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Research Note

Implications of Decreased Nitrite Concentrations on *Clostridium perfringens* Outgrowth during Cooling of Ready-to-Eat Meats

MEGAN I. MYERS,[1] JOSEPH G. SEBRANEK,[1]* JAMES S. DICKSON,[1] ANGELA M. SHAW,[2] RODRIGO TARTÉ,[1] KRISTIN R. ADAMS,[1] AND STEVE NEIBUHR[1]

[1]*Department of Animal Science and* [2]*Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa 50011, USA*

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ABSTRACT

Increased popularity of natural and organic processed meats can be attributed to the growing consumer demand for preservative-free foods, including processed meats. To meet this consumer demand, meat processors have begun using celery juice concentrate in place of sodium nitrite to create products labeled as no-nitrate or no-nitrite-added meat products while maintaining the characteristics unique to conventionally cured processed meats. Because of flavor limitations, natural cures with celery concentrate typically provide lower ingoing nitrite concentrations for ready-to-eat processed meats than do conventional cures, which could allow for increased growth of pathogens, such as *Clostridium perfringens,* during cooked product cooling such as that required by the U.S. Department of Agriculture. The objective of this study was to investigate the implications associated with reduced nitrite concentrations for preventing *C. perfringens* outgrowth during a typical cooling cycle used for cooked products. Nitrite treatments of 0, 50, and 100 ppm were tested in a broth system inoculated with a three-strain *C. perfringens* cocktail and heated with a simulated product thermal process followed by a typical cooling-stabilization process. The nitrite concentration of 50 ppm was more effective for preventing *C. perfringens* outgrowth than was 0 ppm but was not as effective as 100 ppm. The interaction between nitrite and temperature significantly affected ($P < 0.05$) *C. perfringens* outgrowth in both total population and number of vegetative cells. Both temperature and nitrite concentration significantly affected ($P < 0.05$) *C. perfringens* spore survival, but the interaction between nitrite and temperature did not have a significant effect ($P > 0.05$) on spore outgrowth. Results indicate that decreased nitrite concentrations (50 ppm) have increased potential for total *C. perfringens* population outgrowth during cooling and may require additional protective measures, such as faster chilling rates.

Increased consumer interest in natural and organic foods has contributed to increased popularity of natural and organic meat products, which in turn has led to the development of naturally cured or "uncured" meat products. However, to be considered natural or organic, the addition of chemical preservatives such as nitrate and nitrite is not permitted in these products. This requirement poses a problem, because nitrite is an irreplaceable ingredient in cured meats. However, as an alternative to nitrate and nitrite, processors began using vegetable juices and powders, most commonly from celery, which contain measurable amounts of nitrate and nitrite and produce characteristics similar to those of conventionally cured meat products. This alternative to conventional nitrate and nitrite meets the labeling regulations for natural and organically produced processed meats *(12)*.

Product safety becomes a key issue with naturally cured meat products because natural cures, such as celery juice powder, typically do not provide the same amount of nitrite as found in conventionally cured products *(7, 9),* depending on the formulation. Most celery powders contain 15,000 to 20,000 ppm of nitrite, and when these powders are added to meat products at the normal concentration of 0.3 to 0.4%, the resulting nitrite concentration in the product is about 60 to 80 ppm *(8)*. The amount of celery powder that can be used also is limited by flavor *(9)*. However, in-going nitrite concentrations are critical for controlling microbial growth in cured meat products, and in numerous studies naturally cured or "uncured" meat products do not provide the same level of safety as do conventionally cured products with regard to foodborne pathogens such as *Clostridium perfringens* and *Listeria monocytogenes (3, 7)*.

Prevention of *C. perfringens* growth is important because this pathogen is responsible for type A foodborne illness. *C. perfringens* can be a problem in both conventionally cured and "uncured" meat products because it is a spore-forming bacterium that is ubiquitous throughout the environment *(4)*. Improper heating and cooling of foods is a major factor in foodborne outbreaks due to *C. perfringens* infection because of the pathogen's ability to form spores capable of surviving thermal processing, which makes proper cooling, storage, reheating, and holding of food products critical to product safety *(10)*. Therefore, guidelines such as the U.S. Department of Agriculture (USDA), Food Safety and Inspection Service Appendix B *(11)* for the cooling and stabilization of cooked meat and poultry

* Author for correspondence. Tel: 515-294-1091; Fax: 515-294-5066; E-mail: sebranek@iastate.edu.

products have been implemented to assure the control of *C. perfringens*.

Because of the reduced nitrite concentrations typical of naturally cured natural and organic processed meats, a better understand is needed of how these natural cures affect *C. perfringens* under the current Appendix B ready-to-eat meat cooling and stabilization requirement. The objective of this study was to investigate the implications of reduced nitrite concentrations for the potential survival of *C. perfringens* under conditions similar to those associated with these naturally cured products.

MATERIALS AND METHODS

Spore preparation. Three *C. perfringens* strains (ATCC 10258, 13124, and 12917, Food Research Laboratory [FSRL], Iowa State University) were grown in conditions capable of inducing sporulation, as described by Juneja et al. *(5)*. The three strains were individually grown in freshly steamed fluid thioglycollate medium (10 ml/tube; Sigma-Aldrich, St. Louis, MO) under anaerobic conditions and stored at 37°C for 24 h followed by two consecutive 24-h transfers into freshly steamed fluid thioglycollate medium, each stored anaerobically at 37°C. One milliliter of each culture was then inoculated into a 100-ml bottle of modified Duncan and Strong medium (Himedia Laboratories, Mumbai, India) to induce sporulation *(1),* incubated at 37°C for 48 h, and then centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 2 ml of chilled ethanol at 4°C for 2 h. This suspension was then centrifuged at 10,000 × *g* for 10 min, the supernatant was discarded, and the pellet was vortexed thoroughly (Vortex Genie 2, Fisher Scientific, Pittsburgh, PA) and washed twice with 0.1% peptone water (Difco, BD, Franklin Lakes, NJ). The final pellet was suspended in physiological saline (0.85%, wt/vol, sodium chloride) and stored in the FSRL pathogen cooler at 4°C until used for sample inoculation.

Sample preparation. Eighty samples were prepared using 6 ml of *C. perfringens* inoculum, 3 ml of fluid thioglycollate medium, and 1 ml of nitrite treatment. Because a broth medium was used, the nitrite treatments consisted of conventional nitrite at concentrations similar to those often found in formulations for naturally cured meat products. A 1:1 ratio of *C. perfringens* vegetative cells and spores was utilized for each strain, resulting in the total 6 ml (3 ml of vegetative cells and 3 ml of spores) of *C. perfringens* inoculum per sample. *C. perfringens* vegetative cells were prepared by inoculation of freshly steamed fluid thioglycollate medium with each of the strains, and the cultures were then incubated for 24 h at 37°C. Each strain received two consecutive 24-h transfers into freshly steamed fluid thioglycollate medium and were then anaerobically stored at 37°C. Samples were prepared by vortexing together 1 ml of each spore strain to create a three-strain spore cocktail, and then 1 ml of vegetative cells from each strain and 3 ml of freshly steamed fluid thioglycollate were added. Finally, 1 ml of each treatment solution (containing 0, 50, or 100 ppm of nitrite) was added to the broth system. The control sample (0 ppm) was created by adding sterile distilled water. Once all components were added, the samples were thoroughly mixed and placed into test tube racks in a programmable water bath (RTE-211, NESLAB Instruments, Newington, NH), which was tempered to 12.8°C. The water bath was capable of achieving specific temperature points (±0.01°C) via a built-in heating and refrigeration system. The water bath was programmed to simulate a thermal processing cycle typical for hams followed by the 15-h USDA Appendix B cooling-stabilization cycle, resulting in a 20-h total heating and cooling cycle.

Heating and cooling cycle. Samples were heated using a thermal processing treatment that simulated a commercial thermal process. Thermal processing data were collected from a boneless ham process used at the Iowa State University Meat Laboratory, and these data were used as a template for the heat treatment in this experiment. The heat treatment consisted of four 60-min intervals, with each interval meeting a temperature set-point and finally reaching the optimal temperature of 71.1°C, which represents a 4-h thermal processing cycle. Once 71.1°C was reached, samples were held for 5 min at this temperature before beginning the cooling process. The water bath was programmed to begin cooling samples for 55 min (0.5°C/min) according to USDA Appendix B guideline 2 *(11)* to reach 54.44°C (the start of the Appendix B guideline 3). Products cured with a minimum of 100 ppm of sodium nitrite may enter a slow cooling cycle from 54.44°C to reach 26.67°C within 5 h (0.1°C/min) and from 26.67°C to reach 7.22°C within 10 h (0.03°C/min), resulting in a 15-h total cooling cycle.

To monitor *C. perfringens* growth, 11 sample times were established during heating and cooling. Sample 1 was taken at 0 h (the cold sample, 12.77°C) to obtain the starting *C. perfringens* population (~7 log CFU/g). Sample 2 was taken at the height of the heating cycle after the 5-min holding period (71.1°C). Sample 3 was collected at the beginning of the Appendix B cooling guideline (54.4°C). Samples 4 through 6 were collected at 90-min intervals after reaching 54.4°C. After the initial 5-h cooling period, the 10-h cooling cycle began at 26.7°C. Sample 7 was then collected 2 h after the start of the 10-h cooling period, and samples 8 through 11 were then collected at 2-h intervals, with sample 11 collected at 7.2°C, which was the end of the cooling cycle. Two samples for each treatment (0, 50, and 100 ppm) were collected at each of the 11 sample times for microbiological analysis. Three independent replicate experiments were conducted.

Microbiological analysis. Collected samples were vortexed and then diluted to appropriate 10-fold dilutions with 0.1% peptone water. From each dilution, 0.1 ml was spread plated with a glass rod in duplicate on Perfringens Agar Base (Oxoid, Basingstoke, UK) with tryptose sulfite cycloserine (TSC; Oxoid) supplement and subsequently labeled plate A. The original samples were then placed into an 80°C water bath for 10 min to kill all vegetative cells, leaving only spores. These samples then vortexed and diluted with 0.1% peptone water into designated 10-fold dilutions. From each dilution, 0.1 ml was spread plated with a glass rod in duplicate on Perfringens Agar Base with TSC supplement and labeled plate B. Both A and B plates were overlaid with Perfringens Agar Base and TSC supplement at ~8 ml per plate. All plates were then placed into an anaerobic jar that included a Gas Pak anaerobic system envelope (BD) and incubated at 37°C for 24 (±2) h, after which colonies were counted.

Viable *C. perfringens* spore populations were determined by counting colonies from the B plates, representing only spore populations. Vegetative cell populations were determined by counting colonies from the A plates (total population) and then subtracting the spore population (from the B plates). Total populations of viable *C. perfringens* were determined by colony counts from the A plates.

Statistical analysis. Viable *C. perfringens* total population, number of vegetative cells, and number of spores were analyzed using the statistical system WINKS SDA software (Texasoft, Cedar Hill, TX). A one-way analysis of variance (ANOVA) was

TABLE 1. *Mean total* C. perfringens *populations during cooling and stabilization in medium with different nitrite concentrations*[a]

	C. perfringens counts (log CFU/ml)[b] with:		
Temp (°C)	0 ppm	50 ppm	100 ppm
71.1	6.00	6.17 A	5.47 A
54.4	5.87	6.10 B	5.93 B
46.1	5.97	5.23 C	4.83 C
37.2	5.40 X	4.10 DXY	2.80 DY
28.9	5.47 X	4.37 DX	2.47 DY
22.8	5.47 X	3.83 DY	3.77 CY
18.9	5.33 X	3.60 DY	2.03 DZ
15.0	5.30 X	3.80 DY	2.90 DY
11.1	5.67 X	3.63 DY	1.97 DZ
7.2	5.40 X	4.20 DXY	3.20 DY

[a] Standard error of the means = 0.68.
[b] Within a column, means with different letters A through D are significantly different ($P < 0.05$). Within a row, means with different letters X through Z are significantly different ($P < 0.05$).

used to determine differences among the mean values for each treatment (nitrite concentrations) within the total population. When significant effects were found, a Newman-Keuls multiple comparison test was used for pairwise comparisons. A two-way ANOVA was used to determine the significance of effects of treatment (nitrite concentration), temperature, and the interaction of nitrite and temperature for viable *C. perfringens* spores, vegetative cells, and total populations. When significant effects were found, a Newman-Keuls multiple comparison test was used for pairwise comparisons. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Table 1 and Figure 1 illustrate the survival of the total *C. perfringens* population (vegetative cells and spores) throughout the cooling-stabilization cycle. At 0 ppm, there was no significant change ($P > 0.05$) in the total population of *C. perfringens,* with a slight but nonsignificant decline occurring throughout the cooling cycle (Table 1). At both 50 and 100 ppm of nitrite, a significant decrease ($P < 0.05$) in total population occurred from the beginning to the end of the cooling cycle. The temperatures of 46.1, 54.4 and 71.1°C each resulted in significantly higher counts ($P < 0.05$)

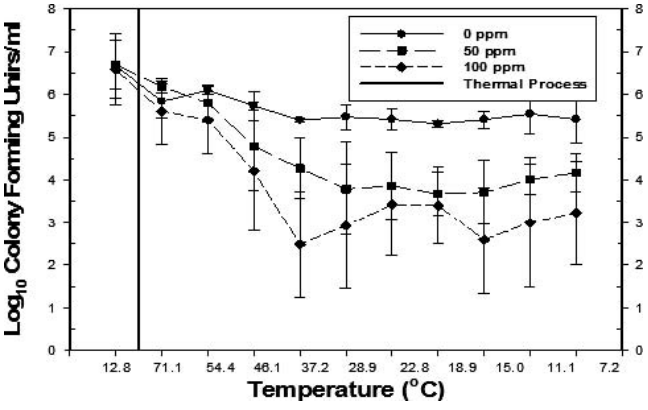

FIGURE 1. *Survival of the total* C. perfringens *population during cooling-stabilization in medium with different nitrite concentrations.*

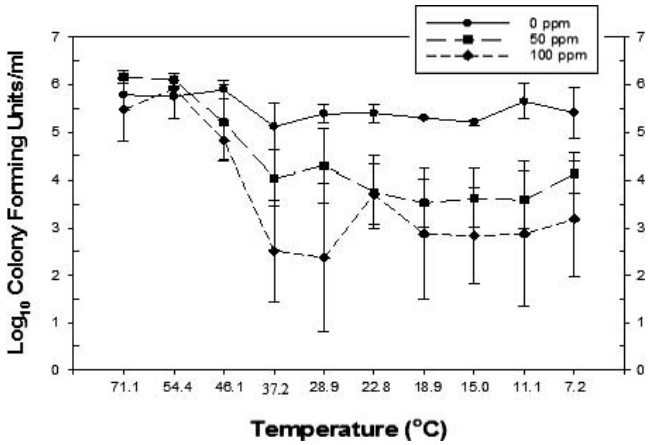

FIGURE 2. *Survival of* C. perfringens *vegetative cell population during cooling-stabilization in medium with different nitrite concentrations.*

within treatments, but counts were not different ($P > 0.05$) between treatments (Table 1). Despite the higher populations at these temperatures, a steady decline in population occurred for the 50- and 100-ppm treatments. The total population differed significantly ($P < 0.05$) between all three nitrite treatments (Fig. 1). As expected, 0 ppm had the highest *C. perfringens* total population ($P < 0.05$). Both nitrite treatments (50 and 100 ppm) significantly reduced ($P < 0.05$) *C. perfringens* survival throughout stabilization and cooling, but 100 ppm had the greatest overall inactivation effect ($P < 0.05$) on the total *C. perfringens* population throughout the cooling cycle. Thus, even at low concentrations such as 50 ppm, nitrite can affect the outgrowth of *C. perfringens,* but with increased nitrite concentrations greater inactivation is achieved. This finding supports those of King *(6),* who also evaluated 0, 50, and 100 ppm of nitrite, both with and without a cure accelerator, for the effect on *C. perfringens* and found results similar to ours. The concentration of nitrite, regardless of the source (conventional or natural), is critical for the prevention of *C. perfringens* outgrowth during cooling. Therefore, naturally cured meat products, which have lower ingoing nitrite concentrations than do conventionally cured products, have an increased risk of pathogen growth and consequently of causing foodborne illness *(3, 7).*

Separating the total population into vegetative cells and spores in this study revealed that the percentage of surviving vegetative cells and spores was not significantly different ($P > 0.05$) between treatments. The treatments with 50 and 100 ppm of nitrite were more effective ($P < 0.05$) for preventing the outgrowth of *C. perfringens* vegetative cells during cooling compared with the 0-ppm treatment (Fig. 2). No significant difference ($P > 0.05$) was found between 50 and 100 ppm of nitrite. The only significant difference ($P < 0.05$) between 50 and 100 ppm of nitrite occurred at 37.2 and 28.9°C (Fig. 2). At these temperatures, 100 ppm had the greatest effect on vegetative cells, suggesting that this difference is likely due to interaction between nitrite and temperature, which was significant ($P < 0.05$) for *C. perfringens* vegetative cell growth.

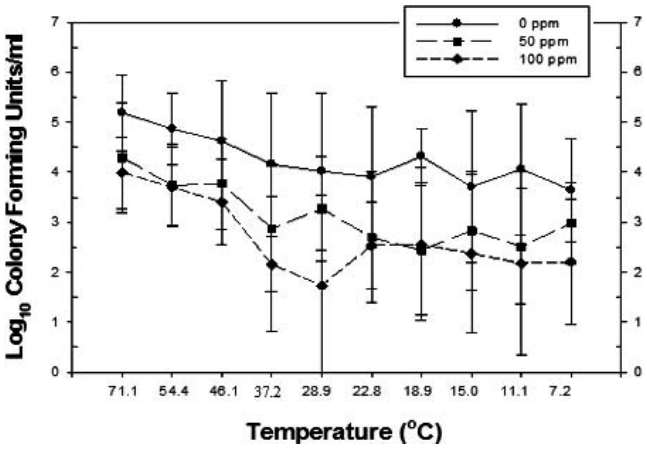

FIGURE 3. *Survival of* C. perfringens *spore population during cooling-stabilization in medium with different nitrite concentrations.*

For the *C. perfringens* spore population (Fig. 3), 50 and 100 ppm of nitrite significantly inhibited ($P < 0.05$) the outgrowth of spores during chilling compared with 0 ppm; however, similar to the vegetative cell data, there was no significant difference ($P > 0.05$) in results between the treatments with 50 and 100 ppm. Temperature also had a significant effect ($P < 0.05$) on *C. perfringens* spores; as temperature decreased, the number of spores also decreased. No interaction was found between nitrite and temperature ($P > 0.05$) for spores.

Evaluation of the individual components of the total *C. perfringens* population revealed found that vegetative cells were significantly affected ($P < 0.05$) by the interaction between nitrite and temperature. However, *C. perfringens* spores were not affected ($P > 0.05$) by this interaction, although temperature and nitrite concentration each had significant effects ($P < 0.05$) alone. These effects on vegetative cells and spores have been well documented, indicating that vegetative cells are sensitive to high and low temperatures and to antimicrobial agents such as nitrite *(2)*. Consequently, the interaction between nitrite and temperature is important for the inhibition of *C. perfringens* vegetative cells. However, *C. perfringens* spores, unlike of vegetative cells, can survive under harsh conditions such as high and low temperatures and the presence of curing salts. Although 50 and 100 ppm of nitrite significantly inhibited spores when compared with 0 ppm, the effects of the two nitrite treatments were not significantly different. Spores will not germinate and grow unless they are under optimal conditions; therefore, temperature was an important factor for spore outgrowth and inhibition throughout this study. Spore survival is a critical factor in naturally cured products because the decreased nitrite concentration may lead to decreased inhibition and subsequent spore germination and outgrowth under temperature abuse conditions.

The results of this study suggest that decreased nitrite concentrations, such as 50 ppm, contribute to suppression of *C. perfringens* populations during slow cooling when compared with 0 ppm. However, 50 ppm of nitrite was not as effective as 100 ppm of nitrite for suppression of the *C. perfringens* population. Therefore, to assure the safety of naturally cured meat products that may not contain as much formulated nitrite as conventionally cured products, additional hurdles such as addition of antimicrobial agents or shortening of the cooling cycle during periods of optimal *C. perfringens* growth should be considered.

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