, ascorbate, and their isomers, erythorbic acid, and erythorbate were authorized for use in cures by the USDA since the 1950s. USDA FSIS states that since cure accelerators are used in conjunction with nitrite and nitrate, they are not permitted for use in non-meat curing systems (USDA, 1995).

Curing accelerators are an important part of the curing process since nitrite must be reduced to nitric oxide and the iron portion of muscle pigments be reduced from the ferric (+3) to the ferrous (+2) state. The curing accelerators have diverse functions, but their primary function is to promote the reduction of nitrite to accelerate curing reactions and catalyze the formation of cured meat pigments. The reductants are capable of donating electrons speeding up the curing related reactions readily and rapidly. Cure accelerators also provide suitable conditions for the chemical change of nitrous acid to nitric oxide and the subsequent reactions with reduced myoglobin. The secondary function of cure accelerators is as an antioxidant to help stabilize the color and flavor of cured meat products as they act as oxygen scavengers preventing fading of cured meat color. Ascorbic acid can be added alone and also along with erythorbate to cured meat at a maximum concentration of

469 ppm, based on the weight of the meat. Sodium ascorbate and sodium erythorbate are limited to 547 ppm (USDA-FSIS, 1995).

Factors affecting cured meat color

The color of cured meat products is essential for customer perception. Several factors are affecting cured meat color, biological factors, and other extrinsic factors such as light, air causing undesirable fading or discoloration. The most common biological factor affecting cured meat color stability is microbiological contamination and growth. Extrinsic factors include oxygen, light, and dehydration (Draudt & Deatherage, 1956). The intermediate nitrite compound formed during the curing process such as nitrosyl hemochrome and nitrosyl myoglobin are unstable and are vulnerable to oxidation in the presence of oxygen and light. The oxygen is significant for the color stability of cured meat products (Gotterup et al., 2008). The researchers observed that oxygen present in the headspace of packaging material affects the color stability of the product. The oxygen in the headspace is responsible for color fading in the modified atmosphere packaged ham. To counter the oxygen impact, the researchers suggested the use of vacuum packaging, and it was observed that discoloration was four times less than that of modified atmosphere packaging (Eleftheriadou et al., 2002).

Nitrates, Nitrites, and Humans

Nitrate is an important constituent of a human's chemical environment. The major source of human exposure is from food and drinking water. Nitrates might occur naturally in food or as an additive for food safety purposes within the limit strictly controlled by regulation. Vegetables are the primary source of dietary nitrate while the level of nitrate present in vegetables varies depending on the type of vegetable, its source, conditions of cultivation, and storage. Nitrate in mammalian systems is readily converted to nitrite either by bacterial or enzymatic action and subsequently

reacts with amines, amides, and amino acids to form N-nitroso compounds. Nitrate is considered to be of comparatively lower toxicity than nitrite and N-nitroso compounds.

Nitrite is synthesized endogenously by enzymatic action or bacterial reduction in the human body. Nitric oxide synthesized enzymatically from nitrites plays a significant role in normal body functions, control of blood pressure, in immune response and wound repair, and neurological functions (Archer, 2002). Nitric oxide is also known for its antioxidant properties. Nitric oxide is suggested to inhibit metabolic pathways to block growth of bacterial cells that cause certain diseases to act as a defensive wall (Cassens et al., 1979).

The World Health Organization has estimated the mean daily take of nitrate at 43 to 141 mg (Sindelar & Milkowski, 2012).The primary source of nitrate human intake is derived from vegetables which account for 80-90% of daily intake while drinking water also accounts for 10-14% of the portion of daily intake. Spinach, beets, radishes, celery, lettuce, cabbage, and collard greens are a few examples of various vegetables found to contain high concentrations of naturally occurring nitrates. Leafy vegetables such as lettuce and spinach tend to have higher levels of nitrate than seeds or tubers. The nitrate accumulation in vegetables depends on several factors such as applied fertilizers that result in a higher uptake of nitrogen, thus higher nitrate content. Nitrate uptake, nitrate reductase activity, growth rate and growth conditions like soil temperature, the intensity of light, level of rainfall, all significantly affect the ultimate nitrate content of vegetables (Sindelar & Milkowski, 2012).

Concerns about using Nitrates/Nitrites

In the late 1960s and mid-1970s, nitrite utilization in cured meats became the source of grave concerns to the extent that the U.S. government even viewed as an absolute restriction on its use in meat items. Investigators publishing on the role of nitrite in the formation of N-nitrosamine in meat products have been concerned about the intake of nitrates and nitrites in humans and the likelihood they form nitrosating compounds which subsequently develop into toxic carcinogenic

N-nitroso compounds such as N-nitrosamines.

Regardless of all of its desired effects, nitrite can react, under specific conditions, with amines and amino acids during thermal processing of cured meats producing N-nitrosamines. The compounds with carcinogenic, mutagenic, and teratogenic properties are formed such as N-nitrosodimethylamine (NDMA) and N-nitrosopyrrolidine (NPYR) which are experimentally confirmed to be carcinogenic in animals. In light of their conceivable connection to cancers in man, significant consideration has been centered on the presence of N-nitrosamines in nitrite cured meat products (Pegg & Shahidi, 2008).

Nitrate/Nitrite Poisoning

Nitrite and nitrate can be a precarious and lethal chemical ingredient, if not used in a limited amount. The lethal dose of nitrite is reported to be above 300 mg/kg (ppm) of body weight. It is the same reason that USDA-FSIS has a restriction in the limit of using nitrates/nitrites in meat products (Pierson et al., 1983). Nitrite has been known to be toxic at high concentrations and direct consumption of sodium nitrite can cause cyanosis due to the formation of methemoglobinemia. The other toxic effect of nitrite is lowering the oxygen transport in the bloodstream due to the oxidation of hemoglobin to methemoglobin (Gautami et al., 1995). Cyanosis is the characteristic blue shade of the skin seen when the measure of un-oxygenated hemoglobin is extremely high. This means insufficient oxygen is circling in the blood to organs and tissues in the body. Methemoglobinemia is where hemoglobin is oxidized to the methemoglobin in which the iron in the heme component of the atom has been oxidized from the ferrous (+2) to the ferric (+3) state. At the point when this happens, the hemoglobin is unable to successfully move and discharge oxygen to organs and tissues in the body. The tissues and organs are then "starved" for oxygen and can slow or stop their function contingent upon the seriousness of the episode. Dietary methemoglobinemia is called ‘‘Blue Baby syndrome’’ in infants under six months where the oxidation of hemoglobin to methemoglobin in erythrocytes due to nitrite poisoning causes skin and organs to turn blue (Archer, 2002).

Nitrosamine and Cancer Connection

Nitrite became a problem in cured meats when the carcinogen nitrosopyrrolidine was found in bacon (Archer, 2002). Nitrite is a reactive chemical, acting as an oxidizing agent, and in the low pH conditions of meat it forms nitrous acid which subsequently forms reactive species like nitric oxide to become the active nitrosating agent. The ability of nitrite to form nitrosamine in cured meats first came to light in the early 1970s, and they form in specific conditions such as in presence of secondary amines, nitrite availability, at neutral pH, and thermally processing temperatures greater than 130^{o}C.

Nitrite use as a preservative in foods, mostly meats, has captured attention in its role in the formation of nitrosamines, as nitrite reacts with secondary and tertiary amines present in foods. An early concern of nitrosamines began as it was found to be associated with liver damage in animals due to the ingestion of food containing dimethylnitrosamine. More than 65 cases of nitrosamines detected in multiple foods such as cheese, meats, mushrooms, and alcoholic beverages raised a serious concern against nitrite(Pierson et al., 1983). The World Cancer Research Fund (WCRF) made a statement that there is a shred of conclusive evidence associated with the consumption of red and processed meat with the emergence of colorectal cancer in 2007. Along with World Cancer Research Fund (WCRF), the International Agency for Research on Cancer (IARC) also showed in their study that with every increase of 50 g of processed meat consumption per day, the risk of colorectal cancer rises by 18%, while with every increase of 100 g of red meat consumed per day the risk of colorectal cancer rises by 17% (Crowe et al., 2019).Though nitrite and processed meat are linked with cancer there is a lack of enough evidence and consensus opinion among the scientific literature. To minimize the risk association of nitrite with cancer, the regulatory agencies like USDA-FSIS has limited the ingoing nitrite in processed meat products. The limited use of nitrite does limit the nitrosamine formation and allows it to impart its characteristic and antimicrobial activity.

Alternatives to Nitrates/ Nitrites

Nitrite has been added to a variety of meat products due to its ability to preserve microbial quality, flavor, and color and to prevent lipid oxidation. Consumer awareness about the health concerns linked to synthetic additives has created a demand for more organic and natural meat. Thus, meat industries are focused on the development of nitrite alternatives. The major obstacle for the meat industry is to seek ways to reduce supplemented and residual nitrite in cured meat to minimize the nitrite intake. The continuous interest in the development of alternatives from natural sources which are proved to comparatively healthier as the consumer demands for salt and nitrite reduced in meat products (Alahakoon et al., 2015).

The risks of botulinum poisoning in cured meat products are prevented by nitrite. The meat industries due to the lack of any suitable alternatives are still committed to the use of nitrite. The Food and Drug Regulations in the USA and other regulatory bodies in Canada and Europe have negated the use of any food additive which in itself is carcinogenic or produces carcinogens in foods. Hence, the use of nitrite in cured meats must be reduced with the availability of effective and safe alternatives (Pegg & Shahidi, 2008).

Chemical Alternatives

The chemical alternatives to nitrates/nitrites as preservatives may have similar harmful effects on the health of consumers. Though, there have been several studies suggesting the use of chemicals like sulfur dioxide, ethylene diamine tetraacetic acid (EDTA), butylated hydroxyanisole (BHA), fumarate esters, and sodium hypophosphite are capable of pathogen inhibition and prevention of lipid oxidation. The risk associated with the use of chemicals and consumer's demand for natural and safer alternatives contradicts the use of chemicals (Gassara et al., 2016).

The nitrite with its multifunctional properties is highly unlikely to be replaced with a single compound. Efforts have made to develop alternative products that can perform a selected function

of nitrite. For example, the use of colorants to substitute nitrite which can give a similar characteristic pink color to cured meat, but toxicity problems persist as a problem in meat processing (Shahidi & Pegg, 1995). Also, vitamins are a possible effective alternative to nitrates and nitrites in meat products such as alpha-tocopherol. Further, studies have proven its inhibitory action against the growth of pathogens. The use of vitamins is not cost-effective and it could make meat processing even more expensive (Gassara et al., 2016). Chemical alternatives have their pros and cons yet, more natural and organic alternatives for nitrite are preferred over chemicals.

Natural Source of Alternatives to Nitrates/Nitrites

According to the USDA Food Standards and Labeling Book (USDA, 2005), products are labeled "natural" if the meat components and their ingredients are minimally processed and are safe for human consumption and any of the process doesn't change the raw product, or the product is intact. The use of natural and organic ingredients during meat processing has gained popularity due to consumer preference for organic and natural foods. Naturally and organically processed meat may be labeled as 'No added preservatives' or 'No nitrites added'. Naturally processed meat products include natural ingredients, such as vegetable powder with high nitrate/nitrite content. A few of them are currently commercially available such as celery and cabbage powder. In addition to the use of natural nitrite alternatives, cherry powder and tomato pulp powder use in meat products have been examined to counter the potential health-risk associated with nitrates due to the formation of nitrosamines (Ko et al., 2017). Alternative sources of nitrite such as celery juice concentrate, celery juice powder (CJP), sea salt, and evaporated cane juice are replacing synthetic nitrite in naturally cured meat products. Vegetables are reported to have high levels of natural nitrate, such as celery (3,151 ppm), turnip greens (9,040 ppm), beets (3,288 ppm), spinach (2,470 ppm) and melon (4,932 ppm) (Djeri & Williams, 2014). Currently, celery powder is used extensively as a vegetable nitrate source in natural and organic meat processing due to its subtle flavor and the minimal color effect on the product. Therefore, more studies and research is required

to identify other natural alternatives of nitrite from vegetable sources to replace traditionally used sodium nitrite in processed meat products as suggested by Ko et al. (2017).

Nitrate is not a reactive curing compound, thus vegetable nitrate reduction to natural nitrite is essential for curing reactions. The natural curing process using naturally obtained nitrate is carried out with an additional step of bacterial reduction of vegetable nitrate into nitrites by specific nitrate-reducing bacteria. Celery juice and its powder are known to have high nitrate content and thus are used in combination with lactic acid starter culture in the production of naturally cured meat. Currently, pre-generated nitrite (i.e. natural nitrate is converted to nitrite by microorganisms) is utilized to ease and speed up curing time (Djeri & Williams, 2014). Nitrate to nitrite reduction can be achieved with a bacterial starter culture, such as *Staphylococcus xylosus* or *Staphylococcus carnosus*, possessing a specific nitrate-reducing ability (Hwang et al., 2018).

The challenging aspect of using vegetable sources of nitrate is to control the level of the nitrate/nitrite utilized in meat processing, the color, flavor, and characteristics associated with nitrite as well to meet the consumer demands in the market. The pathogen reduction achieved using synthetic nitrite might be difficult to achieve by natural sources of nitrite. When celery nitrate was first used as a source of nitrite, it was sold as such and companies would self-ferment the product to generate vegetable-derived (fermented) nitrite. However, this resulted in variable levels of nitrite in the resulting products. Ingredient companies therefore pre-fermented the vegetable extracts and standardized the nitrite levels to eliminate these variabilities in product nitrite levels.

Clean label or Green label concept

The ‘clean label’ bears notions of quality, trust, and transparency. Food labeling has a significant impact on consumer perception and choice of food products. Food mantras such as “you should not eat anything with more than five ingredients you can’t pronounce” or “don’t buy it if you cannot pronounce it” are trending among consumers. Food bloggers recommend consumers that “a ‘clean label’ placed on the pack means the product can be sited as ‘natural’, ‘organic’ and/or

'free from additives/preservatives'." Numerous food producers are adapting to consumer demands for 'clean labels'. If the ingredients in the label are short, simple and feature minimally processed ingredients, and sounds like items that can be found in customer's kitchen as well as do not include names that sound like chemicals can be called a 'clean label'. Clean labeling has become a standard practice in the food industry, with consumers demanding shorter and more familiar ingredients lists and industries answering with highlights of the naturalness of their product.

According to the United States Department of Agriculture (USDA-FSIS), Food Safety and Inspection Service (USDA-FSIS) labeling standards as defined in 21 CFR 101.22 that meat and poultry products labeled "natural" are not permitted to contain any artificial flavoring. 'Clean-label' foods are not constrained to FSIS 'natural' or 'organic' definitions but have simple ingredients that are familiar to consumers and perceived as being derived from a nonchemical source, such as vinegar, flavorings, cultured sugars or dairy ingredients, or ingredients derived from plant material. Nitrates/Nitrites are reckoned as chemical preservatives based on the definition of "natural" and are explicitly listed as banned ingredients for products following organic labeling criteria (Mcdonnell et al., 2013).

The processed meats that are labeled "natural" are required to comply with the definition by the USDA Food Standards and Labeling Policy Book (USDA, 2005). This definition obliges that a natural product:

> "does not contain any artificial flavor or flavoring, coloring ingredient, or chemical preservative (as defined in 21 CFR 101.22), or any other artificial or synthetic ingredient; and the product and its ingredients are not more than minimally processed".

The term "minimally processed" includes:

> "traditional processes used to make the food edible or to preserve it or to make it safe for human consumption, e.g., smoking, roasting, freezing, drying, and fermenting, or those physical processes which do not fundamentally alter the raw product, e.g. grinding meat" (USDA, 2005) (Sebranek & Bacus, 2007).

The lack of a proper definition of "natural" is a growing concern as it needs more clarity and specificity. The use of "natural" on labels has created confusion among consumers, retailers, industry, and regulators as they demand more transparency in food labeling. Sebranek and others have put forward an example such as the use of salt which has dual functions as flavorings or "natural" preservatives are not clearly defined on its labeling requirement as well as beets, a debatable "natural" source of pigment, is disallowed as a coloring agent in natural products, while paprika, also a natural source of pigment, is accepted by USDA as a seasoning ingredient (Sebranek & Bacus, 2007).

Nitrate Reducing Bacteria

Nitrate reducing bacteria (NRB's) are defined as their ability to reduce nitrate into nitrite or other nitrogenous compounds. This reduction is facilitated by the nitrate reductase enzyme produced by these bacteria. Nitrate reducing bacteria comprise of membrane-bound nitrate reductases with an active site in the cytoplasm. *Staphylococcus carnosus* is a popular nitrate-reducing bacteria used in the industry. Coagulase-negative staphylococci (CNS) are commonly found as natural flora of fermented meat products. The role of CNS is significant in the improvement of sensory properties of fermented sausages, by reduction of nitrates to nitrite and then to nitrous oxide, by preventing rancidity through peroxide decomposition, and by producing flavor and aroma compounds through proteolysis and lipolysis (Casaburi et al., 2005).

Staphylococcus species are used extensively as starters cultures in various types of fermented meat products. The different physiological traits of staphylococci, as well as other lactic acid

bacteria, have an impact on the product quality. The microbial reduction of nitrate and nitrite generates biochemically active nitrous oxide, which may bind with myoglobin more effectively and thus producing a more suitable product for human consumption. Also, hydrogen and organic peroxides are formed recurrently as products of microbial metabolism. Therefore, the presence of catalase-producing species significantly improves the appearance and safety of raw fermented products (Miralles et al., 1996)

Staphylococci are fermentative microorganisms with the ability to survive at low pH, low water activity, high salinity, and osmotic stress. Certain species of staphylococci are found in 'naturally fermented meat products' in high numbers and associated with the development of desired flavors. Staphylococci also have proteolytic and lipolytic activities which results in the formation of low molecular weight compounds, including peptides, amino acids, aldehydes, amines, and free fatty acids responsible for flavor development in fermented meat products. Additionally, they have anti-oxidative enzymes together with catalase and superoxide dismutase (SOD) that reduce the formation of unwanted compounds such as superoxide radicals (O_2) and hydrogen peroxide, precursors of oxidative rancidity in food (dos Santos Cruxen et al., 2017).

Vegetable Nitrate and Nitrate Reducing Bacteria's

A natural curing process entails the use of naturally occurring nitrates, which are reduced to nitrite by specific microorganisms, such as lactic acid bacteria or nitrate-reducing bacteria in the meat products. Celery juice and its powder, are commonly used together with lactic acid starter cultures in the production of naturally cured meat products as an alternative to sodium nitrite (USDA FSIS, 2012). Cherry powder is used as a naturally occurring source of ascorbic acid. Moreover, meat processing is made easier and faster with an application of pre-generated nitrite from a natural source which is celery juice powder (CJP) nitrate converted to nitrite by nitrate-reducing microorganisms (Djeri & Williams, 2014).

Currently, two types of meat and poultry products exist in the market: uncured (without any nitrate or nitrite) and no nitrate/nitrite added (replacing traditional nitrate/nitrite with natural source). A natural nitrate source and reducer are used to avoid the direct addition of sodium nitrite. Vegetables like celery, cabbage, and lettuce are well-known to contain significant amounts of nitrate. However, nitrate is not a prime reactive species for curing reaction thus needed to reduce into nitrite before involved in curing reactions. The crucial step of nitrate to nitrite reduction can be achieved by microorganisms either present in the natural flora of meat or by the deliberate addition of microorganisms with nitrate-reducing properties (Sindelar et al., 2007). If vegetable products with an adequate amount of nitrate are added to processed meats in combination with a nitrate-reducing starter culture, then sufficient nitrite can be produced to achieve curing reactions. Vegetable extracts especially derived from celery containing a significant amount of nitrate (several thousand mg/kg of extract) are commercially available to use in the production of natural and organic processed meats. This process has a limitation as the nitrite concentration produced by nitrate reduction cannot be measured accurately as it depletes as soon as it is produced. Thus, a concern is raised for the safety of these products since nitrite is crucial for antimicrobial effectiveness and its amount correlates with its effectiveness. While, if excess nitrite were produced, it also raises the risk for nitrosamine formation (Krause et al., 2011). Hwang and others have investigated the effects of natural nitrites from fermented vegetables on the shelf stability of raw and cooked pork sausages. They found fermented spinach extract being more useful for retaining color development and for inhibiting lipid and protein oxidation. They concluded that pre-converted nitrite from spinach can be used as a natural nitrite as another potential source or alternatives for processed meat products (Hwang et al., 2018).

Quantification of Nitrate/Nitrite content Using HPLC

A wide array of analytical methods for the determination of nitrate and nitrite are applied to the analysis of food, water, plants, and other matrices. Spectrophotometry, high-performance

liquid chromatography (HPLC), gas chromatography (GC), polar graphic method, and capillary electrophoresis (CE) are a few methods used. Spectrophotometric methods are conventionally used to determine nitrate and nitrite quantity in food but, its lack of the sensitivity for detection of trace levels of analytes is deemed unreliable for results obtained due to sample matrix interferences. Capillary electrophoresis is another separation technique that has the advantage of fast simultaneous detection of a wide variety of anions, small sample size, and low buffer consumption. The ionic chromatography (IC), and HPLC methods are branded by faster, more accurate, and higher sensitivity in comparison to the spectrophotometric methods (Chou et al., 2003).

The excessive intake of nitrite and nitrate in the diet may cause toxic effects since methemoglobinemia is produced by oxidation of hemoglobin by nitrite. As well as nitrite and nitrate are associated with the formation of carcinogens like nitrosamines. Thus, the maximum amount of nitrate and nitrite added to any meat product is limited and controlled by regulatory agencies. The amount of nitrite added to a product also depend on its type.

The spectrophotometric method uses a Griess-Romjin reaction for the determination of nitrite in foods is based upon its ability to convert aromatic amines into diazonium ions, and these ions are coupled to another aromatic compound to produce an azo dye. The most common reaction used is between sulphanilamide and N-(1-naphthyl) ethylenediamine as the target amine and coupler, respectively, with the product of the reaction detected at 540 nm. To detect nitrate, it is first reduced to nitrite and determined by the same type of reaction. The chromatography is regarded as more attractive since it is more rapid, sensitive, and selective than methods based on reduction/colorimetric. Ion-chromatographic methods are widely studied for the separation of nitrite and nitrate. Ion-pair HPLC methods are relatively cheaper in instrumentation and columns as well beneficially used in laboratories conveniently. In addition to that, the theoretical column efficiency of ion-pair HPLC is better than that of an IC column, with an appropriate choice of ion-

pair reagent. Despite that, the use of ion-pair HPLC in terms of residual nitrate and nitrite analysis lacks a clear picture. (Cheng & Tsang, 1998) (Romitelli et al., 2007).

Residual Nitrite Analysis

Nitrites added to meat products are known to depleted rapidly as a result of such factors as heat treatment, product composition, storage temperature, etc. Residual nitrate levels in food products are very crucial due to possible reactions with amines and amides forming carcinogens, as well their role as a source of nitrite in human nutrition(Cassens et al., 1978; Pegg & Shahidi, 2008). Nitrite binds with protein in meat also known as protein bound nitrite (PBN). PBN is a result of nitrite added during the curing process of meat. The recent development in processing technologies is trying to minimize the residual nitrate content in finished meat products. The nitrite interactions with the different biological complexes are studied to understand the impact of residual nitrite in a health hazard.

Application of vegetable-based nitrite in hotdogs

The manufactures of hotdogs with an application of vegetable-based nitrite which are labeled as “uncured products” require the proper amount of ingoing nitrates/nitrites. Celery powder manufacturers have suggested inclusion percentages based on the total meat weight per batch of 0.2-0.4% of celery juice powder is a commonly used amount in meat processing (Ruiz-Capillas et al., 2007).

Celery contains naturally occurring nitrates and celery juice is treated with nitrate-reducing bacteria that convert the nitrates into nitrites and is then dried to make cultured celery powder. These products can contain a significant amount of naturally fermented nitrites that are standardized with sea salt. Florida Food Products (Eustis, FL) sells VegStable® 506 which is a water-soluble dried powder consisting of celery powder and sea salt is one of the sources of naturally occurring nitrite. The amount of celery juice powder used is based on meat green weight along with the

desired quantity of nitrite enough to provide antimicrobial properties to the meat product. Cherry powder obtained from cherries is known to have a high amount of ascorbic acid (Vitamin C). The dried cherry powder is used as a cure accelerator in an amount ranging from 250 ppm to 469 ppm. Florida Food Products sells Veg Stable® 525 as one source of dried cherry powder (Ballard & David, 2019).

Sindelar et al., (2007) have studied the treatment combinations containing 0.20% vegetable juice powder (VJP) and starter culture *S. carnosus*. They've found the treatment to be comparable to sodium nitrite added control. The color, cured pigment, residual nitrate, and residual nitrite for the treatments were measured for validation of the occurrence of curing reactions. They've observed a limited difference between treatments and control. The ingoing nitrate/nitrite levels also affect the residual nitrate/nitrite as well as off-flavors of frankfurters (Sullivan et al., 2012b).

CHAPTER III

ISOLATION, CHARACTERIZATION, AND HPLC QUANTITATION: NITRATE REDUCING BACTERIA AND THE FERMENTATION OF NITRATE TO "NATURAL VEGETABLE NITRITE"

Abstract:

Nitrate reducing bacteria (NRB) are used to produce vegetable-derived nitrite allowing clean label status as per USDA-FSIS definition of 'natural nitrite' which can be used in place of sodium nitrite and is responsible for cured meat color, microbial inhibition of spores, and flavor. The current method for detecting the conversion of nitrate to nitrite is an 'in-liquid' assay using 2 added chemical reactants. Screening isolates for ability to reduce nitrate is tedious with this method because it has to be administrated to pure cultures. We developed an 'on-agar' colony-screening assay using the principle of the liquid nitrate reduction assay to detect the conversion of nitrate to nitrite on agar plates using M17 agar base plates (1.5%; Samples that might have NRBs were spread-plated onto this base agar, and overlaid with sterile M17-Nitrate agar as the source of nitrate. After incubation and development of colonies embedded in the sandwich overlay, nitrite was detected using a thin (plain) overlay agar layer containing sulfanilic acid followed by a second overlay agar layer containing alpha-naphthyl amine; the appearance of red color zones above colonies indicated the presence of nitrite. Nitrate reducing bacteria were then examined in broth

extracts derived from vegetables and the ability to ferment nitrate to nitrite was quantified by C8 reversed-phase ion-pairing HPLC analysis.

Staphylococcus carnosus, the industry default strain for nitrate reduction, was able to convert average of 1100 ppm nitrate in broth into 916 ppm nitrite (83% conversion). However, *Staphylococcus caprie* and *Panteoa agglomerens*, which isolated with the M17-Nitrite agar assay, were able to comparably reduce nitrate (ferment) to nitrite in broth to 915 ppm (83% conversion) and 866 ppm (79% conversion) nitrite, respectively. This is the first report of an on-agar colony screening assay for the detection of nitrite and isolation of nitrate-reducing bacteria allowing NRB to be readily isolated. Examination of new isolates will allow optimization of the fermentation process that can make nitrate reduction to nitrite more efficient as applications for natural vegetable nitrite become more applicable in the processed meat industry. NRBs were screened from different sources i.e. five hundred (500) culture isolates from Muriana culture collection, out of which six (6) isolates were NRB positive. Screening of animal and food samples using the agar plate method, identified eleven (11) isolates that were NRB positive.

Introduction

Traditionally, meat curing is defined as direct addition of nitrite to the product. The current U.S. Department of Agriculture (USDA-FSIS) guidelines strictly regulate the use of sodium nitrate and/or sodium nitrite in cured meat. Whereas, for the products cured with natural sources of nitrate or nitrite, such as vegetable juice or celery juice powder, regulations are less clear (Sebranek & Bacus, 2007). The USDA regulations have prohibited the use of artificial flavoring, color, chemical preservatives, or synthetic ingredients for production and of foods labelled ‘natural’. Sodium nitrite and nitrate are not acceptable for use in natural products. The meats are processed without the addition of sodium nitrite and replaced while natural nitrate or nitrite sources with maintaining characteristics similar to conventionally cured products. But the USDA regulations necessitate

these products to be labeled as “uncured” and allows labelling as “no nitrate or nitrite added except those naturally occurring in added ingredients”(Sullivan et al., 2012a).

Natural alternatives sources of nitrite such as celery juice concentrate or celery juice powder (CJP), are replacing synthetic nitrite in naturally cured meat products. Vegetables are reported to have natural nitrate at high levels, such as celery (3,151 ppm), turnip greens (9,040 ppm), beets (3,288 ppm), spinach (2,470 ppm) and melon (4,932) (Djeri & Williams, 2014). Currently, celery powder is used extensively as a vegetable nitrite source in processed meat labelled ‘natural’ or in organic processed meat due to its subtle flavor and the minimal color effect on the product. (Ko et al., 2017).

According to the United States Department of Agriculture (USDA-FSIS), Food Safety and Inspection Service (USDA-FSIS) labeling standards as defined in 21 CFR 101.22 that meat and poultry products labeled “natural” are not permitted to contain any artificial flavoring. ‘‘Clean-label’’ foods are not necessarily constrained to FSIS ‘‘natural’’ or ‘‘organic’’ definitions but have simple ingredients that are familiar to consumers and perceived as being derived from a nonchemical source, such as vinegar, flavorings, cultured sugars or dairy ingredients, or ingredients derived from plant material. Thus, vegetable-derived nitrite allows the use of a “clean label” advantage to the meat industry than ingredients from chemical origin (Mcdonnell et al., 2013).

Nitrate is not a reactive curing compound, thus vegetable nitrate reduction to natural nitrite is essential for curing reactions. The natural curing process using naturally obtained nitrate is carried out with an additional step of bacterial reduction of vegetable nitrate into nitrites by specific nitrate-reducing bacteria. Celery juice and its powder are known to have high nitrate content and thus are used in combination with lactic acid starter culture in the production of naturally cured meat. Currently, pre-generated nitrite (i.e. vegetable nitrate is converted to nitrite by microorganisms) is utilized to ease and speed up curing time (Djeri & Williams, 2014). Nitrate to

nitrite reduction can be achieved with a bacterial starter culture, such as *Staphylococcus xylosus or Staphylococcus carnosus,* possessing specific nitrate-reducing ability (Hwang et al., 2018).

Nitrate reducing bacteria (NRB's) are defined by their ability to reduce nitrate into nitrite or other nitrogenous compounds. This reduction is facilitated by the nitrate reductase enzyme produced by these bacteria. Nitrate reducing bacteria possess membrane-bound nitrate reductases with an active site in the cytoplasm. *Staphylococcus carnosus* is a popular nitrate-reducing bacteria used in industry. Coagulase-negative staphylococci (CNS) are commonly found as natural flora of fermented meat products. The role of CNS is significant in the improvement of sensory properties of fermented sausages, by reduction of nitrates to nitrite and then to nitrous oxide, by preventing rancidity through peroxide decomposition, and by producing flavor and aroma compounds through proteolysis and lipolysis (Casaburi et al., 2005)

Materials and Methods

Bacterial Cultures and Media

Active cultures were grown in Tryptic Soy Broth (TSB, BD Bacto, Franklin Lakes, NJ, USA) in 9-mL tubes at 37°C. Cultures were maintained for storage by centrifugation (6,000xg, 5°C) of 9 mL of fresh, overnight cultures and cell pellets were re-suspended in 2-3 mL of fresh sterile TSB containing 10% glycerol. Cell suspensions were placed into glass vials and stored in an ultra-low freezer (-80°C). Frozen stocks were revived by transferring 100 µL of the thawed cell suspension into 9 mL of TSB, incubating overnight at 37°C, and sub-cultured twice before use. Cultures screened for nitrate reducing activity included 500+ cultures from the FAPC Gilliland Culture collection, the Muriana Culture Collection, and bacteria screened from food (retail) and animal (FAPC slaughter facility) samples.

The Nitrate Reduction Assay (in Broth)

The nitrate reduction assay is a qualitative procedure for determining the ability of bacteria to reduce nitrate into nitrite. Bacteria are cultured in Nitrate Broth (Hi-media) containing nitrate and then the culture is tested for the presence of nitrite. Determination of nitrate reduction to nitrite is a two-step process. First, the reduction of nitrate to nitrite is determined by the addition of Nitrate Reagent A (sulfanilic acid) and followed by Nitrate Reagent B (alpha-napthylamine). If a red color occurs, then this confirms the presence of nitrite; if there is no color change, then either nitrate remains unreduced (determined by addition of zinc dust, if this turns the culture red, then it confirms unreduced nitrate); if there is still no color change, then the only explanation is that nitrate was reduced to nitrite, and then further reduced to other nitrogen compounds.

a. Nitrate reagent A: Sulfanilic acid 5 gm in 1000 ml (30% acetic acid)
b. Nitrate reagent B: Alpha-napthylamine 8 gm in 1000 ml (30% acetic acid)
c. Reagent C: Zinc dust.

The Nitrate Reduction Assay (on Agar)

The Nitrate Test was slow and tedious in liquid broth cultures, for screening individual bacterial isolates for NRBs from food and animal samples. We therefore developed an agar version of the liquid method, the Nitrate agar colony plate assay method, in order to screen colonies from assorted food and animal samples. The agar plate nitrate assay uses a similar principle as the Nitrate reduction test but is performed on agar colonies on petri plates. A dilution series of test samples are surface plated on pre-poured M17 agar plates, allowed to dry (adsorb), and then overlaid with Nitrate Agar (Hi-Media) to entrap the plated colonies in a sandwich overlay technique. The overlaid plates are allowed to incubate overnight at 37^0C, and those plates with a countable range of 25-250 were selected. A plain agar layer containing addition of Nitrate Reagent A (2mL, 5N Sulfanilic acid is added in 50 mL soft agar (0.5% agar) and overlaid onto the previous overlaid plate. Another

plain agar medium containing Nitrate Reagent B, Alpha-napthylamine (2mL is added in 50 mL soft agar (0.5%) and is overlaid in top of the Nitrate Reagent A layer. The chemicals mix by diffusion and zones of red color observed around colonies indicates nitrate-reducing bacteria.

Isolation and Identification of Nitrate Reducing Bacteria

Colonies with red color zones were isolated from agar, cultured, and confirmed for nitrate reducing activity in nitrate broth. The bacterial colony picked from agar plate was grown in TS or MI7 broth media overnight at 30^{o}C. Prior to identification of bacterial isolates from screening using 16s rRNA PCR, bacterial DNA had to be extracted. First, bacteria were grown at 30°C for 16 hours before extraction. Cultures were vortexed to ensure the homogenization of the mixture, and 1.4-mL were transferred to a 1.5- mL Eppendorf microcentrifuge tube. Contents were centrifuged at 12,000xg in an Eppendorf 5424 centrifuge for 90 seconds and supernatant was discarded. A total of 500 µL of deionized water (DI) was added to the tube, and the contents were vortexed to resuspend the pellet. Samples were centrifuged again at 12,000xg for 90 seconds, before discarding the supernatant and repeating the same step with another 500-µL of DI water.

After a second wash with DI water, the supernatant was manually pipetted and discarded. Preparation of DNA was performed by the bead collision method described by Coton and Coton (2005) (Coton & Coton, 2005). Depending on the size of the resulting pellet, 100- 120-µL of 10 mM Tris buffer at pH 7.4 was added. After resuspension, sterile silica beads were added, then samples were subjected to several rounds alternating between vortexing and cooling on ice, intended to shear cells and expose the contents within the cell. After a final cooling period, samples were centrifuged at 12,000xg for 2 minutes. The supernatant containing the extracted DNA was then pipetted and added to another sterile micro-centrifuge tube before placing the sample on ice to prevent DNAses from damaging the product.

Identification was obtained by PCR amplification of 16S rRNA sequences using 'universal primers' and the amplified products were submitted to the OSU DNA Sequencing Core Facility. The identification of bacteria was determined using PCR amplification with universal 16S ribosomal RNA primers designated 8-Forward (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541-Reverse (5'-AAGGAGGTGATCCAGCCGCA-3') primers to provide a long stretch of 16S rRNA sequence for bacterial identification (Henning, Gautam, et al., 2015). Thermal cycling was performed using a PTC-200 Peltier Thermal Cycler (MJ Research/Bio-Rad Laboratories, Hercules, CA, USA;): initial denaturation at 95°C for 4 min, followed by 30 cycles of 94°C for 1 min (denaturation), 60°C for 45 sec (annealing), 72°C for 1 min (extension), followed by a final extension cycle at 72°C for 4 min, and a final hold at 4°C. DNA sequences that were obtained were searched using NCBI's BLAST program. Isolates of similar bacteria were compared using the MEGA-X software tool for sequence alignment to determine if sequenced information was identical (i.e., likely re-isolated the same strain) or showed differences (i.e., could be different strains).

Vegetable Juice Extraction and Nitrate Detection

A commercial 'juicer' was used to extract juice from celery, white cabbage, and iceberg lettuce. Extracted juices were centrifuged at 10,000 rpm for 10 min to get rid of residual solids and the liquid extract was decanted and autoclaved to prevent microbial growth. The presence of nitrate was confirmed using the Nitrate reduction test.

Fermentation Using Nitrate-Reducing Bacterial Isolates

Vegetable juice extracts were inoculated with bacterial isolates to test their fermentation ability. The fermentation ability test showed the bacterial isolate's ability to reduce nitrate into nitrite. Celery, lettuce, and cabbage juices were used as both nitrate source. The 9 mL of juice in

test tubes were inoculated with 1 ml of bacterial culture and incubated overnight at 37°C. The nitrate reduction test was used as a primary test for the presence of nitrite post-incubation.

Quantitation of Nitrate and Nitrite using High Performance Liquid Chromatography (HPLC)

Based on the method of Cheng and Tseng (1998), octylamine orthophosphate was used as an ion-pairing agent in aqueous methanol (10% v/v) mobile phase using C8 Phenomenex Luna HPLC column as the stationary phase for the simultaneous quantification of nitrite and nitrate(Cheng & Tsang, 1998). A diode array detector was used for signal detection of nitrate and nitrite at 210 nm. Standards (Nitrate, Nitrite) of varying concentrations (10-, 50-, 100-, 200-, 300-, 600-, 800- and 1000-ppm) were used for standard curve and calibration.

The HPLC mobile phase was prepared using octylamine orthophosphate and phosphoric acid. Octylamine orthophosphate (1.65 ml) was added into 800 ml of HPLC-grade water (cloudy solution). The pH was then adjusted to pH 7.0 with the addition of ~450 ul of phosphoric acid (clear solution). The volume was then adjusted to 900 mL and 100 mL of methanol was added to result in a 10% (v/v) methanol mobile phase. The prepared mobile phase was then vacuum filtered and sonicated under vacuum for 10 min to degas dissolved gases.

An HP (Agilent) 1050 HPLC system comprised of a solvent degasser, a quaternary pump capable of pumping 4 different solvents, an automatic sampler, a diode array detector, and a computer workstation running HP Chemstation software was used for analysis.

Preparation of Nitrate/Nitrite samples for HPLC analysis:

1. Liquid samples. A 2-ml aliquot was added into 28 ml of deionized water (pre-heated) and held at 80°C in a hot water bath for 30 min.
2. Meat products. A 2-gm sample was stomached with 28 ml of deionized water and then centrifuged. The supernatant was heated at 80°C in a hot water bath for 30 min.

3. Vegetable juice. A 2-ml of juice was added into 28 ml deionized water, preheated to 80^{o}C and held in a hot water bath for 30 min.

After heating, 600 ul of each sample was filtered through 0.2-micron Whatman syringeless filter vials. During HPLC analysis, 20 ul sample volume was injected into the column with an isocratic solvent flow rate of 0.6 ml/min for the solvent.

Statistical Analysis

Experimental challenge trial were performed in triplicate replication in accordance with validation testing criteria established by the NACMCF (National Advisory Committee on the Microbiological Criteria for Foods, 2010) and accepted by USDA-FSIS (USDA-FSIS, 2015).

Results and Discussion

Using the Nitrate Reduction Assay (Liquid) with Modified-Nitrate Broth, 500 bacterial cultures were screened from culture collections (Table 1) using our modified nitrate broth using M17 broth (i.e. M17 broth with 0.1 % potassium nitrate) our M17-nitrate broth was developed since a commercial nitrate broth was not conducive to growth of many Gram (+) bacteria resulting in the inability to properly screen them for nitrate reduction. The use of M17 nitrate broth allowed the growth of bacteria that would not grow well in commercial Nitrate broth and resulted in the successful screening of Gram (+) bacteria in these collections to evaluate their ability to reduce nitrate into nitrite. The red color in test tubes after the addition of Nitrate Reagent A and Nitrate Reagent B confirmed the presence of nitrite after bacterial reduction of nitrate (Figure 1). With M17-Nitrate broth, six (6) bacterial isolates were identified as nitrate reducers (*Lactobacillus reuterii* PIG1-2, PIG1-3, PIG3-1; *Clostridium bifermnetums* P-5, P-42; *Lactobacillus plantarum* ML811) (Table 2). Nitrate reduction was confirmed using the Nitrate Reduction test on the liquid broth cultures.

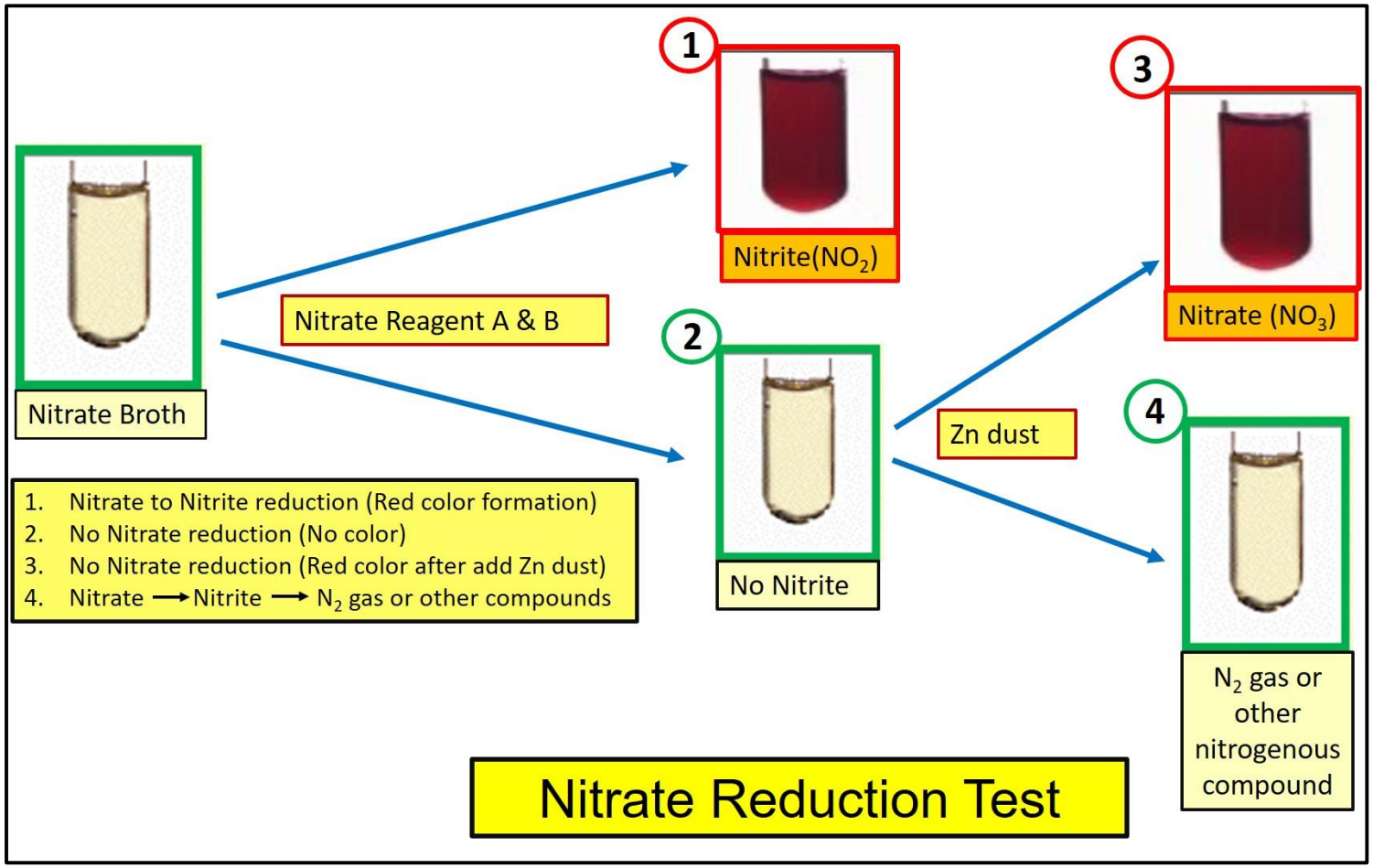

Figure 1. Nitrate Reduction Test using the broth method. 1, 'Red color' after addition of nitrate reagents A and B is positive. 2, 'No color' after addition of nitrate reagents A and B is negative. 3, No Nitrate reduction (red color after Step 2 and addition of Zn dust). 4, No color after addition of Zn dust signifies formation of gaseous nitrogen compounds.

Figure 2. Nitrate Reduction assay performed on M17-Nitrate broth culture broths of various strains from culture collections including: *Staphylococcus carnosus* (positive control), *Clostridium bifermentums* P-5, *Clostridium bifermentums* P-42, *Lactobacillus reuterii* PIG 1-3, *L.actobacillus reuterii* PIG 1-2, *Lactobacillus reuterii* PIG 3-1, *Lactobacillus planturum* ML811, and *Lactobacillus sakei* (negative control). The red color generated after addition of sulfanilic acid and alpha-napthylamine signifies the presence of nitrite (reduced from nitrate). The names in the figure legend are based off of definitive 16S rRNA identifications from DNA sequence and differ from the names attributed to the bacteria (on tubes) from the culture collection.

Table 1. FAPC culture collections screened for Nitrate reduction property using nitrate reduction test in broth method.				
S.N.	Genus	Species	Strain	#Strain
1.	*Streptococcus*	*cremoris*	290-P	1
2.	*kluyveromyces*	*fragilis*	50-16, 55-1, 72-297, C-106, Y1156, Y1171, Y108, Y1196, C-21	9
3.	*Streptococcus*	*thermophilus*	6, 143, 143 S+143, S-1, OSU-4, S-1 S+S1, S-2, SAN3-2, OSU-1, OSU-3, ST143, OSU-2, F-3, ??	15
4.	*Propionibacterium*	*acidipropionici*	P-5, Unknown	2
5.	*Lactobacillus*	*acidophilus*	B12, B13, B15, B16, B17, B18, B20, B3, C7, D2, D3, D4, D5, D6, D7, D8, D9, C11, C14, C17, C18, C19, D10, D13, D14, D15, D16, D17, D19, D20, B4, B5, B6, B7, B8, B9, DKW10, DKW2, DKW3, DKW5, DKW6, DKW8, DKW9, G16, G19, G20, G6, H11, H3,H4,H5, J15, J10, K16, J17, J18, J6, K15, J19, H9, K14, K10, K12, O17, O6, O2, O14, O10, O18, J12, DKW7, G5, 16, 107, 145, 223, 606, 4356, 4962, 107A, 1-F, 1G, 1-I, 2-5, 25-SD, 2-A, 2-R, 3062D, 3073D, 30-S-C, 310-3D, 381-1L-28, 381-1L-23, 381-IL-25, FR-5, 396-IL-28, 149C, 3811C2D, 3-A, 3-B, 3-D, 3-H, 3K, 614, 6-S4, A4, B-L+ (1), D1, C-28, CL3, CL-5, CL-6, C-2, C2-5, CF101, CF102, CF1R1, CF1R2, CF201, CF202, CF2R1, FR2, FR4, FR5, FR-6, GP3B, GP1B, GP2A, ,GP3A, GP3B, GP4A, HR11, HCO, MC1-811, L-1, K-4, HLA-1, R-2, R-1, NCPM, PLB-10, RAT-1, R-3, P-16, PA3, T-3, NCFM-L, NCFM-M, NCFM-F, 30SC, C1-3, C1-6, A-6, GPIC, L-23, ML811, 1	159
6.	*Pseudomonas*	*aeruginso*		1
7.	*Lactobacillus*	*animalis*	35046	1
8.	*Bifidobacterium*	*bifidium*	15696	1

9.	*Bifidobacterium*	*breve*	15700, T11, Unknown	3
10	*Lactobacillus*	*bulgaricus*	10422,33409, LB-18, LB-10422	4
11	*Xanthomonas*	*campesteris*	A-10, A-11, A-12, A-14, A-15, A-16, A-19, A2, E-10, E-5, GG, L-15, O1, A-17, N-7, M-5, Unknown	1
12	*Lactobacillus*	*casei*	L-13, L-4, L-14	17
13	*Lactobacillus*	*cellubiosus*	CAE	3
14	*Leuconostoc*	*citro*		1
15	*Bacillus*	*coagulans*		1
16	*Streptococcus*	*faecium*		1
17	*Propionibacterium*	*fluorescens*		2
18	*Pseudomonas*	*fluorescens*		1
19	*Propionibacterium*	*freudenreichii*	P-41	1
20	*R*	*gracilis*	Y-1092	1
21	*Bifidobacterium*	*infantis*	15697	1
22	*Propionibacterium*	*jensenii*	P-42	1

23	*Lactobacillus*	*lactis*	8000, 12315, 39A-1, 39A-2, 4035-15, B, C2, 403515 L+2, 800 L+2, FARR, LB-1, T4-1,RM2-5, A, FARR L+1, AY29LB, 39-A2 L+1, 4	20
24	*Lactobacillus*	*leichmannii*	4797	1
25	*Bifidobacterium*	*longum*	B-9, L-5, K-5, S-9, S-12, YID, Y1E, Y2Q, Y2D, Y1B, Y1D, Y2N, Y2M, Y1A, Y1F, Y2C	16
26	*Lactobacillus*	*plantarum/casei*	E0-3, E-11, E-12, E-13,E-14, E-15, E-16, E-17, E-18, E-19, E-20, E-2, E-6, E-7, E-8, E-9, E-4, E-15,P-15, P-13	20
27	*Lactobacillus*	*reuterii*	23272, 55739, X-18, X-27	4
28	*Lactobacillus*	*salivarius*	PIG3-3, PIG 3-1, PIG1-2, PIG1-3, 459	5
29	*T*	*saphaerica*	Y-1098, Y-1104	2
30	*MISC (unidentified)*		C4409-16, BIM-1 Vol-6, BIM-1 Vol-7, BIM-1 Vol-8, BIM-1 VOL-9, BIM-2 VOL-5, BIM-2 VOL-6,BIM-2 VOL-7,BIM-2 VOL-8,BIM-2 VOL-9, BIM-3 VOL-5, BIM-3 VOL-6,BIM-3 VOL-7,BIM-3 VOL-8,BIM-3 VOL-9, BIM-4 Vol-3,BIM-4 VOL-5, BIM-4 VOL-6,BIM-4 VOL-7,BIM-4 VOL-8,BIM-4 VOL-9, BIM-5 VOL-5, BIM-5 VOL-6,BIM-5 VOL-7,BIM-5 VOL-8,BIM-5 VOL-9, LBS-2 VOL-4,LBS-1 VOL-9, LBS-4 VOL-4, LBS-4 VOL-7, LBS-3 VOL-7, LBS-2 Vol-8,	115

			LBS-3 Vol-4, LBS-4 VOL-8, LBS-2 VOL-7, LBS-3 VOL-8, LBS-5 VOL-7, LBS-6 VOL-3, LBS-4 VOL-9, MRS-3 VOL-7, LBS 2 VOL-5, LBS-1 VOL-6, LBS-4 VOL-6, LBS-5 VOL-5, LBS-3 VOL-5, LBS-5 VOL-9, LBS-3 VOL-9, LBS-1 VOL-8,MRS-5 VOL-3, MRS-4 VOL-4, MRS-4 VOL-3, MRS-3 VOL-9, MRS-4 VOL-7, MRS-3 VOL-8, MRS-2 VOL-7, LBS4 VOL-3, MRS-3 VOL-3, LBS-1 VOL-4, MRS-4 VOL-8, MRS-3 VOL-4, LBS-1 VOL-5, MRS-2 VOL-6, MRS-4 VOL-6, MRS-2 VOL-8, LBS-2 VOL-6, MRS-4 VOL-5, MRS-5, VOL-4, MRS-2 VOL-9, MRS-3 VOL-5, MRS-3 VOL-6, MRS-4 VOL-9, MRS-2 Vol-5, MRS-5 VOL-5 , T1-5, T2-5, T1-1, T3-9, T2-2, T1-3, T4-4, Y3C, T4-8, MRS-1 VOL-9, MRS-1 VOL-5, MRS-5 VOL-6, MRS-1 VOL-8, MRS-5 VOL-8, MRS-1 VOL-6, MRS-1 VOL-7, MRS-5 VOL-7, MRS-1 VOL-4, YIF, Y2I, Y1C, Y3A, 173, LBS-1 VOL-7, T6-1, TS-2, LBS-5 VOL-4, T6-S, A-18, BIM-6 VOL-3, T3-1, T3-2, MRS-2 VOL-4, LA-42, 176, BIM-2 VOL-3, LBS-3 VOL-5, Y5M, F2A, FIA, S+2, N19	

Screening of Bacterial Samples from Foods and Animals using Nitrate Assay Modified for On-Agar Use

The traditional Nitrate Reduction assay was easy to use for screening individual cultures from a culture collection because they had to be grown anyway in order to obtain the culture from frozen stocks. However, trying to evaluate mixed samples of bacteria found in foods or animal's intestinal contents was tedious using the liquid test that worked well for individual cultures. Since there was no agar plate method to conveniently screen mixtures of different bacteria from food and animal sources for nitrate reduction, we formulated our own on-agar version considering how well M17 nitrate broth worked for the broth assay. The on-agar version of the nitrate reduction assay worked equally well with commercial nitrate agar that was better suited for Gram (-) bacteria as with our version with M17 nitrate agar that worked better with Gram (+) bacteria (Figure 3). Using this method, we isolated additional bacteria from animal sources and retail foods (Table 2).

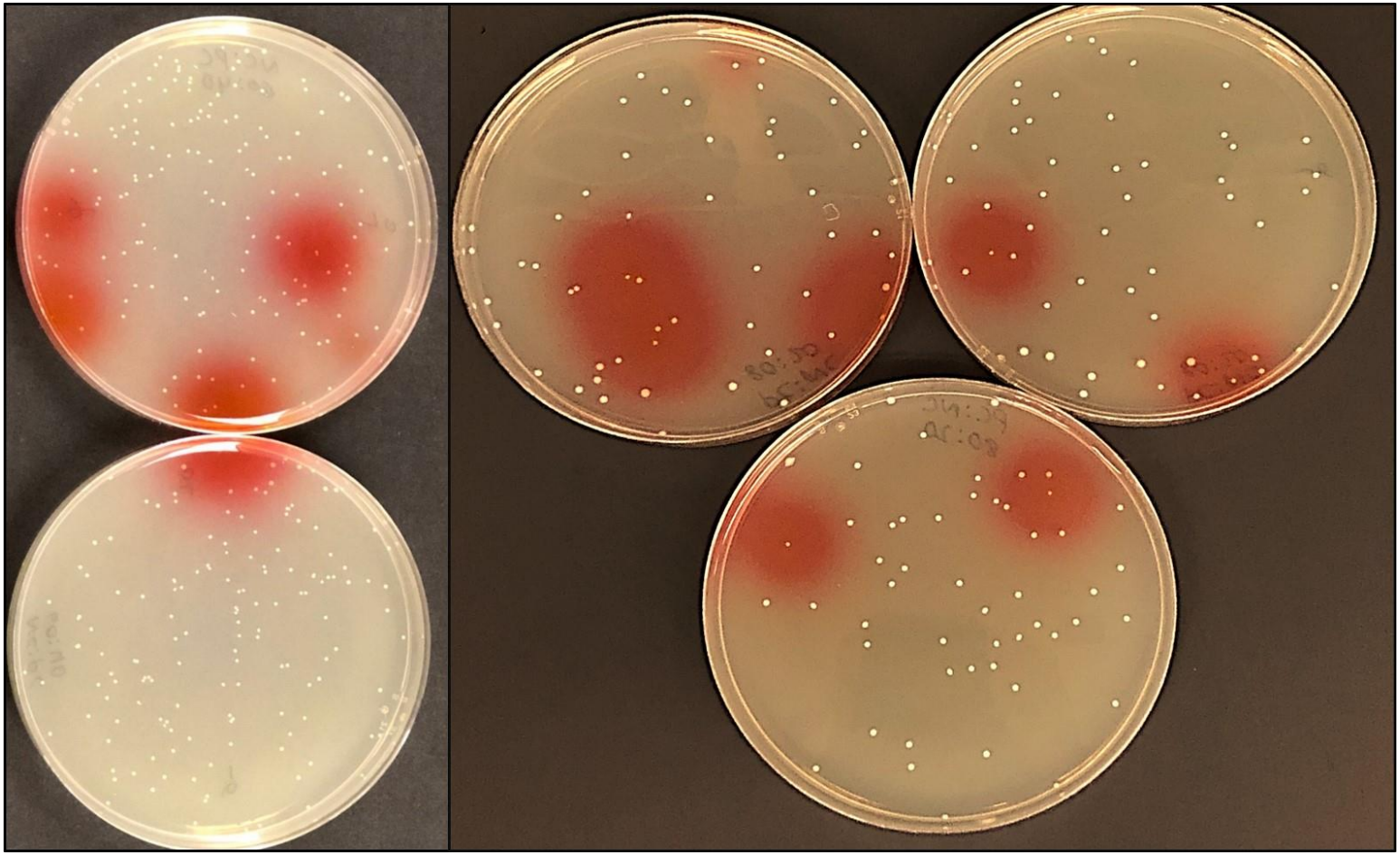

Figure 3. Nitrate reducing bacterial colonies showing red color zones after being overlaid with soft agar overlays containing Nitrate Reagents A and B. Colonies with surrounding red color were isolated using the inverted agar method and streak plated on new plates and confirmed for nitrate reduction using the nitrate broth method.

Table 2. Nitrate reducing bacteria detected in culture collections or food/animal samples.

Isolates	Source
Escherichia coli 309-7	Hog small intestinal sample
Escherichia coli 69	Hog small intestinal sample
Shigella flexneri SFL1520	Hog small intestinal sample
Escherichia coli NCYU-26-73	Hog small intestinal sample
Escherichia fergusonii Z6	Hog small intestinal sample
Escherichia coli PL-AGW6	Hog small intestinal sample
Escherichia coli F9792	Hog small intestinal sample
Lactobacillus reuterii PIG1-2	FAPC culture collection
Lactobacillus reuterii PIG1-3	FAPC culture collection
Lactobacillus reuterii PIG3-1	FAPC culture collection
Clostridium bifermentums P-5	FAPC culture collection
Clostridium bifermentums P-42	FAPC culture collection
Lactobacillus plantarum ML811	FAPC culture collection
Streptococcus hyointestinalis 1336	Hog small intestine sample
Streptococcus hyointestinalis 1340	Hog small intestine sample
Staphylococcus caprie Cab1	Food sample (white cabbage)
Pantoea agglomerans Lett1	Food sample (iceberg lettuce)
Staphylococcus carnosum	Commercial strain

HPLC Analyis of Nitrate and Nitrite in Nitrate Broth, and After Bacterial Fermentation

HPLC analysis allowed us to quantify nitrate and nitrite after fermentation in liquid media, or vegetable extracts (Figure 4), and using standard commercial strains or our bacterial isolates. HPLC analysis was examined at various wavelengths given the multi-wavelength capabilities of the diode array detector. The peaks for nitrite and nitrate were sharper and more prominent at the lowest of the 4 wavelengths examined (254-, 214-, 210-, and 204-nm) and the use of isocratic solvent parameter kept the baseline level. The selectivity of different HPLC column packings (C_{18}, C_8) we tested were conditionally acceptable and provided for either a shorter or longer run; the suitability of any of these will be dependent on the additional peaks that might interfere with quantitation when extracts obtained from vegetables (sources) and hotdogs (processed meat applications) are tested.

Reversed phase (RP) HPLC analysis of nitrate and nitrite with 10% MeOH mobile phase containing octylamine orthophosphate (pH 7.0) as the ion-pairing agent using a Phenomenex 250-mm x 4.0mm Luna C8 end-capped 5µ packing with UV-detection at 210-nm seems our current preference and HPLC standard curves have very good linearity ($r^2 = \geq 0.99$) (Figure 4). The approxmiate elution time for nitrite and nitrate was 15 and 22 minutes respectively. Because of the biochemistry of nitrate-to-nitrite conversion, we did not think end-point analysis would provide the best conversion rate and therefore we chose to follow periodic sampling during the time course of fermentation; this demonstrated that dependent on when you pulled a sample or stopped a fermentation, you could have significantly higher or lower values at one time than another (Table 3). This would be important if one were to design a fermentation for an optimum recovery of nitrite from nitrate reduction.

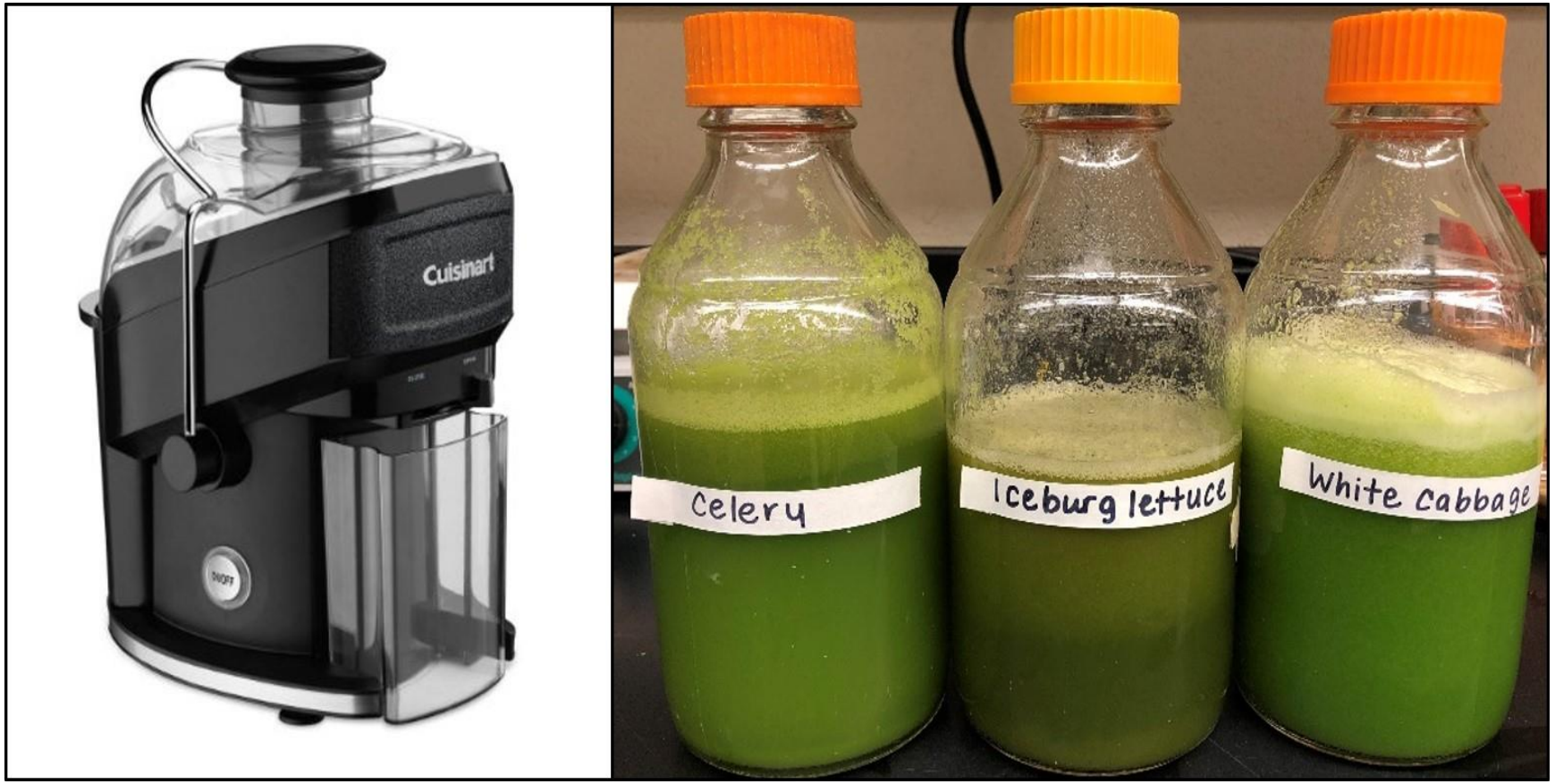

Figure 4. Extraction of juices from celery, iceberg lettuce, and white cabbage for use in bacterial fermentation and nitrate reduction assays using a commercial 'juicer'.

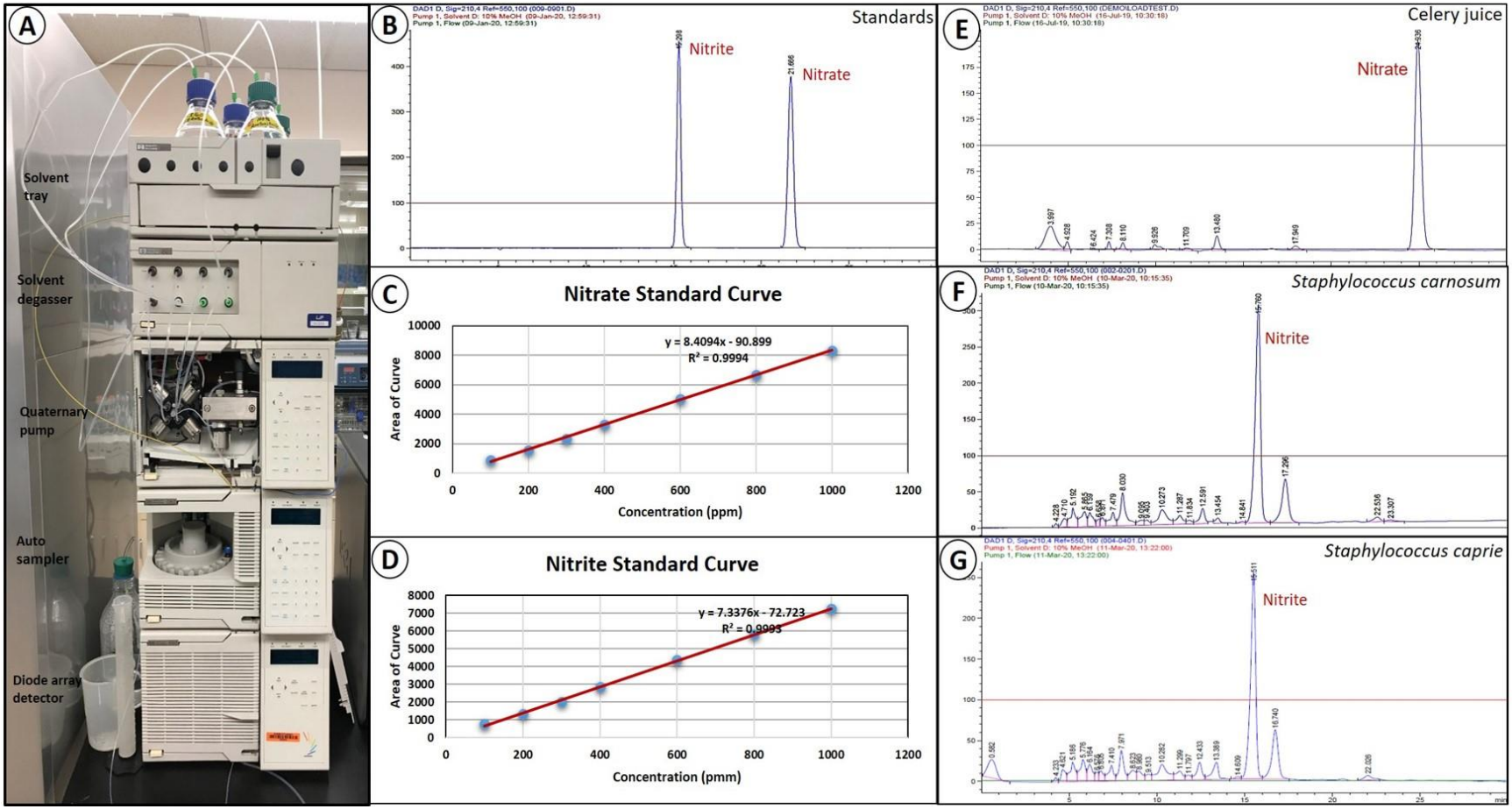

Figure 5. HPLC analysis of nitrate and nitrite using C_8-RP ion-pairing chromatography. Panel A, HPLC system. Panel B, chromatogram of nitrite and nitrate standards. Panel C, standard curve of nitrate standards. Panel D, standard curve of nitrite standards. Panel E, celery juice extract. Panel F, celery juice extract fermented with *Staphylococcus carnosum*. Panel G, celery juice extract fermented with bacterial isolate, *Staphylococcus caprie*. Chromatogram were detected at 210 nm.

Table 3. Time course conversion of nitrate (Nitrate broth) to nitrite by *Staphylococcus aureus*, *Staphylococcus caprie,* and *Pantoea agglomerans* as quantified by HPLC; starting nitrate concentration was 1,100 ppm.

Time (hr)	***Staphylococcus carnosus*** **Nitrite (PPM)**	***Staphylococcus caprie*** **Nitrite (PPM)**	***Pantoea agglomerans*** **Nitrite (PPM)**
0	0.00±0	0.00±0	0.00±0
12	821.5±5.1	690.2±11.2	700.9±11.5
16	917±4.6	787.2±8.4	866.6±6
20	895.9±4.5	661.7±3.9	799.2±8.9
24	905.7±5.4	727.8±7.7	685.6±12.5
36	888.8±1.7	915.8±9	760.4±5.4
48	844.7±5.0	641.1±6.1	712.8±8.8
72	809.3±3.5	553.5±4.7	686.7±11.7

Bacterial species produce nitrate reductase enzyme to enable them to reduce nitrate into nitrite. This activity can be readily confirmed using a simple biochemical nitrate reduction tube assay to detect nitrite, nitrate, or absence of both. Some bacteria can reduce nitrate to nitrite and then further, to nitrogen gas. Commercial 'Nitrate Broth' is not suitable for Gram-positive or lactic acid bacteria, so we adapted M17 broth as a more suitable medium to test these organisms (by addition of 1gm/L potassium nitrate as the nitrate source). We developed a modified agar plate colony screening assay to increase our chances of screening nitrate reducing bacteria from a mixture of bacteria plated onto agar plates and readily found such bacteria from animal and food sources. The qualitative analysis of nitrate content in vegetable juices was confirmed using the simple nitrate reduction test. Celery, cabbage, and lettuce used in this study are well-known sources of nitrate. HPLC quantification of nitrate content in vegetable juice extracts proved the presence of high nitrate content (i.e. approximately 2000ppm). An alternative source of nitrite is 'natural nitrite' as derived from vegetables. Nitrate present in vegetable extracts or juice can be fermented using nitrate-reducing organisms resulting in 'natural nitrite'. The vegetable sourced nitrite is considered 'natural nitrite' and allows a 'clean label' tag in processed meats. New sources of nitrate-reducing organisms shall provide more efficient nitrate fermenting bacteria to generate nitrite.

Staphylococcus carnosus is a standard commercial strain used to produce vegetable-based nitrite. As observed it has converted nitrate into nitrite efficiently. Similarly, *Staphylococcus caprie* was able to efficiently reduce nitrate broth into nitrite.

The food samples used for isolating bacteria were cabbage, lettuce, and carrot. As cabbage and lettuce are high in nitrate content, it is plausible that nitrate-reducing bacteria may be present. The animal samples used were mainly intestinal, thus the animal gut is rich in *E. coli* and other coliforms bacteria capable of reducing nitrate. *E. coli* are known to have nitrate reductase enzyme; thus some were isolated in our study and were identified as positive nitrate reducers in the agar plate colony method.

Conclusion

The data presented herein demonstrates that a variety of bacteria can reduce nitrate to nitrite. Some of our data with direct fermentation of vegetable extracts did not lead to as high a conversion rate to nitrite as obtained with nitrate broth. Perhaps because of the nutrient content of vegetable extract is not as bacteriologically conducive to growth and reductase activity as is nitrate broth media? This could be subject to future optimization. HPLC analysis with C_8 Reversed Phase columns using ion-pairing mobile phase lends itself towards quantification of nitrate and nitrate. Vegetable derived nitrite is a 'natural nitrite' and nitrate reducers play a significant role in the production of natural nitrite from vegetable derived nitrate. The optimization of the fermentation process for nitrate reduction with new isolates can make production of vegetable nitrite more efficient. The modified nitrate broth using M17 broth (0.1% KNO_3) facilitated the detection of Gram-positive nitrate reducing bacteria. Further study of nitrate reducing bacteria is needed to enhance and optimize the nitrate to nitrite conversion in vegetable extracts and studies on especially their application toward the inhibition of spore germination in *Clostridium spp*, especially *Cl. perfringens* and *Cl. botulinum* would be useful to the processed meat industry.

CHAPTER IV

COMPARISION OF SODIUM NITRITE AND CELERY NITRITE ON THE INHIBITION OF SPORE GERMINATION OF CLOSTRIDIUM SPOROGENES IN LOW AND HIGH FAT ALL BEEF FRANKFURTERS

Abstract: Nitrite is a regulated ingredient used to prevent the germination of *Clostridium* spores in processed meat products. The use of nitrite as a food preservative got a boost when USDA-FSIS considered vegetable-sourced nitrite (i.e., vegetable nitrate fermented to nitrite) as 'natural nitrite' whereby products can be labelled as having 'no added preservatives'. This designation and the labelling allowance provides for a 'clean label' application of nitrite. Our objective was to identify permissive conditions that facilitate validation of spore inhibition during the comparison of sodium and vegetable (celery) nitrite in cooked meat products. A three-strain spore crop from *Clostridium sporogenes* (ATCC 3584, ATCC 19404, and ATCC BAA-2695) was applied during ingredient formulation of low and high fat hotdogs that were divided into 3 batches (control without nitrite, hotdogs with sodium nitrite, hotdogs with celery nitrite). In both processes, sodium nitrite was compared at comparable levels of celery nitrite. Hotdogs followed a standard preparation and cook process (nitrite was used at 156 ppm). Heat treatment during hotdog cooking process killed any remaining vegetative cells and initialized spore germination and growth. In our assays, we allowed spore germination to occur at 35^{o}C as well as 5^{o}C and 15^{o}C so that we could best observe inhibition of spore germination by nitrite under the most permissive conditions, compared to controls processed without nitrite.

Celery nitrite was equally as good as than sodium nitrite in low fat and high fat all beef hotdogs and spore outgrowth was only observed at 35°C abuse temperature conditions. The nitrite validation assay described herein allows easy determination if nitrite levels can prevent spore germination under the most permissive conditions to help keep processed meat safe. All treatments were performed in duplicate replication with triplicate samples and comparisons were analyzed by repeated measures analysis of variance to determine significant differences ($p < 0.05$) between the treatments.

Introduction

Clostridium species (*Cl. botulinum*, *Cl. perfringens*) are Gram-positive, spore-forming, anaerobic bacteria known for toxic pathogenicity in certain foods when spores germinate under permissive conditions and illness arises due to consumption of preformed toxin in food (*Cl. botulinum*) or by consumption of vegetative cells that sporulate in the gut and produce toxin (*Cl. perfringens*). *Cl. botulinum* has notably been a problem with canned products (i.e., low acid vegetables) for which 12-log reduction of spore count in commercial thermal processing (i.e., '12-D process') has been defined to prevent such occurrences (Anderson et al., 2011). *Clostridium* spp. are well known as spore-formers and most spores of spore-forming bacteria are more heat resistant than the vegetative cells themselves (Olguín-Araneda et al., 2015).

Bacterial spores are also commonly associated with soils whereby spore forming bacteria that end up in soil may find this an inhospitable environment, they then sporulate, the vegetative cells degrade, and this leads to an accumulation of spores in soils over time since the spores are relatively indestructible under natural conditions. This feature is why anything grown in the agricultural environment can potentially be contaminated with spores. Processed foods, whether vegetable or animal-derived foods, could have spores associated with them. Another characteristic of spores is that heating serves to stimulate them to germinate. This is usually done on purpose prior to microbial sampling of spores in order to a) eliminate co-contaminating vegetative cells

while simultaneously b) inducing synchronous germination of the spores in a sample. Since *Clostridium* spp. are strict anaerobes, they require an anaerobic environment. Therefore, heating followed by bottling, canning, or vacuum packaging can have dire consequences if nothing is done to prevent spores from germinating such as low temperature, low pH, and nitrites.

Chemical nitrite (or vegetable nitrate that is converted into nitrite) is an indispensable ingredient in cooked processed meats. It is responsible for obvious changes in cured meats as noticed by consumers, such as the color of 'cured' meats (i.e., the pink color of cured hams). What may not be so obvious is that the main reason for introducing nitrite into cured/processed meats is to inhibit the germination of *Clostridium* spores (*Cl. botulinum, Cl. perfringens*) (Pierson et al., 1983). Residual spores from harvested vegetable or animal-derived food products could contaminate the finished product, since both types of products are either grown in soil, or have extended contact with soil. The process of cooking and vacuum-packaging RTE processed meats introduces both spore-germinating stimulants such as the heating process and anaerobic packaging. Most food process validation studies are preferably performed with foodborne pathogens, but this requires the procedures to be done in a protected BSL-2 laboratory and the equipment used is often restricted or dedicated for use with pathogenic microorganisms (or non-edible products) for safety reasons. The use of 'surrogate microorganisms', or those that are closely related to pathogens but are not pathogenic (BSL-1), allows such procedures to be performed directly with real commercial equipment in food manufacturing plants (Brown et al., 2012). 'In-plant studies' are often preferred to laboratory test runs because it is often questionable whether the transition from laboratory to commercial process provides an equitable evaluation of the processing equipment used in commercial food manufacture.

In this study, we used 3 *Clostridium sporogenes* strains as surrogate microorganisms for *Cl. botulinum / Cl. perfringens* to perform food processing trials on high and low fat hotdogs. The production of hotdogs for this study follows industry standards. To constitute low fat, 21CFR101.62 was referenced to assure commercial hotdog guidelines were met. Hotdogs were

measured to have a core temperature of 165+°F during the cook process. Low and high fat hotdogs all beef, were made with either chemical or vegetable nitrite, and controls without nitrate were rinsed with brine chill solution, removed from casings, vacuum packaged, and then held at 5°C (refrigerated temperature), 15°C (accelerated shelf life temperature), and 35°C (permissive temperature). Samples were taken at periodic interval to compare outgrowth of spores in hotdogs held under these varied conditions.

Materials and Methods

Bacterial Cultures and Media

Three strains of *Clostridium sporogenes* (ATCC 3584, ATCC 19404, ATCC BAA-2695) were used in this study to propagate spore crops for use as a 3-strain spore inoculum for hotdog meat matrix prior to stuffing and cooking. All 3 strains are non-pathogenic and considered BSL-1 for biosafety purposes. Strains of *Cl. sporogenes* were grown anaerobically in Reinforced Clostridium Broth without agar (RCB; HiMedia, Mumbai, India) at 37°C, induced to sporulate by extended incubation (5-6 days) at 30°C (sub-optimal temperature) in broth tubes or on Reinforced Clostridium Agar (RCA; RCB + 1.5% agar). Broth tubes, with loosely seated caps, and agar plates were incubated anaerobically in anaerobe jars (BD Labs, Franklin Lakes, NJ, USA) using Anaero-Pack CO_2-generating envelopes and cartridges (Mitsubishi Gas Chemical America, Inc., New York, NY).

Antibiotics, Disc Assay, and Media Validation of Resistance of Strains of *Clostridium sporogenes*.

Clostridium sporogenes ATCC 3584, ATCC 19404, and ATCC BAA-2695 were tested for innate antibiotic resistance using BD BBL Sensi-Discs (BD Labs) consisting of sterile paper discs impregnated with specific levels of antibiotic (Sandle, 2016). Bacterial lawns were obtained for

individual *Clostridium* serovars by seeding 0.1 mL of overnight culture into 10 mL molten/tempered RCA (0.75% agar), mixed, and overlaid onto pre-poured RCA (1.5% agar) in 150-mm petri plates. When the overlay was solidified, antibiotic discs were aseptically dispensed onto the bacterial lawns and plates were incubated overnight at 37 °C. Following incubation, cultures were evaluated for resistance (no zone) or degree of susceptibility subjective size of the inhibitory zone (slightly sensitive, sensitive, very sensitive).

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

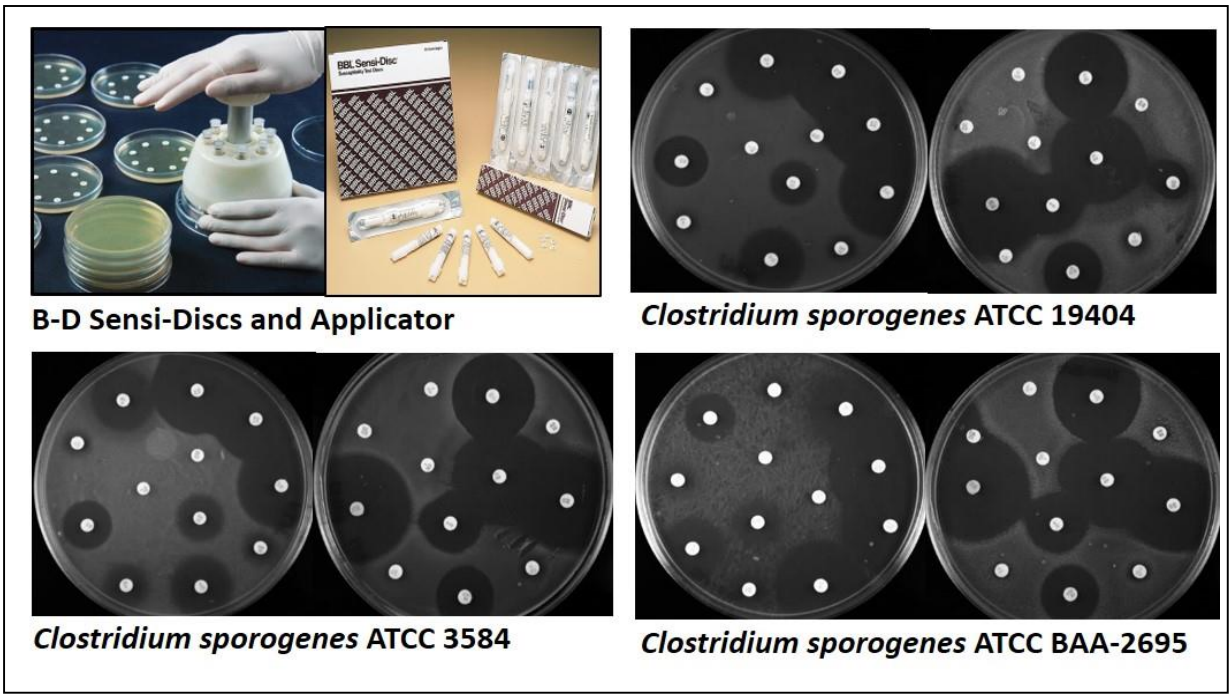

Figure 1. Determination of antibiotic resistance for use in selective media. B-D Sensi-Discs and applicator and various reactions on bacterial lawns of different strains of *Clostridium sporogenes*. Antibiotics to which the challenge organisms are resistant would be suitable for use in selective media to eliminate indigenous bacteria during product sample enumeration.

Antibiotic resistance was confirmed on agar by plating individual cultures grown in RCB (without antibiotics) for quantitative enumeration onto RCA plates, with and without, individual antibiotics. Quantitative enumeration was then performed with multiple antibiotics. This was especially important for combinations of antibiotics to insure the absence of synergistic inhibitory activity when multiple antibiotics are combined. All assays were performed in triplicate replication. The use of antibiotics in selective media ensures microbial enumeration of our spore inoculum strains and not other bacteria/spores that might also be indigenous to the non-sterile meat matrix.

Inducing Sporulation of Vegetative Cells and Harvesting Spore Crops for Use as Challenge Inoculum

Two methods of sporulation for the 3 strains of *Clostridium sporogenes* were used in this study: sporulation in broth (W.-W. Yang et al., 2009) and sporulation on agar (Janganan et al., 2016). Spores from *Cl. sporogenes* ATCC 3584, ATCC 19404, and ATCC BAA-2695 were harvested after sporulation in broth and on agar (Figure 2) to determine if one was better than the other. Briefly, for sporulation in broth, individual cultures were grown anaerobically overnight in RCB at 37^{o}C and transferred as 10% inoculum to 90 mLs of sporulation medium (3% trypticase, 1% peptone, 1% ammonium sulfate), heat shocked at 80^{o}C for 15 min (to induce synchronous germination prior to sporulation), and then incubated anaerobically with gentle shaking (~180 rpm) for 5-6 days at suboptimal temperature of 30^{o}C. Yang et al. (W. W. Yang et al., 2009)found that the combination of heat shock and anaerobic incubation at suboptimal temperature produced the greatest percentage of endospores. Sporulation on agar was also performed by a modification of the agar method of Janganan et al. (Janganan et al., 2016) by anaerobic incubation of individual cultures in RCB overnight at 37^{o}C and then spread plating each culture on 6 petri plates containing RCA and again incubating anaerobically at 30^{o}C for 6 days. After 6 days, lawns on the *Clostridium* sporulation plates were resuspended with 6 mLs of sterile water using a plastic rake and transferred to sterile Oak Ridge tubes. At this point, the remaining procedure was the same for both methods,

the post-sporulation cultures or lawn-resuspended sporulated cells were centrifuged at 12,850 xg, decanted, and resuspended with deionized water. The pellets were resuspended and the centrifugation was repeated 3x whereby the final pellet was resuspended with 20 mLs sterile 1x phosphate buffered saline (PBS: 137 mmol NaCl, 2.7 mmol KCl, 10 mmol Na_2HPO4, pH 7.4) containing filter-sterilized lysozyme solution in PBS (final lysozyme concentration was 0.5 mg/mL) and incubated at 37^{o}C for 2 hrs (Figure 2). After lysozyme incubation, the sporulated cells were sonicated for 5 min at high setting in a sonication bath and then centrifuged at 2,050 xg at 5^{o}C for 20 min, decanted, resuspended with 20 mLs sterile deionized water, and repeated 2 more times. Spore crops were then distributed into Eppendorf tubes and frozen at -20^{o}C until needed.

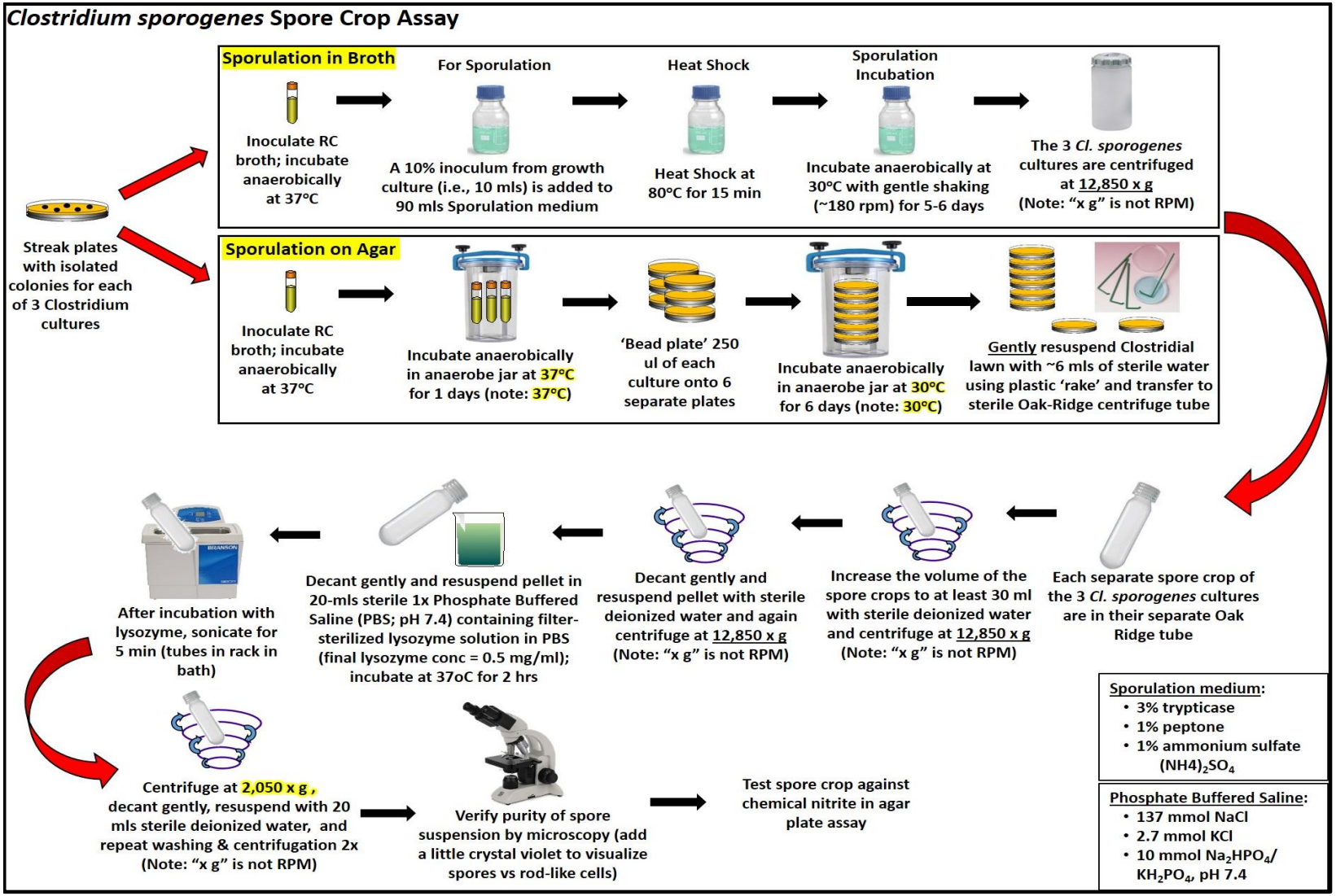

Figure 2. Schematic of 2 different spore crop assays used in this study to obtain spores for challenge studies with hotdogs. The 2 methods differ in the way spores are stimulated to germinate, one in broth and the other on agar plates, after which both methods are identical.

Electron Microscopy of *Clostridium sporogenes* and Spore Crops

Bacteria and spore crops were grown and harvested as described above and were examined by scanning electron microscopy (SEM) at the Oklahoma State University Electron Microscopy Core Facility. Bacteria and spore crops were centrifuged (12,857 x g, 4^{o}C, 10 min) and suspended in 0.1% sterile BPW in preparation of adherence to glass cover slips. Poly-L-lysine pre-coated German glass cover slips (Neuvitro Corp, Vancouver, WA, USA) were placed in 24-well Corning non-coated plates and aliquots of culture or spores were spotted (50-200 ul) onto the cover slips and allowed to dry for 30 min. Approximately 200 ul of 2% glutaraldehyde fixative was added and allowed to react for 2 hrs and then rinsed 3x with buffered wash for 15 min. Cover slips were then fixed with 1% osmium tetroxide (OsO_4) for 1 hr and rinsed 3x again with buffered wash for 15 min. Slides were then dehydrated with an ethanol series: 50%, 70%, 90%, 95% for 15 min each, and then 3x with 100% ethanol for 15 min. Hexamethyldisilazane **(**HMDS**)** was used as an alternative to critical point drying by washing 2x for 5 min and the glass slips were then allowed to dry/set overnight. Double-sided carbon tape was used to adhere the slips to the SEM stubs which were then sputter coated with gold-palladium. SEM was then performed on a FEI Quanta 600 FEG ESEM instrument (ThermoFisher Scientific, Hillsboro, OR, USA).

Screening of Bacteriocin-Producing Strains against *Clostridium sporogenes* (ATCC 19404, ATCC BAA-2695, ATCC 3584)

Four strains of bacteriocin-producing LAB cultures that previously demonstrated exceptional inhibitory activity against *Listeria monocytogenes* were spot-tested on lawns of our strains of *Cl. sporogenes* to confirm antagonistic activity. These included *Lactobacillus curvatus* Beef3 and FS37, *Streptococcus sp.* 323, and *Pediococcus acidilactici* Bac3. These strains were then grown in MRS broth media at 30^{o}C and after growth for 16 hours, bacteriocin-producing LAB cultures were centrifuged at 10,000 rpm in a Sorvall® RC 5C Plus centrifuge with a SS-34 rotor

for 10 minutes, followed by recovery of the supernatant and pellet. Because the bacteriocins belong to heat-stable class IIa bacteriocins, the bacteriocin supernatants were pasteurized at 80°C for 15 min to eliminate any remaining bacterial cells.

Meat Formulations and Hotdog Manufacture

Hotdog formulations included low and high fat all beef versions (based on FOSS method for fat determination) (Table 1). Each fat level was comprised of multiple batches consisting of control (no nitrite), containing sodium nitrite (and sodium erythorbate), containing celery nitrite (and cherry extract), or containing bacteriocin (no nitrite).

Hotdogs were manufactured with formulations described in Table 1. No antimicrobials were added (i.e., lactate, diacetate) as is commonly done in commercial frankfurters so as not to confuse the source of antimicrobial activity of nitrite during assessment of spore germination. Emulsions were stuffed into Viscofan 24/USA casings and thermally processed (cooked) in an electric-fired, batch oven (Alkar, DEC International, Washington) to an internal temperature of 74^{o}C (165^{o}F). After cooking, hotdogs in casings were chilled with a cold water rinse and then peeled using a peeling machine (PS760L Peeler, Linker Machines, Rockaway, NJ, USA). The formulation above was used for evaluation of spore germination when sodium nitrite, celery nitrite, or when no nitrite was used. The hotdogs manufactured by these different protocols were kept separate from each other, vacuum packaged, chilled down to refrigeration temperature (non-frozen), and were immediately incubated for shelf life storage at refrigerated shelf life storage (5^{o}C), accelerated shelf life storage (15^{o}C), and extreme growth permissive temperature storage (35^{o}C). Evaluation of spore outgrowth was further examined when hotdogs were immediately frozen after manufacture and then placed at 35^{o}C for shelf life testing (extreme temperature).

Statistical analysis

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

Table 1. Hotdog ingredient formulation for low- and high-fat all beef hotdogs.

Ingredients	Formulation %	Low Fat Formulation			High Fat Formulation		
Beef:	72.69%	(Beef 90, beef 50)			(Beef 80, beef 50)		
Ice/Water	24.92%	(80% + 20% portions)*			(80% + 20% portions)*		
AC Legg Bologna/Frank Seasoning w/o Erythorbate:	2.14%						
Salt, dextrose, MSG (2.91%), onion powder, garlic powder, spice extracts, tricalcium phosphate							
		CTL	**Nitrite**	**Celery**	**CTL**	**Nitrite**	**Celery**
Prague Powder (6.25% sodium nitrite)		--	0.18%	--	--	0.18%	--
Sodium Erythorbate		--	0.04%	--	--	0.04%	--
Celery Powder (2.34% sodium nitrite)		--	--	0.48%	--	--	0.48%
Cherry Powder (17% ascorbic acid)		--	--	0.20%	--	--	0.20%
Alkaline Phosphate	0.25%						
Total:	**100%**						
Restricted ingredients:							
- Sodium nitrite (max = 156 ppm)		155			156		
- Sodium erythorbate (max = 547 ppm)		543					
- Ascorbic acid (max = 468 ppm)					468		
- Alkaline phosphate (max = 0.5%)	0.34%						

***Note:** Spores were added in the 80% portion of water that was used for the entire batch mixture; the remaining 20% water was used for specific sub-batch portions (control, sodium nitrite/erythorbate, celery nitrite/cherry powder) within each low/high-fat formulation. Bacteriocin-containing low and high fat all beef hotdogs were formulated the same as controls (no nitrate) except that bacteriocin culture supernatant was added in place of the 20% portion of water.

Results and Discussion

Antibiotic Resistance of Inoculum Strains of *Clostridium sporogenes* and use in Developing a Selective Medium

Antibiotic resistance of *Cl. sporogenes* ATCC 3584, ATCC 19404, and ATCC BAA-2695 was determined using BD Sensi-Disc assays described previously. A qualitative listing of the results was prepared of the reactions we obtained with various antibiotic discs (Table 2). This allowed the comparison of antibiotic resistance and *Cl. sporogenes* ATCC 3584, ATCC 19404, and ATCC BAA-2695 which were shown to be resistant to the same 5 antibiotics (oxacillin, colistin, amikacin, tobramycin, isoniazid). This commonality of antibiotic resistance allows their inclusion in agar plating medium to insure that only these strains would be enumerated even if other indigenous bacteria or spore formers were present in the tested samples. However, such assays are not definitive as the dynamics of microbial plating needs to be quantified with the level and combination of antibiotics that is intended to be used as antibiotic levels and/or combinations can result in inhibitory responses that do not result in enumerative quantification. Therefore, plating on appropriate antibiotic levels in agar, both individually and then in combination, provided confirmation that the chosen levels would allow quantitative enumeration if used as a selective media. Using this approach, we obtained similar quantified enumeration data plating the 3 strains of *Cl. sporogenes* on RCM containing oxacillin (1 ug/ml) and colistin (10 ug/ml) or containing colistin (10 ug/ml) and amikacin (10 ug/ml) (Figure 3). An additional adjustment to lower the levels of oxacillin (0.5 ug/ml) and colistin (5 ug/ml) provided better correlation to control platings than at the higher levels (Figure 4). We chose to use oxacillin (0.5 ug/ml) and colistin (5 ug/ml) for subsequent studies because of antibiotic cost and liquid availability of oxacillin. The lack of inhibitory effect of dual antibiotics assures that microbial counts will be quantitative and excludes indigenous bacteria. The generic media with added antibiotics is considered less harsh than using a selective medium as selective medium might inhibit injured or stressed inoculum cells (Karolenko et al., 2020).

Table 2. Antibiotic disk assay using B-D Sensi-Discs on lawns of various strains of *Cl. sporogenes* to determine susceptibility or resistance.

Antibiotic:	Oxacillin	Colistin	Amikacin	Tobramycin	Isoniazid	Ethionamide	Streptomycin	Vancomycin	Rifampin	Nalidixic Acid	Novobiocin
Sensi-Disc Dose (ug) :	1	10	30	10	5	25	50	30	5	30	5
Sensi-Disc Designation:	OX/1	CL/10	AN/30	NN/10	INH/5	EA/25	S/50	VA/30	RA/5	NA/30	NB/5
Cl. sporogenes 3584	-	-	-	-	-	-	++	++	++	++	+
Cl. sporogenes 19404	-	-	-	-	-	-	+	++	+++	++	+
Cl. sporogenes BAA-2695	-	-	-	-	-	+	+	++	++	++	+

Antibiotic:	Furazolidone	Nitrofurantoin	Cephalothin	Gentamycin	Erythromycin	Chloramphenicol	Cefazolin	Cefoxitin	Tetracycline	Ampicillin
Sensi-Disc Dose (ug) :	100	300	30	10	15	5	30	30	30	10
Sensi-Disc Designation:	FX/100	F/M /300	CF/30	GM/10	E/15	C/5	CZ/30	FOX/30	TE/30	AM/10
Cl. sporogenes 3584	+++	+++	+++	+	++	++	+++	++	+++	+++
Cl. sporogenes 19404	+++	+++	+++	+	++	++	++	++	+++	+++
Cl. sporogenes BAA-2695	+++	+++	+++	+	++	++	+++	++	+++	+++

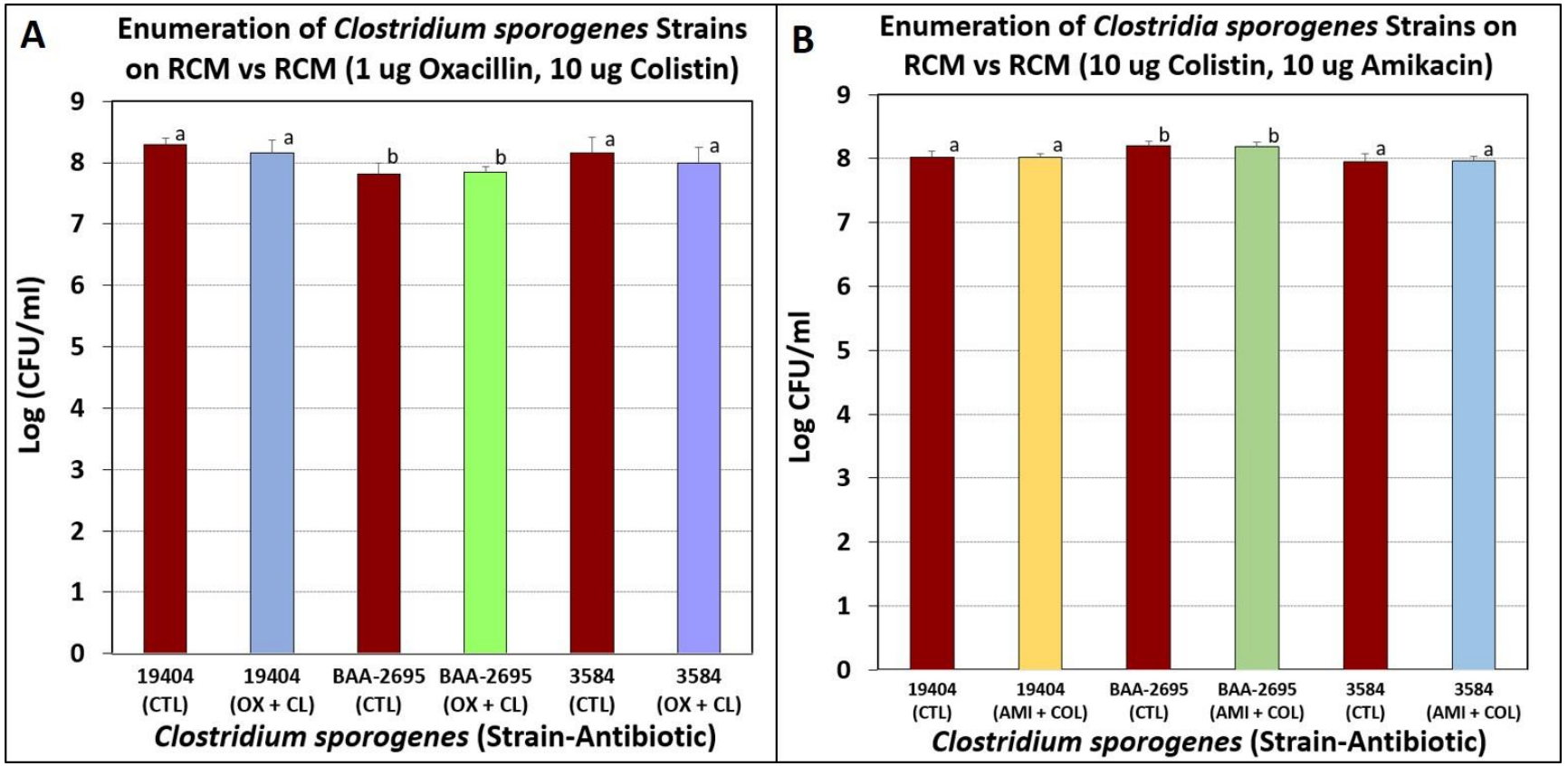

Figure 3. Enumeration of individual strains of *Clostridium sporogenes* on Reinforced Clostridium Medium (RCM) agar containing a combination of 2 antibiotics selected from the disc assay to determine suitability for use in antibiotic selective media for enumeration of *Cl. sporogenes* in this study. Panel A, media prepared with oxacillin (1 ug/ml) and colistin (10 ug/ml); Panel B, media prepared with colistin (10 ug/ml) and amikacin (10 ug/ml). Data are presented as the mean of triplicate replications and error bars represent the standard deviation from the mean. Means with different letters are significantly different, as determined by one-way ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$); means with the same letter are not significantly different ($p > 0.05$).

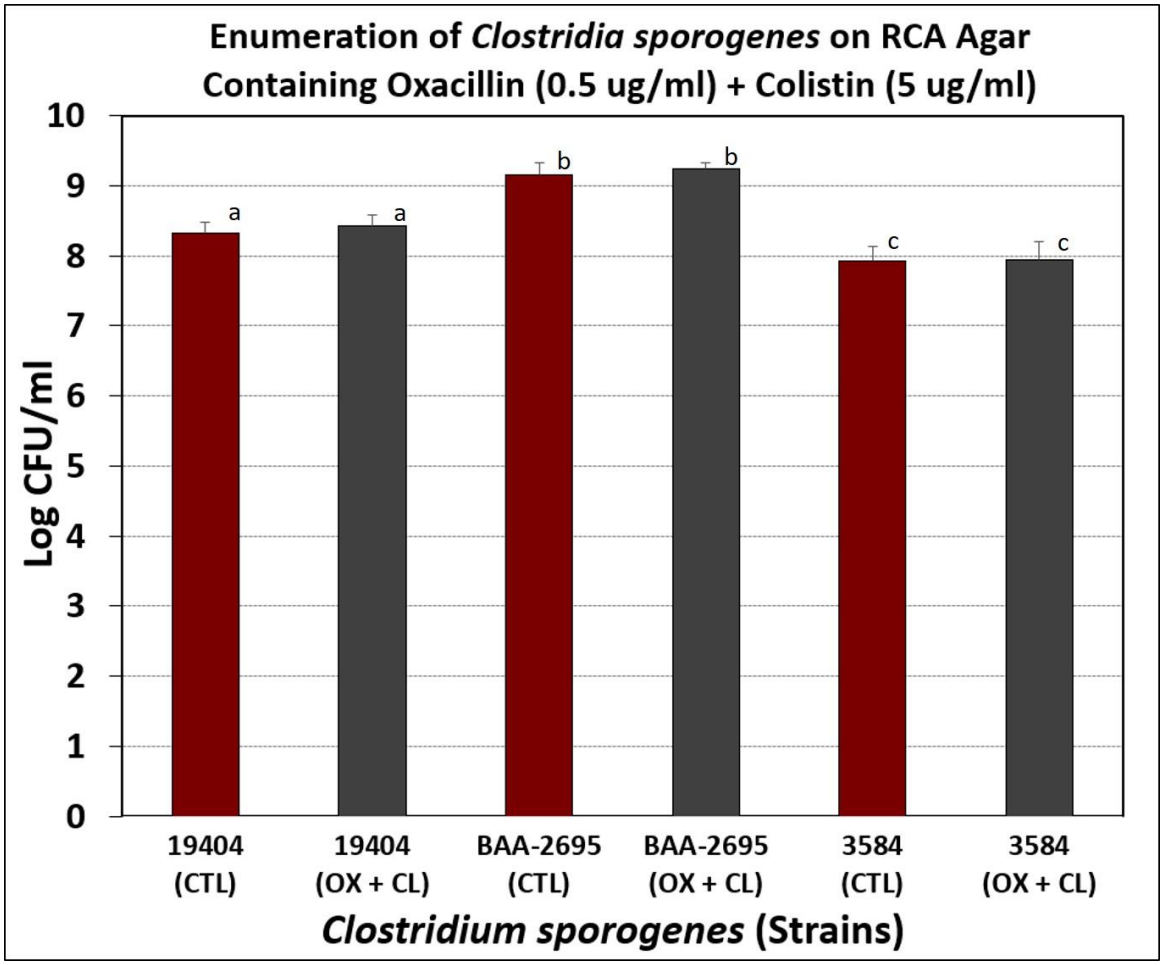

Figure 4. Enumeration of individual strains of *Clostridium sporogenes* on Reinforced Clostridium Medium (RCM) agar containing a combination of 2 antibiotics selected from the disc assay to determine suitability for use in antibiotic selective media for enumeration of *Cl. sporogenes* in this study (oxacillin, 0.5 ug/ml, and colistin, 5 ug/ml). Data are presented as the mean of triplicate replications and error bars represent the standard deviation from the mean. Means with different letters are significantly different, as determined by one-way ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$); means with the same letter are not significantly different ($p > 0.05$).

Electron Microscopy of *Clostridium sporogenes* and Spore Crops

Diagrams and SEM images of *Clostridium* endospores including the outer 'exosporium' obtained from the internet show typical endospore exosporium 'wrapper' surrounding the internal spore and germinating vegetative cells emerging from the exosporium (Figure 5, top row).

Examination of vegetative cells of *Cl. sporogenes* was confusing at first because cells taken from broth were morphologically significantly smaller rods than those recovered from agar that appeared as long, extended rods, making us think that one or the other was perhaps a contaminant. After reexamination of our procedures and repeated isolation and testing, we concurred that growth on agar vs broth provided different vegetative cell morphology (Figure 5, middle row). Examination of sporulating cells show the typical terminal endospore 'club shaped' vegetative cells as one would expect in this state (Figure 5).

During the recovery of spore crops, we mostly observed endospores encased in their exosporium sheath in spite of the fact that we used lysozyme and multiple centrifugations during harvesting of spore crops (Figure 5, bottom row). Although there are extended tedious procedures to recover pure spores, we did not feel this was necessary as the exosporium-coated endospores did not appear to interfere with our use for enumeration nor when used as challenge inoculants in experiments with food products. On occasion we did observed small, circular shapes scattered among some preparations that are presumed to be spores, but because of their uncharacteristically plain circular shape, they could also have been artifacts of the scanning electron microscopy sample treatment protocol (Figure 5, last image, bottom row).

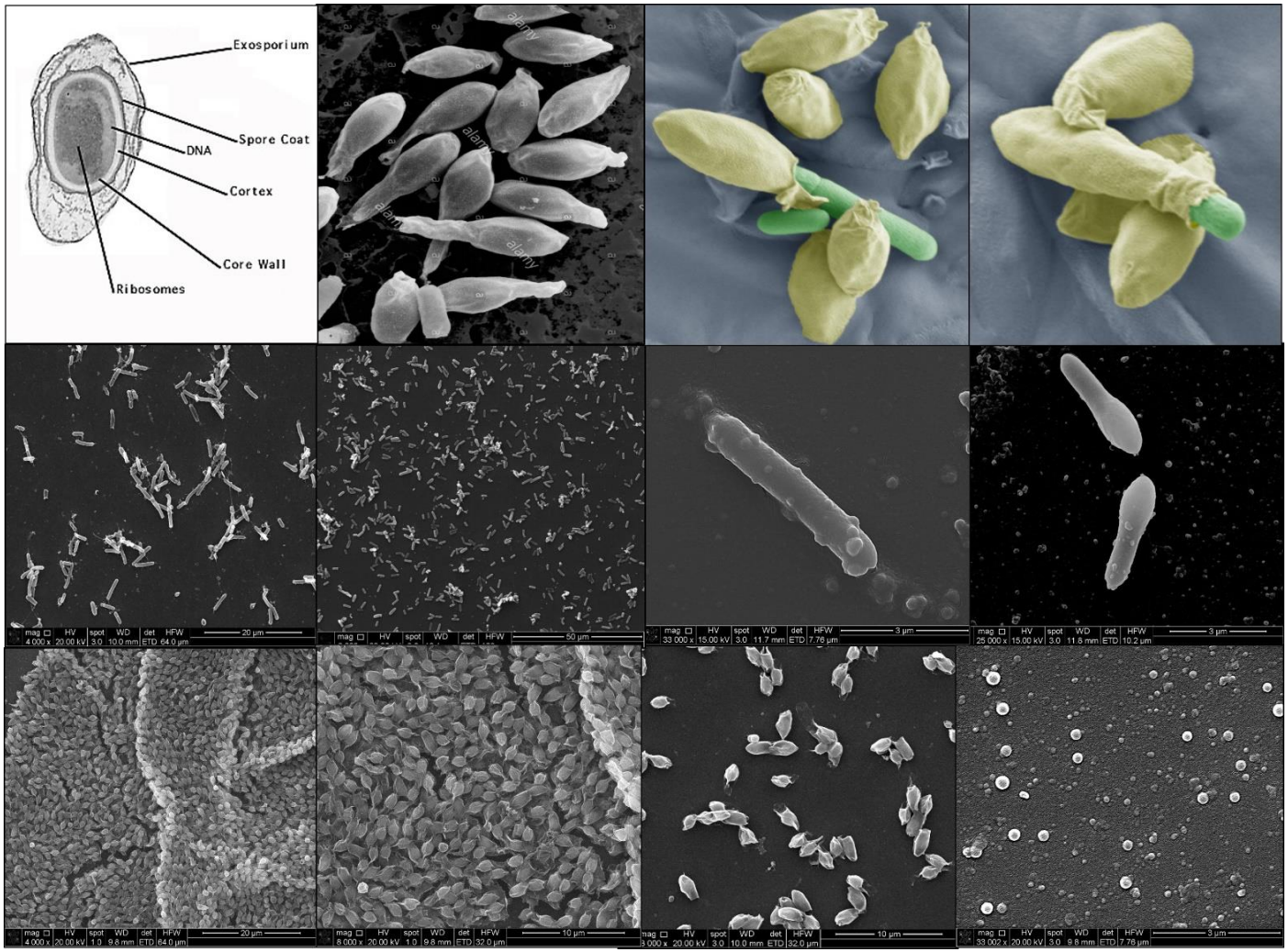

Figure 5. Diagram of spore coat/spore and SEM images of Clostridium endospores (from internet, top row). Images from OSU Microscopy Core Facility (middle and bottom rows): SEM images of Clostridium sporogenes from agar (long rods), from broth (short rods), undergoing sporulation (club-shaped), and concentrated endospores with exosporium and without with spores (bottom row).

Manufacturing of Low Fat All Beef Hotdogs Challenged with Spores from *Clostridium sporogenes*

Hotdogs were manufactured with low- and high-fat formulations; each formulation was split into 3 equivalent portions after emulsifying with spores and then the final portion of water was formulated without nitrites (controls), or with sodium nitrite/erythorbate, or celery nitrite/cherry extract (Table 1,Figure 6).

After manufacture of various hotdogs, they were vacuum packaged, refrigerated, and placed at 5^{o}C, 15^{o}C, and 35^{o}C to examine the effects of different shelf life temperatures. Additional portions were frozen (-45^{o}C) for several hours and then placed at 35^{o}C to compare the effects of freezing on retardation of germination. The hotdogs held at 5^{o}C, 15^{o}C, and 35^{o}C were examined at periodic intervals by plating on RCA containing dual antibiotics (oxacillin, colistin), incubated anaerobically at 37^{o}C, and enumerated after 2 days. The data shows that no outgrowth of spores occurred at either 5^{o}C or 15^{o}C with either sodium or celery nitrite, and even without any nitrite (Figure 7). In every study we performed, samples from hotdogs with either sodium nitrite or celery nitrite were approximately 0.5-log lower than samples from hotdogs manufactured without nitrite (controls). We interpret this to suggest that heating in the prescence of nitrite is inhibitory to the spores. All samples were made from the same batch of meat matrix that was inoculated with the same spore crop, mixed, and then divided into different batch groups dependent on further addition of the 20% remaining water from the formulation (i.e., control water, water containing sodium nitrite, or water containing celery nitrite). Also, since the hotdogs were cooked, the difference is not due to a mixture of vegetative cells and spores as the cooking process should have killed any vegetative cells in all of the meat mixtures and it is only the samples without nitrite that plate out at higher levels. In hindsight, perhaps plating of the raw. Uncooked emulsion would have helped determine if this same phenomenon only occcured after heating (cooking).

Figure 6. Manufacture of all beef hotdogs in the Meat Pilot Plant of the Robert M. Kerr Food & Ag Products Center at OSU. A uniform base of meat was emulsified with 80% of the water, divided into different batches, and the final ingredients added in the remaining 20% water for the various trials (i.e., control with no nitrite, celery nitrite, or sodium nitrite). Hotdogs were stuffed and cooked in an Alkar oven. After cooking hotdogs were cooled by brine spray, peeled, vacuum packaged, and sent directly for shelf life storage at incubators set for 5°C, 15°C, and 35°C.

Although 15°C is considered an abuse temperature for accelerated shelf life studies for some pathogens, it is not effective to demonstrate growth of *Cl. sporogenes* as even samples without nitrite (i.e,. controls) do not show outgrowth at either 5° or 15°C, even after 20 weeks of storage.

However, we evaluated a higher abuse temperature (35°C) for use as a 'permissive' temperature assay to allow spore outgrowth in hotdogs (Figure 8) that provides for better impacts on spore germination and outgrowth. At 35°C, we demonstrated a significant difference between control hotdogs made without nitrite vs experimental hotdogs made with either celery or sodium nitrite (Figure 8A). However, there was no significant difference in spore germination and growth between celery nitrite and sodium nitrite formulated to the same nitrite concentration (Figure 8). Hotdogs were also vacuum-packaged and immediately frozen (-20°C) for 2 hrs after cooking to try to arrest biochemical reactions involved in germination that might be stimulated by the cooking process, and then placed at 35°C (Figure 8B). However, this freezing treatment, may have only slightly reduced their rate of outgrowth (Figure 8B). The presence of nitrite in hotdogs held at 35°C (95°F) may have provided several days of protection from outgrowth to higher numbers compared to samples without nitrite at this extreme abuse temperature. Nitrite is meant to provide safety from minor accidental/unintended temperature abuse that normally would not be expected to occur over prolonged periods, so suppression over such a time period (~48 hrs) at such a high temperature (35°C/95°F) is welcomed, although no one would approve of consumption of such an abused product.

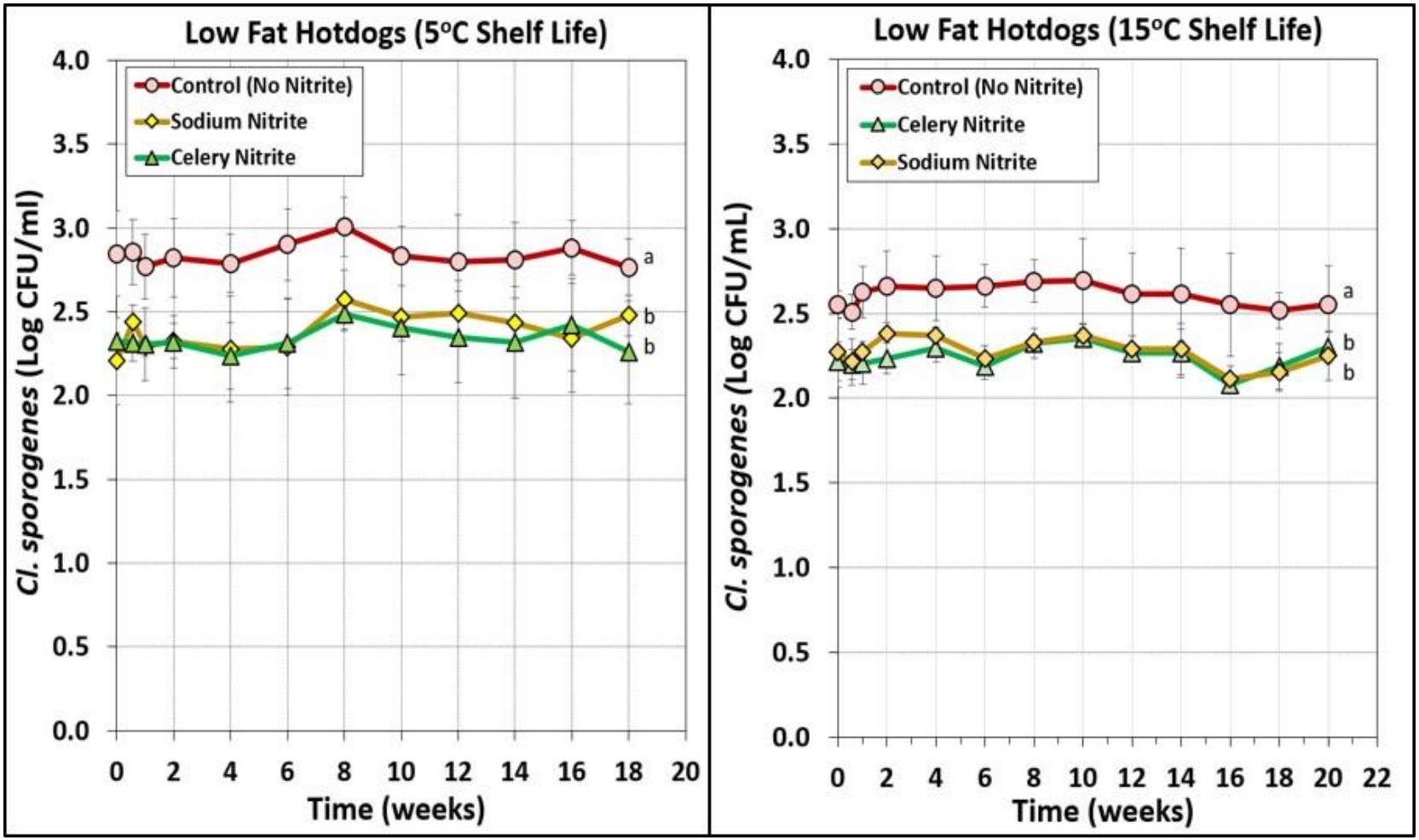

Figure 7. Sampling of low fat hotdogs manufactured without nitrite (Control), with sodium nitrite, or with celery nitrite held at 5°C or 15°C for 18-20 weeks after cooking. Treatments are the means of triplicate samples frrom each of 2 replications (n=6). Treatments were analyzed by RM-ANOVA using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences; treatments with different letters are significantly different ($p < 0.05$); treatments with the same letter are not significantly different ($p > 0.05$).

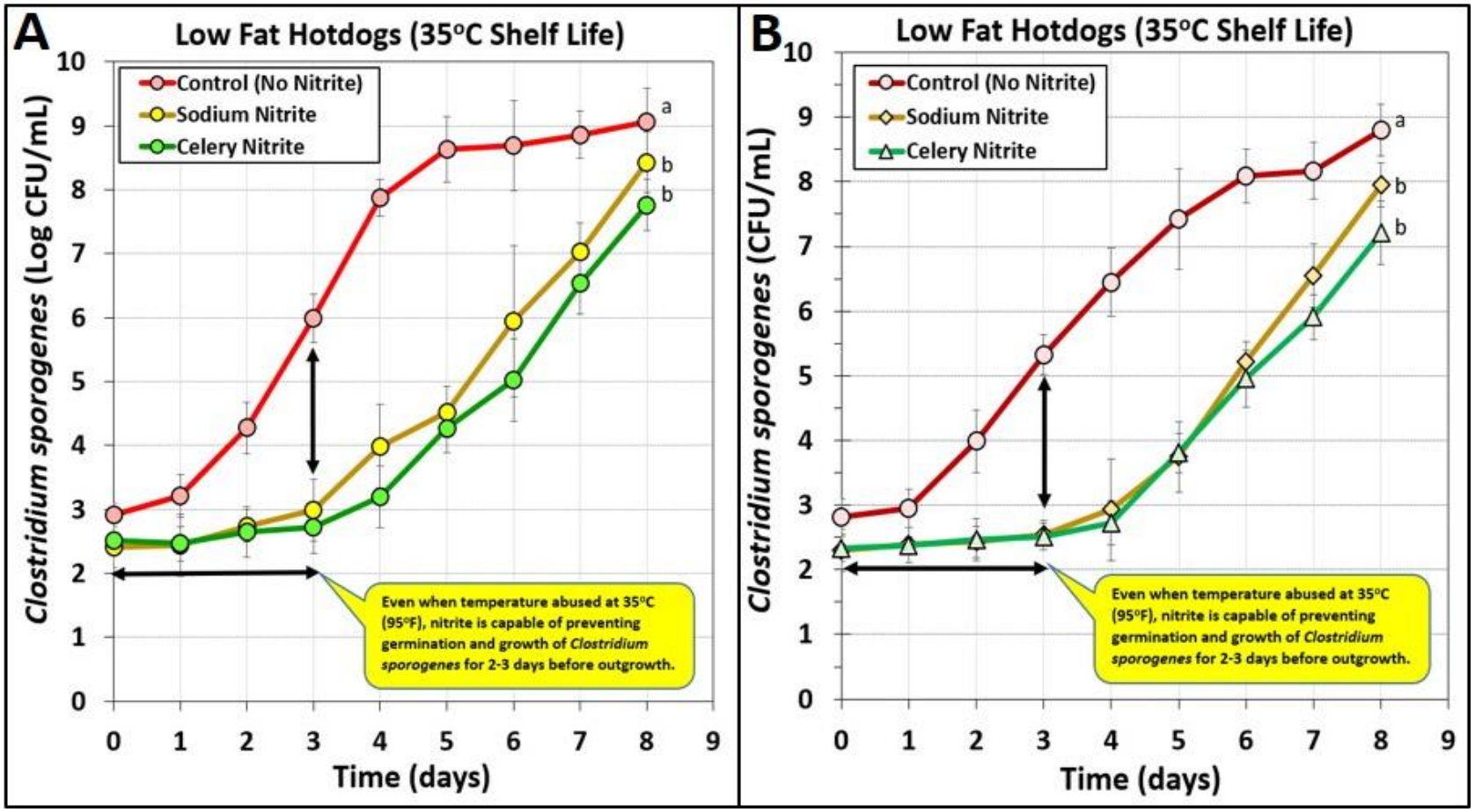

Figure 8. Sampling of low fat all beef hotdogs manufactured without nitrite (Control), with sodium nitrite, or with celery nitrite held at 35°C after cooking for 8 days; this was the 'permissive' assay to allow spore germination and vegetative cell growth to evaluate effectiveness of nitrite in preventing spore germination. Panel A, hotdogs were transferred directly to 35°C incubators after cooking/vacuum packaging. Panel B, hotdogs were frozen (-20°C) for 2 hrs after cooking/vacuum packaging and then transferred to 35°C incubators. Treatments are the means of triplicate samples from each of 2 replications (n=6). Treatments were analyzed by RM-ANOVA using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences; treatments with different letters are significantly different ($p < 0.05$); treatments with the same letter are not significantly different ($p > 0.05$).

Manufacturing of High Fat All Beef Hotdogs Challenged with Spores from *Clostridium sporogenes*

High fat all beef hotdogs were also manufactured in 3 batches (in each of 2 separate trials): control (no nitrite) and hotdogs with celery nitrite or sodium nitrite (Figure 9). The data again shows that no growth occurred at either 5°C (for 18 weeks) or at 15°C for 8 weeks, even when nitrite was not included in the meat matrix (i.e., the controls). Spore outgrowth was only demonstrated at permissive incubation shelf life temperatures of 35°C (95°F). Outgrowth starts immediately within the first 24 hrs in control hotdogs, but in samples with sodium nitrite or celery nitrite, held at 35°C, outgrowth is prevented for up to 2 days even when held at 35°C (95°F). All treatments again show differences in initial numbers between control samples without nitrite (higher counts) than those with sodium or celery nitrite (lower counts) as was also observed with the low fat hotdogs (Figures 8 & 9). In control hotdogs without nitrite held at 35°C, outgrowth starts immediately within the first 24 hrs, but in samples with sodium nitrite or celery nitrite outgrowth is prevented for up to 2 days even when held at 35°C (95°F) (Figure 9).

It is not clear why there is a difference between initial numbers at the start of shelf life between nitrite containing hotdogs (lower counts) and hotdogs without nitrite (higher counts)(Figures 8 & 9). This was consistent with multiple replications and multiple trials for both low and high fat hotdogs. Since these are samples plated immediately after cooking (0 shelf life time), it might be the result of inhibition of 'nitrite plus heat' that could be inhibitory to some portion of spores that were present in the sample. One possibility could be residual nitrite carry over onto plated samples that may have, however, limited inhibition of spore germination. Nitrite levels are significantly reduced by cooking (Tables 4&5) and samples are diluted to 100 fold or more when plated such tha nitrite would not likely be an inhibitory factor on plates. In hindsight, we could have plated a sample of hotdog emulsion of these three treatments prior to cooking to see if they also demonstrated the same difference in counts of *Cl. sporogenes* as observed after cooking.

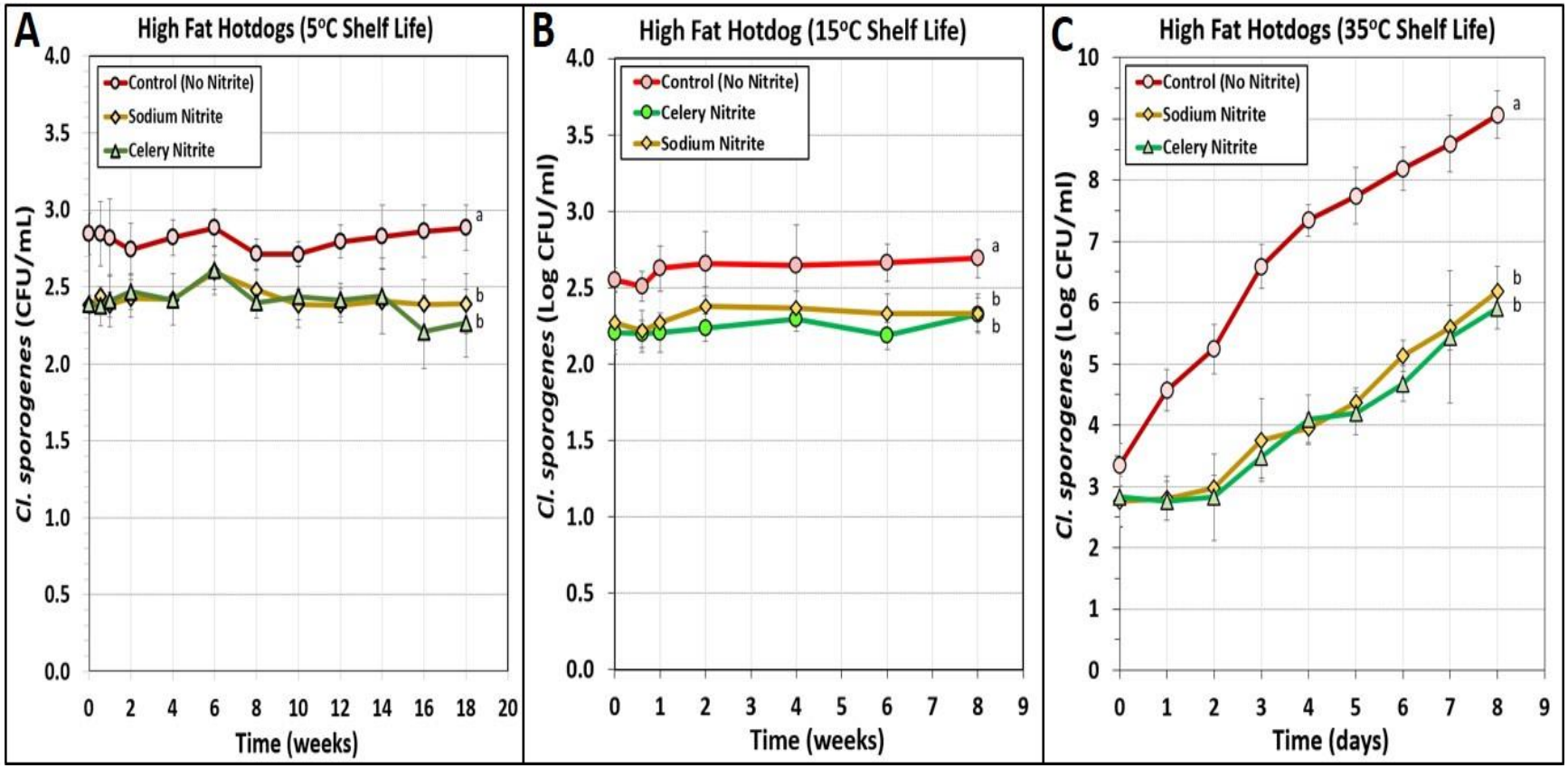

Figure 9. Sampling of high fat all beef hotdogs manufactured without nitrite (Control), with sodium nitrite, or with celery nitrite held at 5°C, 15°C, or 35°C after cooking.. Treatments are the means of triplicate samples frrom each of 2 replications (n=6). Treatments were analyzed by RM-ANOVA using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences; treatments with different letters are significantly different ($p < 0.05$); treatments with the same letter are not significantly different ($p > 0.05$).

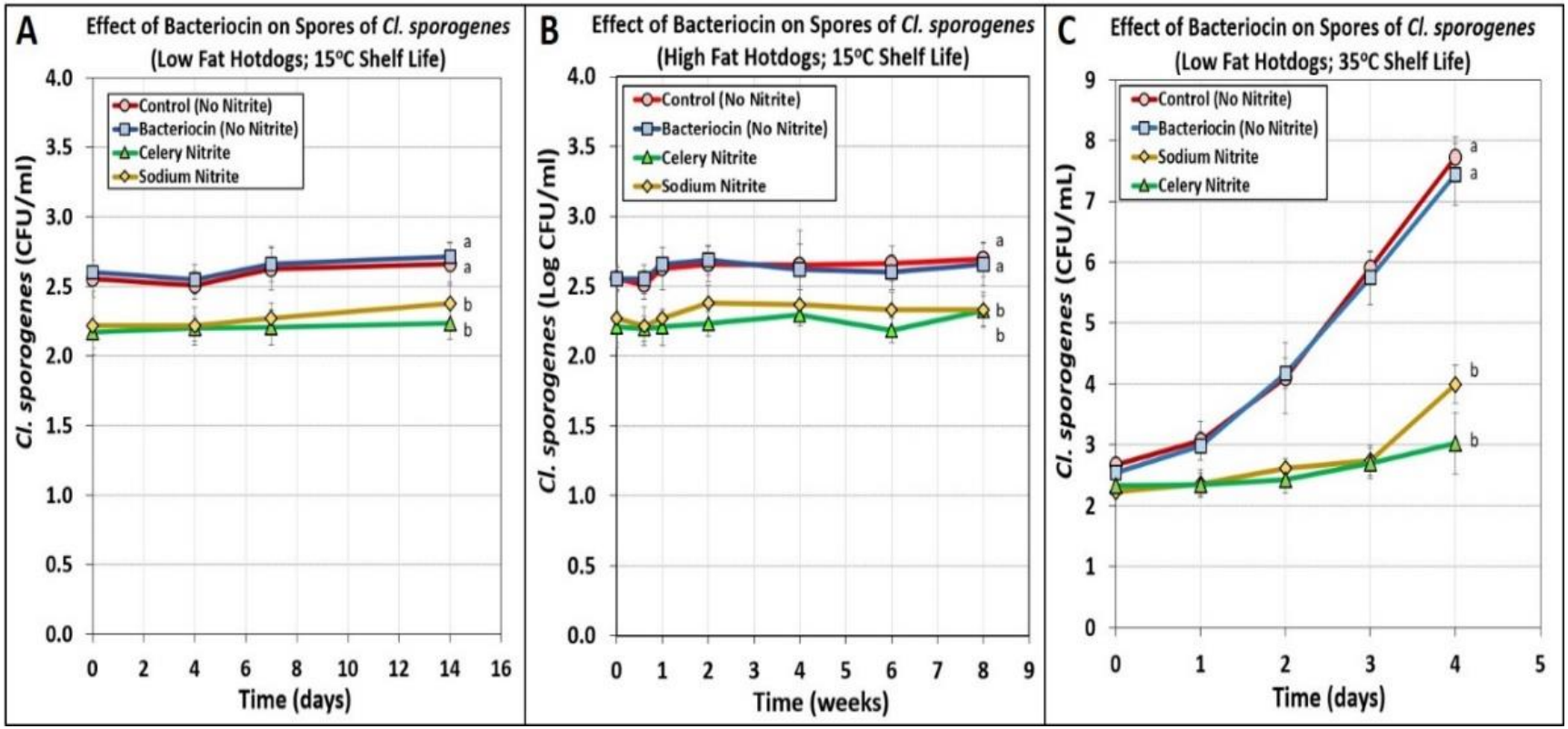

Figure 10. Sampling of low and high fat all beef hotdogs manufactured without nitrite (Control and Bacteriocin), with sodium nitrite, or with celery nitrite held at 15°C or 35°C after cooking to determine if bacteriocin preparations have inhibitory activity against spore germination or vegetative cell growth. Treatments are the means of triplicate samples frrom each of 2 replications (n=6). Treatments were analyzed by RM-ANOVA using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences; treatments with different letters are significantly different ($p < 0.05$); treatments with the same letter are not significantly different ($p > 0.05$).

Manufacturing Low and High Fat All Beef Hotdogs Containing Bacteriocins and Challenged with Spores from *Clostridium sporogenes*

Several trials were performed with both low- and high-fat all beef hotdogs incorporating a bacteriocin cocktail of different bacteriocins (Vijayakumar & Muriana, 2017). The bacteriocins were used in place of nitrite with the intention that the bacteriocins might be inhibitory and prevent spore germination or be inhibitory to *Cl. sporogenes* vegetative cells that might germinate and grow. However, there was no effect observed at 15°C with either low or high fat hotdogs as was observed previously (Figures 10A, 10B). When tested with low fat all beef hotdogs at the permissive temperature of 35°C, the bacteriocin-containing hotdogs showed no significant difference than control hotdogs made without nitrite while nitrite-containing (sodium nitrite, celery nitrite) hotdogs demonstrated the typical 2-3 days of control before outgrowth starts to occur as observed earlier (Figure 10C). In prior testing of batceriocin on our *Clostridium* strains, we observed inhibitory activity when bacteriocin supernatants were spot tested on lawns of *Cl. sporogenes.* In subsequent examination using neutralized supernatants, none of the *Cl. sporogenes* strains were inhibited by the bacteriocin mixture and this was not pursued further. Subsequent screening of additional bacteriocin-producing strains against *Clostridium* spp., using neutralized supernatants, demonstrated that some bacteriocins were capable of a significant inhibitory effect (Table 3). The original bacteriocins producing strains used in this study were screened for activity towards *L. monocytogenes* so the inhibitory activity was not optimized for *Clostridium* spp. (Garver & Muriana, 1993; Henning, Vijayakumar, et al., 2015; Macwana & Muriana, 2012; Vijayakumar & Muriana, 2017). When neutralized supernatants were screened against *Cl. sporogenes* (ATCC 19404, ATCC BAA-2695, ATCC 3584), *Enterococcus faecalis* FS96, *Enterococcus faecium FS97-2*, and *Lactococcus lactis* FS97-1 exhibited activity against *Clostridium sporogenes* (ATCC 19404, ATCC BAA-2695) but, no activity was observed against *Clostridium sporogenes* ATCC 3584 (Table 3). These bacteriocins might be more amenable for this type of approach in future studies.

Table 3. Screening of bacteriocin producing bacteria against *Clostridium sporogenes* strains			
Organism	*Clostridium sporogenes* ATCC 19404	*Clostridium sporogenes* ATCC BAA-2695	*Clostridium sporogenes* ATCC 3584
Enterococcus faecalis FS96	++	++	--
Enterococcus thailandicus FS92	--	--	--
Lactococcus lactis FS90	--	--	--
Enterococcus faecium FS56	--	--	--
Enterococcus faecium FS97-2	++	++	--
Lactococcus lactis FS97-1	++	++	--
Lactococcus lactis FS95	++	--	--
Lactococcus lactis FS93	++	--	--
Lactococcus lactis 91-1	--	++	--
Lactococcus plantarum FS73	--	++	--
Lactobacillus curvatus Beef3 *	--	--	--
Streptococcus sp. 323 *	--	--	--
Pedicoccus acidilactici Bac 3 *	--	--	--
Lactobacillus curvatus FS47 *	--	--	--

HPLC Analysis of Residual Nitrite/Nitrate

Reversed-phase HPLC analysis is a highly selective, sensitive, and convenient method for analysis of residual nitrite/nitrate. Nitrite and nitrate standard curves analyzed by HPLC were highly linear, showing r^2 values of 0.9939 and 0.9955, respectively (Figures 11A, 11B, and 11C). However, the detection of reactive species is tentatively based on their occurrence in the chromatogram at the same residence time as the standards; any reactive interaction with other constituents will change molecular dynamics and change the elution characteristics to something other than that which was determined by molecular standards. This worked well for analysis of nitrate/nitrite from fermented broths of vegetable extracts even though the vegetable extracts were autoclaved (121^{o}C, 15 min, 15 psi). However vegetable juice extracts are significantly lower in concentration of potential reactive constituents than solid meat emulsion.

For hotdogs, the same maximum permissible amount of nitrite (i.e., 156 ppm based on standardized concentrations) for both sodium and celery nitrite was added to their respective uncooked meat emulsions in both low- and high fat all beef hotdogs. Uncooked meat emulsions were sampled, extracted, and analyzed for nitrite/nitrate by HPLC analysis along with samples of hotdogs after cooking (Tables 4 and 5). The array of samples included: a) control samples without spices (to see if spice vegetables contribute nitrate), b) control samples with spices, c) sodium nitrite with spices, and d) celery powder with spices for both low fat and high fat all beef hotdogs were compared (Tables 4 and 5).

No nitrate or nitrate was detected in control samples made without nitrites, whether with or without spices (Tables 4 and 5). The uncooked meat emulsion from low fat hotdogs formulated with nitrites showed appropriate levels of nitrite relative to formulated levels (i.e., sodium nitrite, 168 ppm; celery nitrite, 157 ppm; Table 4) whereas high fat all beef hotdogs showed a bit lower level of nitrite compared to expected (i.e., sodium nitrite, 129 ppm; celery nitrite, 140 ppm; Table 5). It is likely that the lower levels obtained from the high fat hotdogs was the result of either the higher fat levels complexing some of the nitrite or interfering with recovery.

The amount nitrite added in hotdog emulsions was reduced in post-cooked hotdogs. The uncooked emulsion from sodium nitrite-added low fat hotdogs was 166.8 ± 2.05 ppm while levels obtained from post-cooked hot dogs were 34.4 ± 4.6 ppm (Table 4). Similarly, celery nitrite in uncooked emulsion from low fat hotdogs was 156.6 ± 3.7 ppm while levels after cooking dropped to 26.8 ± 4.5 ppm (Table 4). This was not unexpected as reactive nitrite, formulated into complex meat emulsion and subjected to high cooking temperatures (~165^{o}F) is known to form various reactive intermediates (i.e., nitrosamines). Several authors have also suggested that some nitrite added to meat during the curing process may be converted back to nitrate. Nitrite is utilized during the curing reaction and Dethmers and Rock (1975) stated that nitrous acid might yield nitric oxide and nitrate from the oxidation of nitric acid by oxygen to yield nitrite which later reacts with water to form nitrite and nitrate. They suggest that sodium erythorbate, used as cure accelerators, might also play a role in the conversion of nitrite to nitrate (Dethmers et al., 1975). Nitrite may be converted to nitrate due to the inert properties of nitrite in a cooked cure meat system(Price & Schweigert, 1987).

In low fat all beef hotdogs formulated with sodium nitrite, we found low levels of nitrate in the uncooked meat emulsion (6 ppm ± 0.5 ppm) while higher levels in the cooked meat emulsion (82.4 ± 4.7 ppm) (Table 3). This suggests that nitrate is being generated in some manner. In low fat hotdogs formulated with celery nitrite, uncooked meat emulsion was shown to have moderately higher levels of nitrate (47.5 ± 2.4 ppm) than sodium nitrite-added meats, and a bit more in the cooked hotdogs (53.8 ± 2.9 ppm) (Table 4). This could be explained in that we examined the commercial celery nitrate preparation and it was found to contain low levels of nitrate in addition to the high nitrite levels. In high fat hotdog emulsions, we found similar results whereby sodium or celery nitrite significantly decreases after cooking, but we also found moderate levels of nitrate (47-54 ppm) in the post-cooked hotdogs for both preparations formulated with sodium or celery nitrite (Table 5). The nitrite ion is a highly reactive species especially at pH < 7.0, thus, it interacts with various components of meats such as amines, amino acids, sulfhydryl's, phenolic compounds,

myoglobin, ascorbate or erythorbate. Hence, the residual levels of nitrite in the cured meat is expected to be significantly lower in comparison to ingoing nitrite of uncooked meat. This loss of nitrite depends on multiple factors such as the pH of meat, temperatures, processing conditions, and storage time and temperature (Sen & Baddoo, 1997).

HPLC Analysis of Residual Erythorbate/Ascorbic Acid

Sodium erythorbate and Cherry extract (ascorbic acid) are added as curing accelerators and are an important part of the curing process since nitrite must be reduced to nitric oxide and the iron portion of muscle pigments be reduced from the ferric (+3) to the ferrous (+2) state. The curing accelerators have diverse functions, but their primary function is to promote the reduction of nitrite to accelerate curing reactions and catalyze the formation of cured meat pigments. Although we were able to generate exceptional standard curves by HPLC C8 ion pairing analysis of both erythorbate and ascorbic acid (Figures 11D and 11E), we were not able to detect ascorbic acid at appropriate use levels from uncooked hotdog emulsions nor detect any erythorbate at all (Tables 6 and 7).

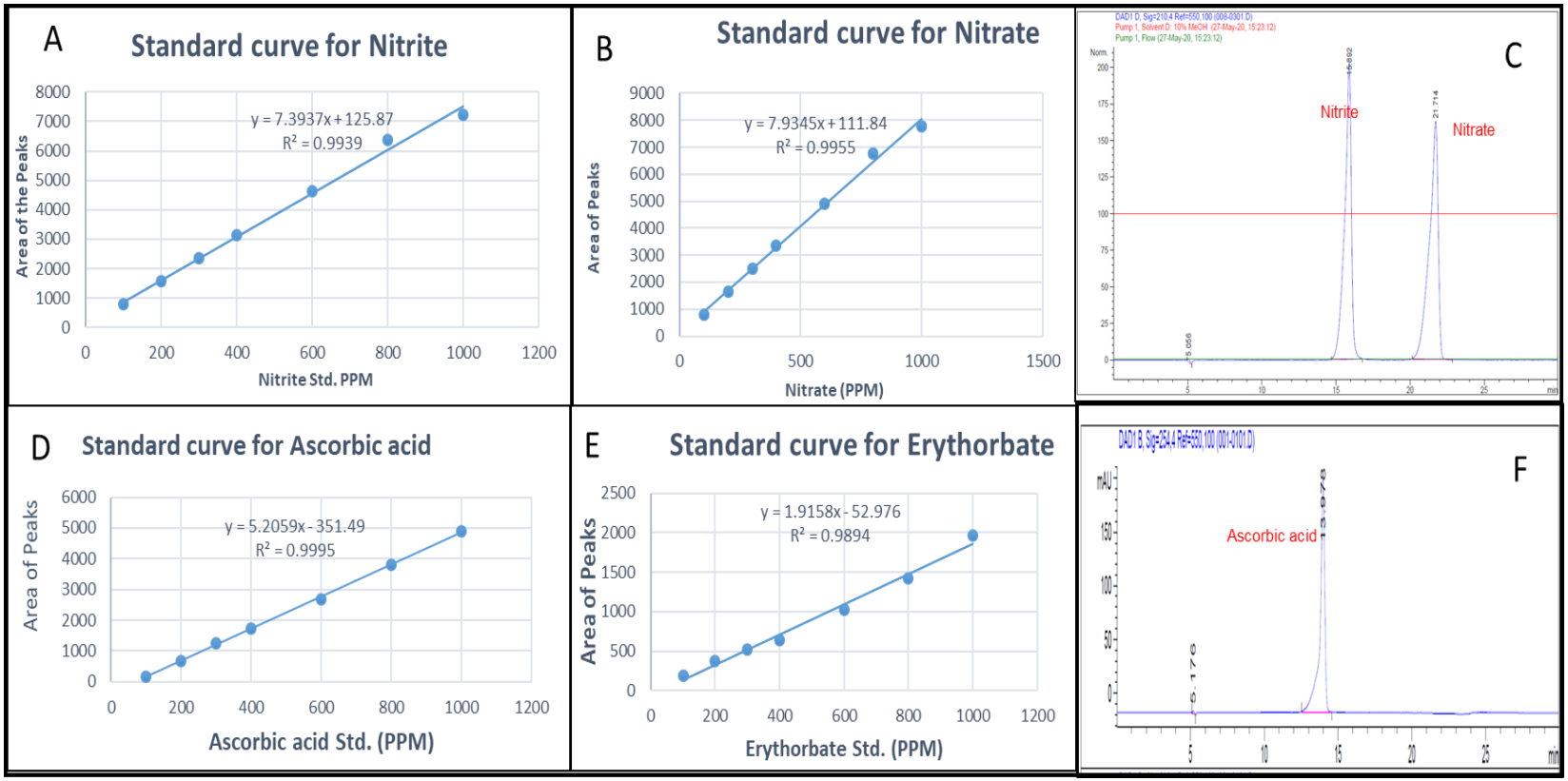

Figure 11: HPLC analysis of nitrate and nitrite using C_8-RP ion-pairing chromatography. Panel A, Standard Curve for Nitrite Stanards. Panel B, standard curve of nitrate standards.Panel C, chromatogram of nitrite and nitrate standards Panel D, standard curve of Ascorbic acid standards. Panel E, standard curve of Erythorbate standards. Panel F,chormatogram for ascorbic acid.

Table 4. Low Fat Hotdogs: residual nitrite and nitrate analysis data obtained by HPLC analysis.

Low Fat	Nitrite (ppm)		Nitrate (ppm)	
	Uncooked	**Cooked**	**Uncooked**	**Cooked**
Control without spice	Not detected	Not detected	Not detected	Not detected
Control with spice	Not detected	Not detected	Not detected	Not detected
Sodium nitrite	166.8 ± 2.0	34.4 ± 4.6	6.0 ± 0.54	82.4 ± 4.7
Celery powder	156.6 ± 3.7	26.8 ± 4.5	47.5 ± 2.4	53.8 ± 2.9

*All Nitrite/Nitrate levels are expressed in ppm (parts per million); 156 ppm of sodium nitrite or celery extract is added to the hotdog emulsion before cooking. Controls do not have added nitrite or celery extract.

Table 5. High Fat Hotdogs: residual nitrite and nitrate analysis data obtained by HPLC analysis.

High Fat	Nitrite (ppm)		Nitrate (ppm)	
	Uncooked	**Cooked**	**Uncooked**	**Cooked**
Control without spice	Not detected	Not detected	Not detected	Not detected
Control with spice	Not detected	Not detected	Not detected	Not detected
Sodium Nitrite	128.6± 4.5	27.3 ± 3.3	29 ± 3.6	15.6 ± 2.2
Celery powder	140 ± 4.7	30.3 ± 4.6	30.65 ± 4.3	32.5 ± 1.7

* All Nitrite/Nitrate levels are expressed in ppm (parts per million); 156 ppm of sodium nitrite or celery extract is added to the hotdog emulsion before cooking. Controls do not have added nitrite or celery extract.

Table 6. Ascorbic acid calculation on celery powder based hotdogs using cherry extract.

Initial level =468 ppm	Low Fat		High Fat	
	Uncooked	Cooked	Uncooked	Cooked
Cherry extract/ Ascorbic acid (with celery nitrite)	90.1±2.3	123.1±3.4	95.4±2.6	187.1±3.8

*All ppm reported is average ppm obtained from samples. 468 ppm of ascorbic acid as a form of cherry extract is added to the hotdog emulsions before cooking. Controls do not have any added cherry extract.

Table 7. Erythorbate calculation for comparison of Uncooked and Cooked Samples.

Initial level = 543 ppm	Low Fat		High Fat	
	Uncooked	Cooked	Uncooked	Cooked
Sodium erythorbate (with sodium nitrite)	Not detected	Not detected	Not detected	99.5±2.7

*All ppm reported is average ppm obtained from samples. 543 ppm of sodium erythorbate is added to the hotdog emulsions before cooking. Controls do not have any added cherry extract.

Conclusions

The data presented herein demonstrates that celery nitrite provides an equivalent level control on germination and outgrowth of *Clostridium sporogenes* spores as does sodium nitrite when used at equivalent levels. Our data suggests that refrigeration temperature alone is capable of preventing outgrowth of *Clostridium sporogenes* even when no nitrite is present, and even as high as 15°C. Food safety measures are often introduced for those random occurrences where physical control measures (i.e., temperature) have mechanical problems and the use of nitrite provides a protective advantage to hotdogs in case such problems were to occur.

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