Isolation and Characterization of Nitrate Reducing Bacteria (NRB) to Generate Vegetable-Derived 'Natural Nitrite' and Validation of its Effectiveness against *Clostridium* Spores in Low- and High-Fat Hotdogs

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ABSTRACT

Introduction. In the US, sodium nitrate is used as a curing agent in processed meats and is therefore a regulated ingredient. Nitrate reducing bacteria (NRB) can convert vegetable nitrate into nitrite allowing green/clean label status in the US as per USDA-FSIS definition of 'natural nitrite'.

Objectives. Our objectives were to isolate and characterize new nitrate reducing bacteria that that can generate nitrite from vegetable extracts and examine celery-derived nitrite to inhibit spore germination by *Clostridium sporogenes* (surrogate for pathogenic *Clostridium* spp.) in processed meats.

Materials and Methods. The traditional qualitative in-liquid nitrite broth test has been used to detect nitrite in individual culture tubes is further not suitable for Gram(+)/lactic acid bacteria (LAB) nor for testing mixtures of bacteria from various food/animal samples. We developed an in-liquid version of nitrate broth using M17 broth (containing 0.1% potassium nitrate) that was suitable for Gram(+)/lactic acid bacteria and further developed an M17-based 'on-agar' colony-screening plate assay to detect the conversion of nitrate to nitrite by bacterial colonies using multiple soft agar overlays. Nitrate to nitrite conversion was quantified by C8 reversed-phase ion-pairing HPLC analysis. Celery-nitrite powder was also used to manufacture low- and high-fat hotdogs inoculated with spores of *Clostridium sporogenes* and incubated at various temperatures.

Results. Several strains isolated using our on-agar nitrate assay were selected and compared to *Staphylococcus carnosus*, a strain commonly used for nitrate reduction. *S. carnosus* was able to convert 1100 ppm M17-nitrate broth to 917 ppm nitrite. *Staphylococcus caprae* and *Panteoa agglomerans*, several NRB isolates, were also able to ferment the same broth to 916 ppm and 867 ppm nitrite, respectively. Vegetable nitrite (celery) was able to prevent spore germination in equivalent fashion as sodium nitrite in both low- and high-fat hotdogs. Although no germination was observed at low temperature, the use of high temperature ('permissive conditions') allows potential *Clostridium* spore activation to be observed.

Significance. We have demonstrated that vegetable/celery-nitrite provides equivalent inhibition of *Clostridium* spore germination as does sodium nitrite when standardized to common levels in hotdogs. This is the first report of an on-agar colony screening assay for the detection of NRBs allowing them to be readily isolated from various mixed bacterial sources. This will allow the identification of new bacteria that may generate nitrite an produce additional inhibitory factors (i.e., bacteriocins) that may contribute to pathogen suppression in foods.

Keywords: Vegetable nitrite, *Clostridium*, spores, nitrate reducing bacteria, hotdogs, sodium nitrite.

Definitions:

1. **Meat curing** is defined as direct addition of nitrite. U.S. Department of Agriculture (USDA) strictly regulates the use of sodium nitrate and/or sodium nitrite in cured meat.
2. **'Natural' meat and poultry products.** Can not have artificial ingredients (food coloring, preservatives).
3. **Vegetables are loaded with nitrate.** This is partly from nitrogen in the soil. USDA considers that 95-98% of our exposure to nitrite comes from microorganisms in the gut that convert vegetable nitrate to nitrite.
4. **'Natural Nitrite'.** USDA deemed that microbially fermented vegetable extracts (vegetable nitrite) can be considered 'natural nitrite', be added to meat/poultry products in lieu of sodium nitrite:
   - **Can now claim 'no added preservatives'**
   - **Can not claim the product is 'cured'**
5. **'Natural Nitrite'** is also allowed for use in other products besides 'natural meats'.

Nitrate content of commonly consumed vegetables.

Food items	N	Nitrate (mg 100 g^{-1}) Mean ± SD	Median
Leafy vegetables			
Basil	19	236 ± 91.9	258
Parsley	30	171 ± 182	129
Coriander	26	237 ± 101	196
Cress	19	225 ± 168	173
Dill	20	183 ± 140	161
Fenugreek	21	656 ± 41.8	55.2
Leek	21	177 ± 96.5	161
Mint	21	279 ± 248	182
Tarragon	19	424 ± 298	355
Spinach	37	183 ± 124	142
Lettuce	41	365 ± 232	313
Celery	21	261 ± 160	206
Cabbage	33	198 ± 146	155

Part 1: Isolation and Characterization of Nitrate Reducing Bacteria and the Fermentation of Nitrate to 'Natural Nitrite'

Part 1 : Isolation and Characterization of Nitrate Reducing Bacteria

Part 2 : Application of 'Natural Nitrite' to Inhibit *Cl. sporogenes* in Hotdogs

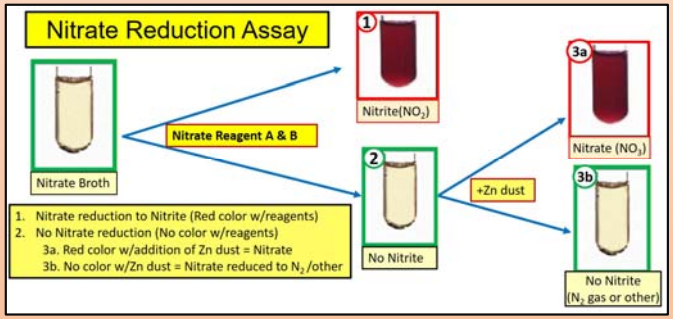
Bacterial Cultures and Media

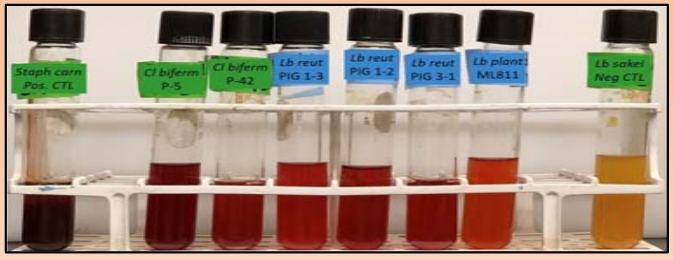
Cultures screened for nitrate reducing activity included 500+ cultures from the FAPC Gilliland Culture collection, the Muriana Culture Collection, and bacteria isolated from food (retail) and animal (FAPC slaughter facility) samples.

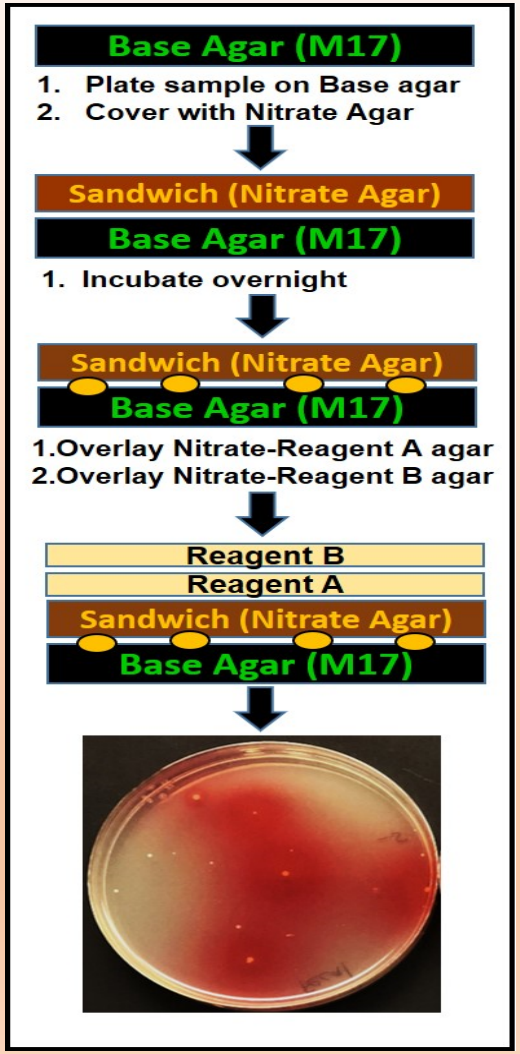
The Nitrate Reduction Assay / NRA (in Broth)

The NRA is a qualitative procedure for determining the ability of bacteria to reduce nitrate into nitrite using Nitrate Broth.

a. **Reagent A**: Sulfanilic acid (0.5% in 30% acetic acid)
b. **Reagent B**: Alpha-napthylamine (0.8% in 30% acetic acid)
c. **Reagent C**: Zinc powder.





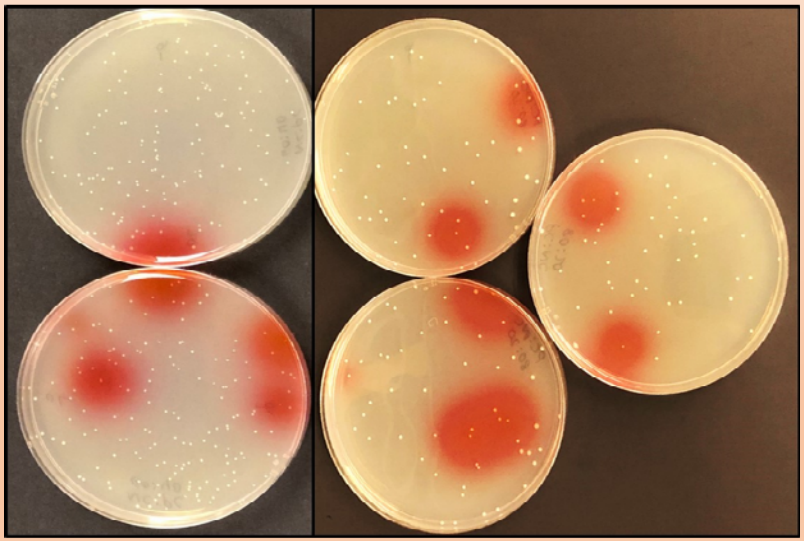


Isolation and Identification of Nitrate Reducing Bacteria

Colonies with red zones were isolated from agar, cultured, and confirmed for nitrate reducing activity in nitrate broth. Positive nitrate reducing cultures were then subjected to 16S rRNA PCR Bacterial DNA was isolated from cell pellets by the bead collision method described by Coton and Coton (Coton & Coton, 2005). Identification was obtained by PCR of 16S rRNA sequences using 'universal primers' and the amplified products were submitted to the OSU DNA Sequencing Core Facility.

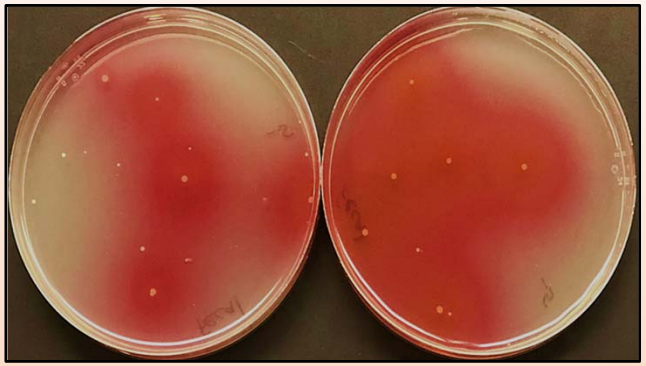
Statistical Analysis

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).



Agar plate Nitrate Assay showing nitrate reducing bacterial colonies (red 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.

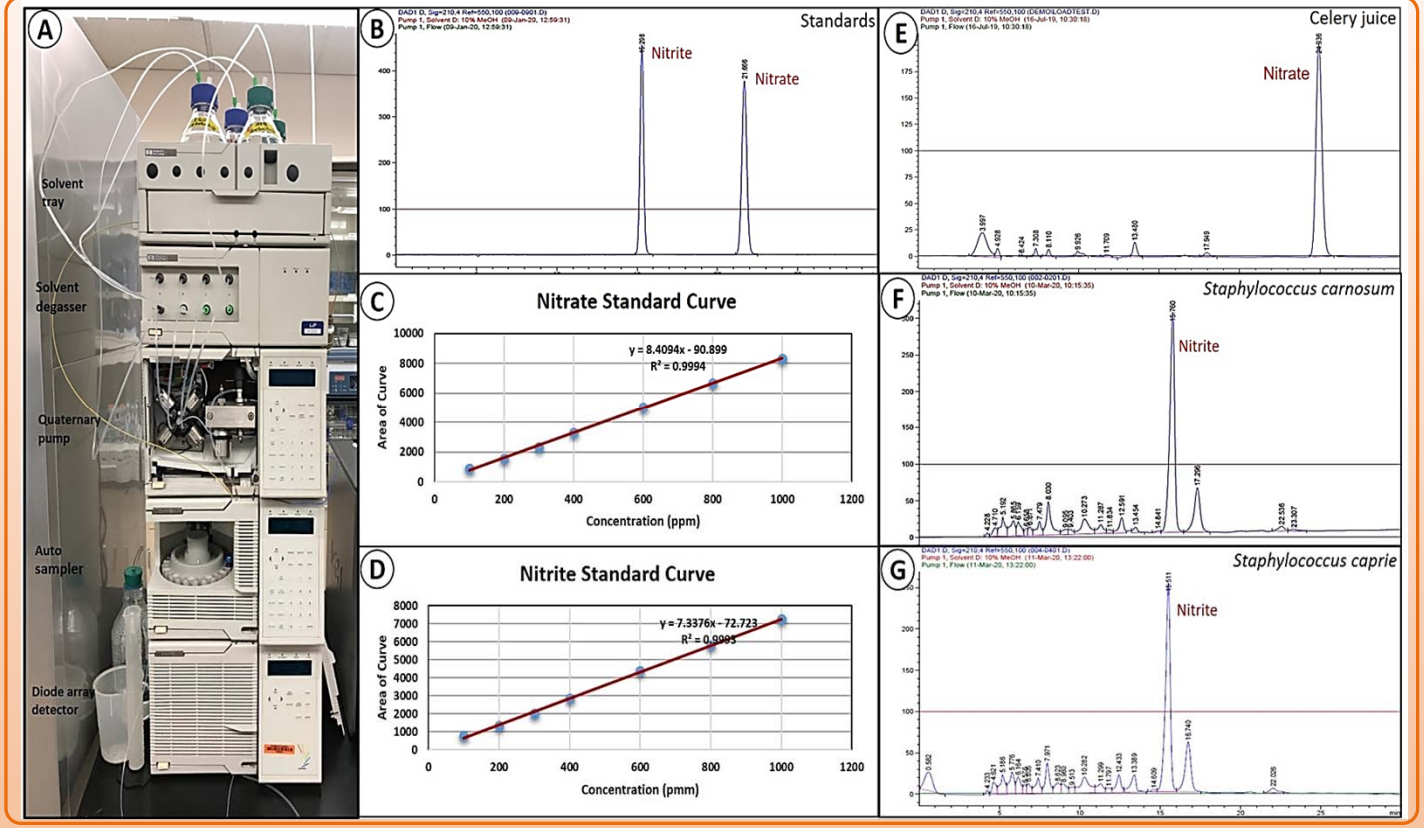
Part 1: Isolation and Characterization of Nitrate Reducing Bacteria and the Fermentation of Nitrate to 'Natural Nitrite'



Using the on-agar Nitrate Reduction Assay, we isolated additional bacteria from animal sources/retail foods using commercial Nitrate Agar as the overlay [better for Gram(-) bacteria] and M17 agar as the base medium [better growth and nitrate reductase activity by Gram(+) bacteria] (Table 1).

Table 1. Nitrate reducing bacteria detected in culture collections or isolated from food and 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



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

HPLC analysis allowed us to quantify nitrate and nitrite after fermentation in liquid media, or vegetable extracts (Figure 4) 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 (various sources) and hotdogs (processed meat applications) are tested.

Part 2 : Application of 'Natural Nitrite' to Inhibit *Clostridium* Spore Germination in Low- and High-Fat Hotdogs

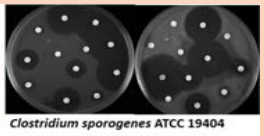
Objectives:

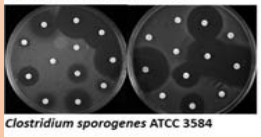
To examine celery-derived nitrite to inhibit spore germination by *Clostridium sporogenes* (surrogate for pathogenic *Clostridium* spp.) in processed meats.

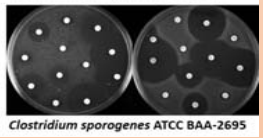
Bacterial Strains:

- *Clostridium sporogenes* **ATCC 3584**,
- *Clostridium sporogenes* **ATCC 19404**, and
- *Clostridium sporogenes* **ATCC BAA-2695**
  - (PA 3679) / (NCA 3679)
- Were obtained from American Type Culture Collection and used in this study to harvest spores for use as a 3-strain spore inoculum
- Characterized for **<u>antibiotic resistance</u>**


B-D Sensi-Discs and Applicator


Clostridium sporogenes ATCC 19404


Clostridium sporogenes ATCC 3584


Clostridium sporogenes ATCC BAA-2695

- *Clostridium* spp. are strict **<u>anaerobes</u>**, that require anaerobic gas environment.

Clostridium sporogenes Spore Crop Assay

Sporulation in Broth

Inoculate RC broth; incubate anaerobically at 37°C

For Sporulation: A 10% inoculum from growth culture (i.e., 10 mls) is added to 90 mls Sporulation medium

Heat Shock: Heat Shock at 80°C for 15 min

Sporulation Incubation: Incubate anaerobically at 30°C with gentle shaking (~180 rpm) for 5-6 days

The 3 *Cl. sporogenes* cultures are centrifuged at <u>12,850 x g</u> (Note: "x g" is not RPM)

Streak plates with isolated colonies for each of 3 Clostridium cultures

Sporulation on Agar

Inoculate RC broth; incubate anaerobically at 37°C

Incubate anaerobically in anaerobe jar at 37°C for 1 days (note: 37°C)

'Bead plate' 250 ul of each culture onto 6 separate plates

Incubate anaerobically in anaerobe jar at 30°C for 6 days (note: 30°C)

<u>Gently</u> resuspend Clostridial lawn with ~6 mls of sterile water using plastic 'rake' and transfer to sterile Oak-Ridge centrifuge tube

Each separate spore crop of the 3 *Cl. sporogenes* cultures are in their separate Oak Ridge tube

Increase the volume of the spore crops to at least 30 ml with sterile deionized water and centrifuge at <u>12,850 x g</u> (Note: "x g" is not RPM)

Decant gently and resuspend pellet with sterile deionized water and again centrifuge at <u>12,850 x g</u> (Note: "x g" is not RPM)

Decant gently and resuspend pellet in 20-mls sterile 1x Phosphate Buffered Saline (PBS; pH 7.4) containing filter-sterilized lysozyme solution in PBS (final lysozyme conc = 0.5 mg/ml); incubate at 37oC for 2 hrs

After incubation with lysozyme, sonicate for 5 min (tubes in rack in bath)

Centrifuge at 2,050 x g , decant gently, resuspend with 20 mls sterile deionized water, and repeat washing & centrifugation 2x (Note: "x g" is not RPM)

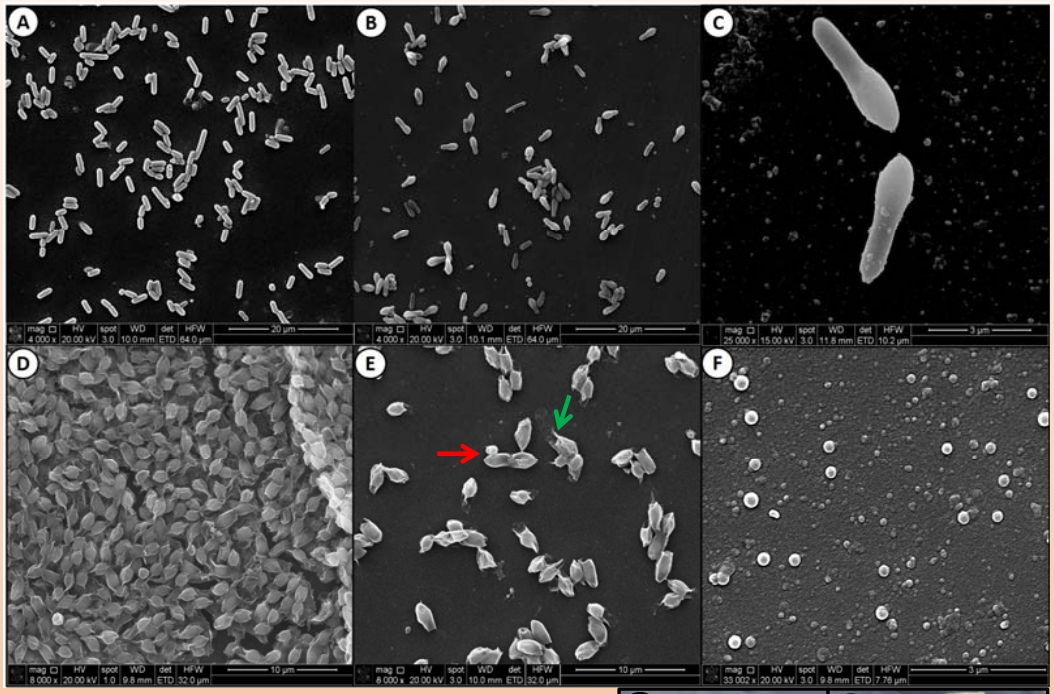
Verify purity of spore suspension by microscopy (add a little crystal violet to visualize spores vs rod-like cells)

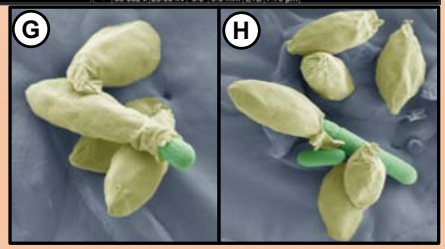
Test spore crop against chemical nitrite in agar plate assay

<u>Sporulation medium</u>:
- 3% trypticase
- 1% peptone
- 1% ammonium sulfate $(NH4)_2SO_4$

<u>Phosphate Buffered Saline</u>:
- 137 mmol NaCl
- 2.7 mmol KCl
- 10 mmol Na_2HPO_4/ KH_2PO_4, pH 7.4

Part 2 : Application of 'Natural Nitrite' to Inhibit *Clostridium* Spore Germination in Low- and High-Fat Hotdogs





Scanning Electron Microscopy (SEM) of *Clostridium sporogenes* spores:

Panel A, *Cl. sporogenes* vegetative cells;
Panel B, vegetative cells w/terminal endospores;
Panel C, close up of terminal endospores;
Panel D, aggregate of exosporium w/spores;
Panel E, dispersed exosporia containing spores;
Panel F, isolated spores;
Panel G, germination (early);
Panel H, germination (later)

'Natural Nitrite' in low- /high-fat hotdogs:

- Batch 1: Controls (spores, but no nitrite)
- Batch 2: Sodium nitrite (+erythorbate) + spores
- Batch 3: Celery nitrite (+cherry extract) + spores

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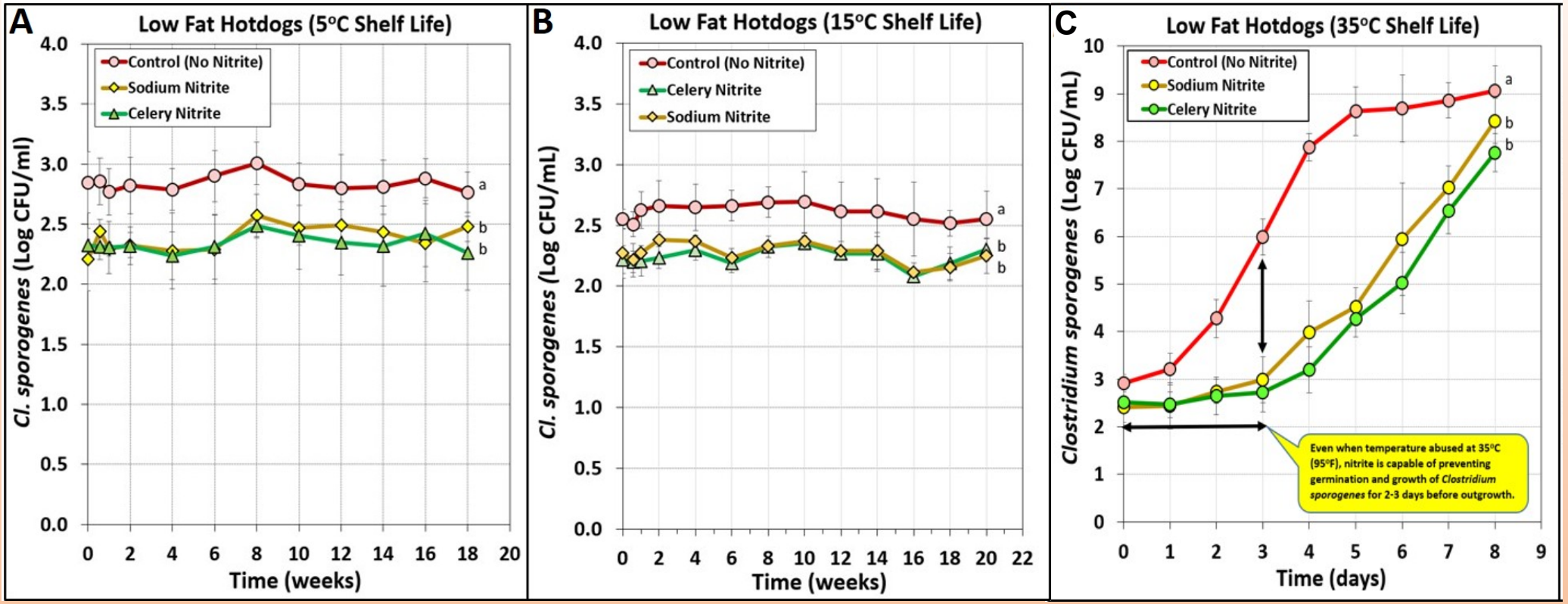
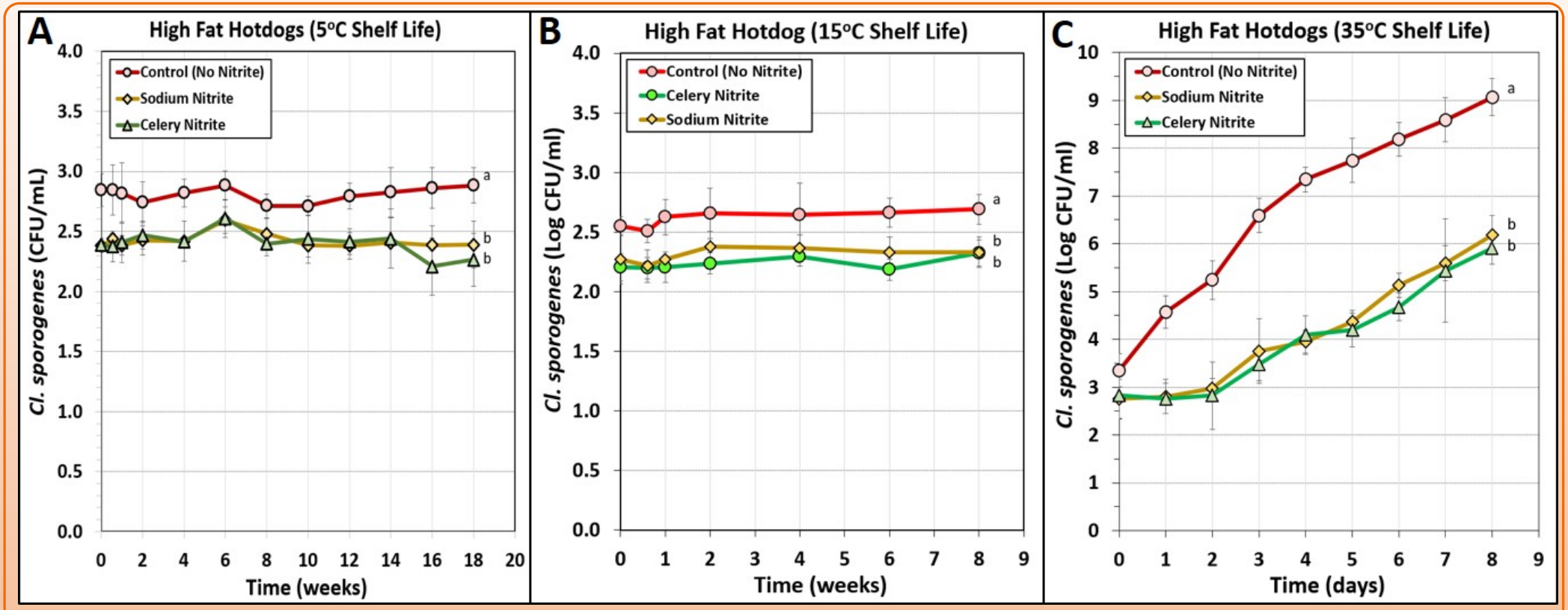


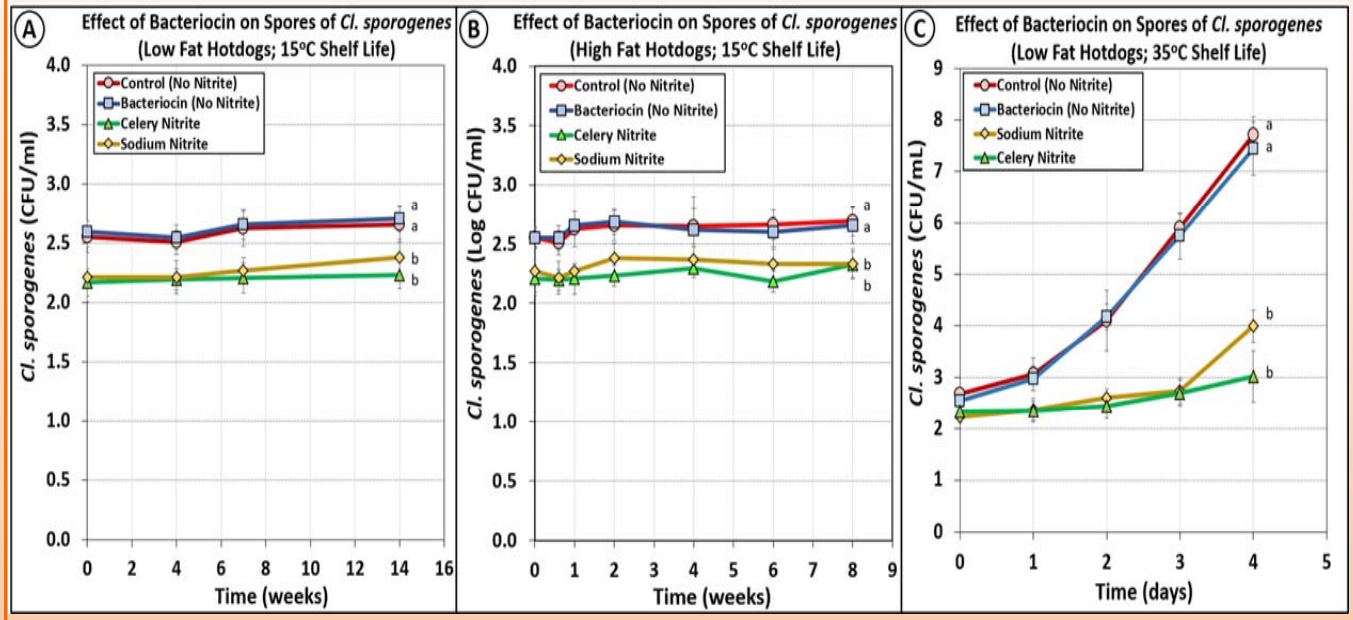
Sampling of low fat hotdogs (containing spores) manufactured without nitrite (control), with sodium nitrite, or with celery nitrite held at 5 °C (Panel A) or 15 °C (Panel B) for 18-20 weeks after cooking, or at 35 °C for 8 weeks (Panel C). Data points are the means of triplicate samples from each of 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 ($p > 0.05$).

Part 2 : Application of 'Natural Nitrite' to Inhibit *Clostridium* Spore Germination in Low- and High-Fat Hotdogs



Sampling of high fat hotdogs (containing spores) manufactured without nitrite (control), with sodium nitrite, or with celery nitrite held at 5 °C (Panel A), 15 °C (Panel B), or 35 °C (Panel C) after cooking. Treatments are the means of duplicate replications testing triplicate samples at each time period (n_{total}=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 ($p > 0.05$).

Future Research for 'Natural Nitrite', Inhibition of *Clostridium*, and Bacteriocins



Sampling of low and high fat hotdogs (containing spores) manufactured without nitrite (control), without nitrite but with bacteriocin preparation, with sodium nitrite, and with celery nitrite held at 15 °C (Panel A, low fat hotdogs), 15 °C (Panel B, high fat hotdogs), or 35 °C (Panel C, low fat hotdogs). Treatments are the means of duplicate replications testing triplicate samples at each time period (n_{total}=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 ($p > 0.05$).

Future Research in this Subject Area:

1) Bacteriocins were anti-Listeria but tested on *Clostridium*; we will screen for bacteriocins effective against Clostridia.

2) Compare the effect of vegetable nitrite vs sodium nitrite on *Cl. botulinum* (non-toxigenic isolates) and *Cl. perfringens*.

3) Try to quantify nitrite (HPLC analysis) from processed meats.

Acknowledgements & Funding Sources:

- Foundation for Meat & Poultry Research & Education (Beef Checkoff; #G20001940)
- USDA-NIFA National Needs Fellowship Grant (USDA #2016-11407)
- Advance Foods/SE Gilliland Professorship in Microbial Food Safety (#21-57200)
- Oklahoma Agricultural Experiment Station Hatch Project (#OKL03090)