Meat and Muscle Biology™

Comparison of Growth Inhibition of *Clostridium perfringens*, *Clostridium botulinum*, and *Bacillus cereus* During Extended Cooling of Uncured Poultry

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Abstract: The 2021 FSIS Appendix B guidelines restrict cooling of meat and poultry products to limit the growth of *Clostridium perfringens* to no greater than 1-log. This study compared the effect of pH and salt on the growth of 3 sporeforming pathogens, *Clostridium perfringens*, *Bacillus cereus*, and *Clostridium botulinum* during extended cooling in an uncured poultry product. Nine turkey treatments (75% moisture) were formulated using a full factorial design (pH 5.8, 6.2, 6.6; salt 1.2%, 1.5%, 1.8%). Treatments were inoculated with 3-log spores/g of *C. perfringens*, *B. cereus*, or *C. botulinum* and vacuum-packaged (25 g/package). Samples were cooked to 73 °C, then cooled from 48.9 °C to 26.7 °C (phase 1) in 1, 1.5, 2, 2.5, or 3 h, from 26.7 °C to 12.8 °C (phase 2) and from 12.8 °C to 4.4 °C (phase 3) in 5 h each. Samples were assayed by enumerating on selective agars at 0-time (post cook before incubation) and at internal temperatures 48.9, 26.7, and 4.4 °C. *C. botulinum* grew <1-log, and no botulinum toxin was detected regardless of cooling rate or formulation tested. *B. cereus* was inhibited in all formulations through the 12.5 h total cool (2.5-h phase 1 cooling), but >1-log increase was observed in pH 6.6 formulations for 13-h total cool. Less than a 1-log *C. perfringens* growth was detected for all treatments when phase 1 cooling was limited to 1 h, with 1.5-h phase 1 cool for pH <6.2 with >1.2% salt, pH 6.6 with >1.5% salt, and 2-h phase 1 cool for pH <5.8 and 1.8% salt. All formulations supported a 1- to 3-log increase of *C. perfringens* when phase 1 cool was 3 h. This study confirms cooling conditions that inhibit *C. perfringens* will likewise inhibit *C. botulinum* and *B. cereus* for the duration of the cooling, and phase 1 cooling can be extended up to 2.5 h depending on salt/pH combinations.

Key words: *Clostridium perfringens*, *Clostridium botulinum*, *Bacillus cereus*, cooling, USDA-FSIS Appendix B, model validation
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Introduction

Sporeforming pathogens, such as *Clostridium perfringens*, *Clostridium botulinum*, and *Bacillus cereus*, form heat-stable endospores that are widely distributed in environments such as soil and water and in live animals, and which survive conventional thermal or high-pressure processing designed to eliminate vegetative cells (International Commission on Microbiological Specifications for Foods, 1996; Sebranek, et al., 2001; Byrne et al., 2006; Li et al., 2020). Surveys reveal 2-4 log per gram presumptive *C. perfringens* or *B. cereus* in meat batter (Abrahams and Riemann, 1971; Konuma et al., 1988; Dodds, 1993; Kalinowski et al., 2003; Taormina and Bartholomew, 2005; Golden et al., 2009; Rahnama et al., 2023). However, a recent market basket survey conducted by the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) of populations of *C. perfringens* spores in commercial ready-to-eat meat products revealed that levels of *C.* perfingens spores are typically less than detectable levels of 0.5 log CFU/g (U.S. Department of Agriculture Food Safety and Inspection Service, 2023).

Clostridium sp. are of particular concern as *C. perfringens* has been observed to have generation times of less than 10 min at optimal growth temperatures, and *C. botulinum* produces a potent neurotoxin resulting in the serious neuroparalytic illness called botulism (Schroder and Busta, 1971; Sobel, 2005; Centers for Disease Control and Prevention, 2012). *B. cereus* is similarly found in meat and poultry products and can produce a heat-stable enterotoxin that remains active after recooking (Granum, 1994; Rahnama et al., 2023). Outbreaks related to these pathogens are often associated with improper hot-holding or storage temperatures in food service operations, restaurants, and homes (Bennett et al., 2013; Wittry et al., 2022). However, the slow chilling of commercially prepared cooked meat products could also present a risk for germination, growth, and toxin production, particularly when the product temperature passes through each pathogen's permissible growth range during cooling (Schroder and Busta, 1971; International Commission on Microbiological Specifications for Foods, 1996; Solomon and Lilly, 2001; Danler et al., 2003; National Advisory Committee on Microbiological Criteria for Foods, 2010; Juneja et al., 2018b).

The USDA-FSIS Compliance Guidelines for Stabilization of Meat and Poultry Products (Appendix B) states that the stabilization treatment must not allow the multiplication of toxigenic microorganisms (U.S. Department of Agriculture Food Safety and Inspection Service, 1999; U.S. Department of Agriculture Food Safety and Inspection Service, 2017; U.S. Department of Agriculture Food Safety and Inspection Service, 2021). The guidelines define multiplication as a mean net growth of no greater than a 1-log increase of *C. perfringens* or ≤0.3 log increase of *C. botulinum*. The 2017 version of Appendix B (U.S. Department of Agriculture Food Safety and Inspection Service, 2017) also identified less than a 3-log increase of *B. cereus* as the maximum growth limit. These values were based on the 2 to 4 log per gram presumptive *C. perfringens* or *B. cereus* in meat batter mentioned previously (Abrahams and Riemann, 1971; Konuma et al., 1988; Dodds, 1993; Kalinowski et al., 2003; Taormina and Bartholomew, 2005; Golden et al., 2009; Rahnama et al., 2023), with populations associated with illness generally 5-log or higher for *C. perfringens* and *B. cereus* (U.S. Food and Drug Administration, 2012; U.S. Department of Agriculture Food Safety and Inspection Service, 2023).

The limit for growth of *C. botulinum* is based on a comparison of enumeration data with toxin production from a 1957 study in laboratory media with non-proteolytic toxin type E strains (Ohye and Scott, 1957). More recent research suggests a 2-log increase is required for detectable toxin production by proteolytic strains at ambient temperatures (20 °C or higher) in processed cheese and meats (Ter Steeg et al., 1995; Ward et al., 2023). However, the correlation between botulinum log growth, total populations, and toxin production is unclear for all strains and temperatures.

The 1999 version of FSIS Appendix B Option 2 previously allowed uncured products to be cooled from 48.9 °C (120 °F) to 12.8 °C (55 °F) in 6 h and continued cooling to 4.4 °C (40 °F), with no intermediate temperature recording required (U.S. Department of Agriculture Food Safety and Inspection Service, 1999). It was recognized that this option had a small margin of safety as it did not specify rates of cooling when products were at temperatures that allowed the most rapid pathogen growth. The 2017 and 2021 FSIS guidelines modified Option 2 to limit cooling times from 48.9 °C (120 °F) to 26.7 °C (80 °F) to 1 h and from 26.7 °C (80 °F) to 12.8 °C (55 °F) in 5 h, with continued cooling to 4.4 °C (40 °F) prior to shipping. The FSIS recognizes that large diameter uncured products fall into a "scientific gap" where previously followed cooling rates could support >1-log growth of pathogens but had not translated into reported illnesses associated with these products.

The FSIS references several cooling models to be considered as food safety documentation (U.S. Department of Agriculture Food Safety and Inspection Service, 2021). However, these models have not been validated under all situations for extended cooling of formulated meat products. ComBase Perfringens Predictor accounts for the effect of both salt and pH, but moisture is not included (Le Marc et al., 2008; Mohr et al., 2015; ComBase, 2023). Using this model for an uncured meat with pH 6.4 and 1.6% NaCl, *C. perfringens* is predicted to increase 2.84-log when cooled from 48.9 °C to 12.8 °C in 6 h (linear curve); in contrast, the 2-phase cooling schedule (48.9 °C to 27.0 °C in 1 h and from 27.0 °C to 12.8 °C in an additional 5 h) results in only a 0.36-log growth. The ComBase models for *B. cereus* and *C. botulinum* do not include the full range of temperatures experienced during cooling. The USDA Agricultural Research Service Pathogen Modeling Program (ARS PMP) for *C. botulinum* was developed for chicken, beef, and pork, but does not allow for input of other formulation characteristics that affect growth (Juneja et al., 2021a; Juneja et al., 2021b; Juneja, et al., 2022; U.S. Department of Agriculture Agricultural Research Service, 2023). The Danish Meat Research Institute predictive model for *C. botulinum* in meat products allows for input of moisture,

pH, and salt, but does not have an option for dynamic temperature conditions (Danish Meat Research Institute, 2023). No cooling model is available to assess potential growth of *B. cereus* in meats, but models are available for cooling of cooked beans, pasta, and rice (Juneja et al, 2018a; Juneja et al., 2019a; Juneja et al., 2019b; U.S. Department of Agriculture Agricultural Research Service, 2023). While these models are helpful, they cannot be used under all circumstances without further validation.

Although the revised options for uncured meats are considered fail safe for all products they restrict manufacturers that require flexibility in cooling times for large diameter products where it is thermodynamically challenging to meet the defined cooling rates. As mentioned above, the cooling rates for these productsfall under the "scientific gaps" identified by the FSIS in the 2021 guidelines that needs additional research (U.S. Department of Agriculture Food Safety and Inspection Service, 2021). To fill current knowledge gaps and address industry needs with respect to time-temperature cooling profiles for RTE meat products based on product formulation, this study was designed to assess whether the initial cooling phase (from 48.9 °C to 12.8 °C) of the 2021 revised Appendix B Option 2 can be extended beyond 1 h based on intrinsic properties of a product formulation. Additionally, data were used to validate the accuracy of available models for the 3 pathogens as an option to assess cooling deviations.

Materials and Methods

Bacterial spore preparation

Spore crops of *Clostridium perfringens* (ATCC 12915, 12916, and 13124), *Clostridium botulinum* (proteolytic type A [56A, 62A, 69A, 77A, and 90A] and type B [53, 113B, 213B, 13983B, and Lamanna-okra B]), and *Bacillus cereus* (ATCC 14579, B4AC, and BC101E) were prepared as previously described (Christiansen et al., 1974; Tanaka et al., 1980; Gonzalez et al., 1999; Kennedy et al., 2013). Prior to inoculation, each spore crop was enumerated using the appropriate media. *C. perfringens* populations were enumerated on tryptose-sulfite-cycloserine agar (TSC) with a TSC overlay (Oxoid Ltd., Basingstoke, UK); *C. botulinum* populations with 5-tube most-probable-number technique in Trypticase-peptone-glucose-yeast (TPGY) extract broth supplemented with cooked meat (BBL Microbiological Systems, Sparks, MD); and *B. cereus* populations on mannitol yolk polymyxin B (MYP) B agar (Difco, BD, Sparks, MD) (U.S. Food and Drug Administration, 2000). Each inoculum was prepared by suspending approximately equal populations of appropriate strains in sterile deionized water to yield a target 3-log spores per gram of inoculated raw batter when using a 1% inoculum (v/w).

Preparation of model poultry formulations

Frozen turkey breasts obtained from a commercial supplier were thawed at 4.4 °C until use (within 3 d of thawing). Base formulation for the 9 model turkey variables included turkey breast (~74% moisture), 1.2% modified food starch, 0.37% sodium tripolyphosphate, 1.2% carrageenan, salt (to meet target level), and water/ice mixture added as balance. Sodium bicarbonate and 0.5N hydrochloric acid were added to select formulations to adjust pH as needed. The 9 treatments represented a full factorial design with endpoint targets of 75% moisture; pH 5.8, 6.2, or 6.6; and 1.2%, 1.5%, or 1.8% salt. For each treatment, nonmeat ingredients were combined until dissolved, and the brine was added to the ground turkey breast (ground through a 4.76-mm plate) and mixed for 3 min in a mixer (model AS 200, Hobart Corp., Troy, OH). Each treatment was packaged in oxygen-impermeable bags (3 mil high barrier pouches; 12.8 cm x 23 cm; oxygen transmission rate 50 to 70 cm^3/m^2, 24 h at 25 °C and 60% relative humidity; water transmission rate 6 to 7.5 g/m^2, 24 h at 25°C and 90% relative humidity; UltraSource, Kansas City, MO), vacuum sealed (Multivac AGW, Sepp Haggemuller KG, Wolfertschewenden, Germany), and stored at −20 °C until inoculation. Independent batches were produced for each replication.

Physiochemical analysis

Triplicate cooked samples for each treatment and trial were analyzed for moisture using the 5 h, 100 °C vacuum oven method AOAC 950.46 (AOAC International, 2000); NaCl was measured as $\%Cl^-$ using $AgNO_3$ potentiometric titration (Mettler G20 Compact Titrator, Columbus, OH), and water activity was measured using a Decagon AquaLab 4TE water activity meter (METER Group, Pullman, WA) (AOAC International, 2000). Triplicate cooked samples were analyzed for pH (Orion Star A111 pH meter and Orion 8104 combination electrode, Thermo Fisher Scientific, Waltham, MA) by creating a slurry from 10 g of cooked sample with 90 ml of deionized water and homogenizing in a stomacher for 1–2 min.

Inoculation

Treatment batches were thawed at 4 °C prior to use (within 2 d). Each raw batter was inoculated to a target 3-log spores per g using a 1% inoculum (v/w) of appropriate pathogen cocktail and mixed for 3 min (KitchenAid stand mixer, Benton Harbor, MI). After mixing, the inoculated meat was portioned into oxygen- and moisture-impermeable bags (25 ± 0.5 g/pouch) and vacuum-packaged. Additional uninoculated samples from each formulation were prepared as described above to monitor background microflora (aerobic plate count: Plate Count Agar, Difco, BD, Sparks, MD; anaerobic plate count: Oxoid Ltd., Basingstoke, UK) and for proximate analysis. Triplicate inoculated raw samples were immediately stored at 4 °C for enumeration of pre-cook populations.

Prepared sample bags were pressed to a uniform thickness (approximately 3 mm), hung onto removable incubator racks using binder clips, and spread evenly to ensure uniform heat distribution. To monitor changes in internal sample temperature in real time, thermocouple probes (traceable thermometer and type K probe, Thermo Fisher Scientific, Waltham, MA) were inserted into individual packaged meat samples using a rubber septum (Chembond round patches, Tru-Flate, Plews and Edelman, Dixon, IL). Prepared samples were cooked within 18 h of inoculation.

Cooking, cooling, and sampling

Packaged meat samples were clipped onto racks and submerged into large water baths (Magni Whirl, Blue M, Blue Island, IL) at 75 °C until the internal sample temperature reached 73 °C (4 min). Samples were removed from the water bath, rapidly cooled in a water bath to 48.9 °C (3–4 min), transferred to a programmable incubator (Freeze/Thaw Chamber Model 7901-25-2, Caron, Marietta, OH), and cooled in 3 phases including phase 1: from 48.9 °C to 26.7 °C in 1, 1.5, 2, 2.5, or 3 h; phase 2: from 26.7 °C to 12.8 °C in 5 h; and phase 3: from 12.8 °C to 4.4 °C in 5 h.

Triplicate samples for each treatment were enumerated for target pathogens at pre-cook and post cook when internal sample temperature reached 48.9 °C, 26.7 °C, 12.8 °C, and 4.4 °C. Samples were diluted (25 g meat with 50 ml with Butterfield's phosphate buffer, pH 7.1 ± 0.1) and homogenized for 1 to 2 min in a stomacher (IUL Masticator 400, Neutec Group Inc., Barcelona, Spain). Samples were serially diluted in 0.1% peptone water and spread plated on duplicate TSC agar, MYP agar, or differential reinforced clostridial agar (dRCA, Oxoid Ltd., Basingstoke, UK) for *C. perfringens*, *B. cereus*, or *C. botulinum*, respectively. TSC and dRCA plates were overlaid with a thin layer of the same media and incubated anaerobically (Anaero Pack System, Mitsubishi Gas Chemical, Tokyo, Japan) at 35 °C for 1 d or 30 °C for 2 d, respectively. MYP plates were incubated aerobically at 30 °C for 1 d. Populations were converted to log CFU/g; the minimum limit of detection by direct plating was 1.48 log CFU/g. For each cooling profile, the same batch of meat, water bath, and programmable incubator was used for all 3 pathogens; different batches of meat and inocula were used for each of the 2 trials. Duplicate uninoculated cooked samples were assayed at 0 h (post cook) and at the end of cooling for populations of background aerobic and anaerobic bacteria and pH.

Due to the lack of published literature or consensus correlating changes in populations of *C. botulinum* to the presence of botulinum toxin, samples were tested using the standard mouse bioassay (Solomon and Lilly, 2001) on all end-of-cooling samples (4.4 °C) and any additional samples during cooling in which ≥0.3 log increase was observed.

Data analysis

Duplicate trials for each pathogen were conducted comparing the effects of salt, pH, and cooling rate (9 formulations x 5 cooling profiles; 4 sampling intervals; 3 samples/interval). Microbiological data were transformed into log values for analysis. Populations of post-cook (48.9 °C) samples were averaged for each trial. Log change for individual samples was calculated by subtracting each data point (log CFU/g of inoculated meat) at 26.7 °C, 12.8 °C, and 4.4 °C from the 0-time average (post cook) of the respective treatment/replication. Data reported on figures for each treatment (formulation/cooling profile/pathogen) are the mean log change and standard deviations for both trials ($n = 6$). Data were also compared to output from predictive models for *C. perfringens* (ComBase, 2023); *C. botulinum* for cooling of pork, chicken, and beef; and *B. cereus* for cooling of rice, beans, and pasta (U.S. Department of Agriculture Agricultural Research Service, 2023).

Experiments had 3 levels of salt, 3 levels of pH, and 5 phase 1 cooling times, resulting in 45 runs that were replicated 2 times, 4 sampling times, with 3 samples each, for a total of 1080 samples for each pathogen. Data were analyzed using least-squares means using a factorial arrangement of treatments. All effects were considered as being fixed effects. LSMeans were calculated using JMP version 17.0.0 (JMP Statistical

Discovery, LLC 2022). Means were separated using Tukey's HSD test; levels sharing the same letter are not significantly different ($\alpha = 0.05$). Note that populations that are less than 0.5 log different from inoculated levels are not of practical (biological) significance because a log value of 0.5 is within inherent error for cultural enumeration techniques, even though they might be statistically different due to low standard deviation (National Advisory Committee on Microbiological Criteria for Foods, 2010).

Results and Discussion

Physiochemical analysis and background microbiota

Results for physiochemical analysis for all trials are reported in Table 1. Average moisture across all treatments and trials was 75.17% ± 0.43%. The pH values for the 3 levels were 5.86 ± 0.02, 6.24 ± 0.01, and 6.58 ± 0.03. Analyzed salt values for the 3 levels were 1.20% ± 0.04%, 1.46% ± 0.05%, and 1.77% ± 0.05%. Populations of aerobic and anaerobic background microflora in uninoculated samples were below the limit of detection (<1.48 CFU/g) in all cooked formulations throughout cooling (data not shown). Additionally, the uninoculated sample pH values remained unchanged to the end of cooling for all trials.

Clostridium perfringens

As expected, high salt, low pH, and short time for phase 1 (48.8 °C to 27 °C) cooling were found to significantly ($P < 0.05$) impact the inhibition of *C. perfringens*. Changes in pathogen populations (log CFU/g meat) for the 9 formulations for each cooling profile are displayed in Figures 1a–1e; least-squares means plots for interaction between cooling and salt, or pH are displayed in Figure 2. Although the majority of the growth, if any, occurred during phase 1 (data not shown), *C. perfringens* continued to grow slowly during phase 2, where temperatures were still within growth range of the pathogen (27.0 °C to 12.8 °C). Therefore, log change data are reported for samples at the end of the full cooling process (Figure 1a–1e). Average populations remained below the 1-log increase threshold for *C. perfringens* in all 9 formulations when phase 1 was limited to 1 h (total 11 h cooling; Figure 1a). When phase 1 cooling was extended to 1.5 h, only treatment 9 (1.2% salt, pH 6.6) supported >1 log growth with an average increase of 1.04 log CFU/g (Figure 1b). Additionally, although one sample for treatment 8 (1.5% salt, pH 6.6) supported a 1.04 log increase (data not shown), the overall average log change for this formulation for the 6 samples tested was 0.84 ± 0.06. When extending the phase 1 cooling to 2 h, all treatments with a pH of 5.8 and/or a salt level of 1.8% inhibited growth (<1-log increase; Figure 1c). Finally, when phase 1 was increased to 2.5 or 3 h (Figures 1d and 1e), all treatments, except for pH 5.8 with 1.8% salt at 2.5 h, supported greater than an average 1-log increase; although the average increase for the pH 5.8 with 1.8% salt was 0.77 log, sporadic samples supported >1-log increase. Based on these data, Table 2 indicates the maximum pH and minimum salt combinations that can be used for specific phase 1 cooling profiles (with additional 5 h cooling from 26.7 °C to 12.8 °C and 5 h from 12.8 °C to 4.4 °C).

Clostridium botulinum

None of the individual samples tested for any of the treatments had populations of greater than a 0.9-log higher than inoculated levels regardless of cooling profiles (Figure 3). Overall, the populations of

Table 1. Proximate analysis average and standard deviation model uncured turkey with 75% target moisture (duplicate trials, triplicate samples per trial, $n = 6$).

Treatment #	Target % NaCl	Target pH	% Moisture	% Salt	pH	a_w
1	1.2%	5.8	75.63 ± 0.22	1.19 ± 0.04	5.87 ± 0.02	0.982 ± 0.003
2	1.5%	5.8	75.34 ± 0.23	1.51 ± 0.04	5.85 ± 0.02	0.980 ± 0.003
3	1.8%	5.8	75.35 ± 0.18	1.81 ± 0.02	5.85 ± 0.03	0.978 ± 0.003
4	1.2%	6.2	75.47 ± 0.32	1.22 ± 0.09	6.25 ± 0.01	0.983 ± 0.002
5	1.5%	6.2	74.96 ± 0.55	1.41 ± 0.03	6.24 ± 0.02	0.982 ± 0.003
6	1.8%	6.2	74.79 ± 0.32	1.76 ± 0.06	6.23 ± 0.01	0.979 ± 0.001
7	1.2%	6.6	75.26 ± 0.27	1.20 ± 0.05	6.56 ± 0.02	0.980 ± 0.003
8	1.5%	6.6	75.05 ± 0.37	1.48 ± 0.05	6.56 ± 0.02	0.976 ± 0.002
9	1.8%	6.6	75.54 ± 0.48	1.77 ± 0.02	6.62 ± 0.03	0.980 ± 0.004

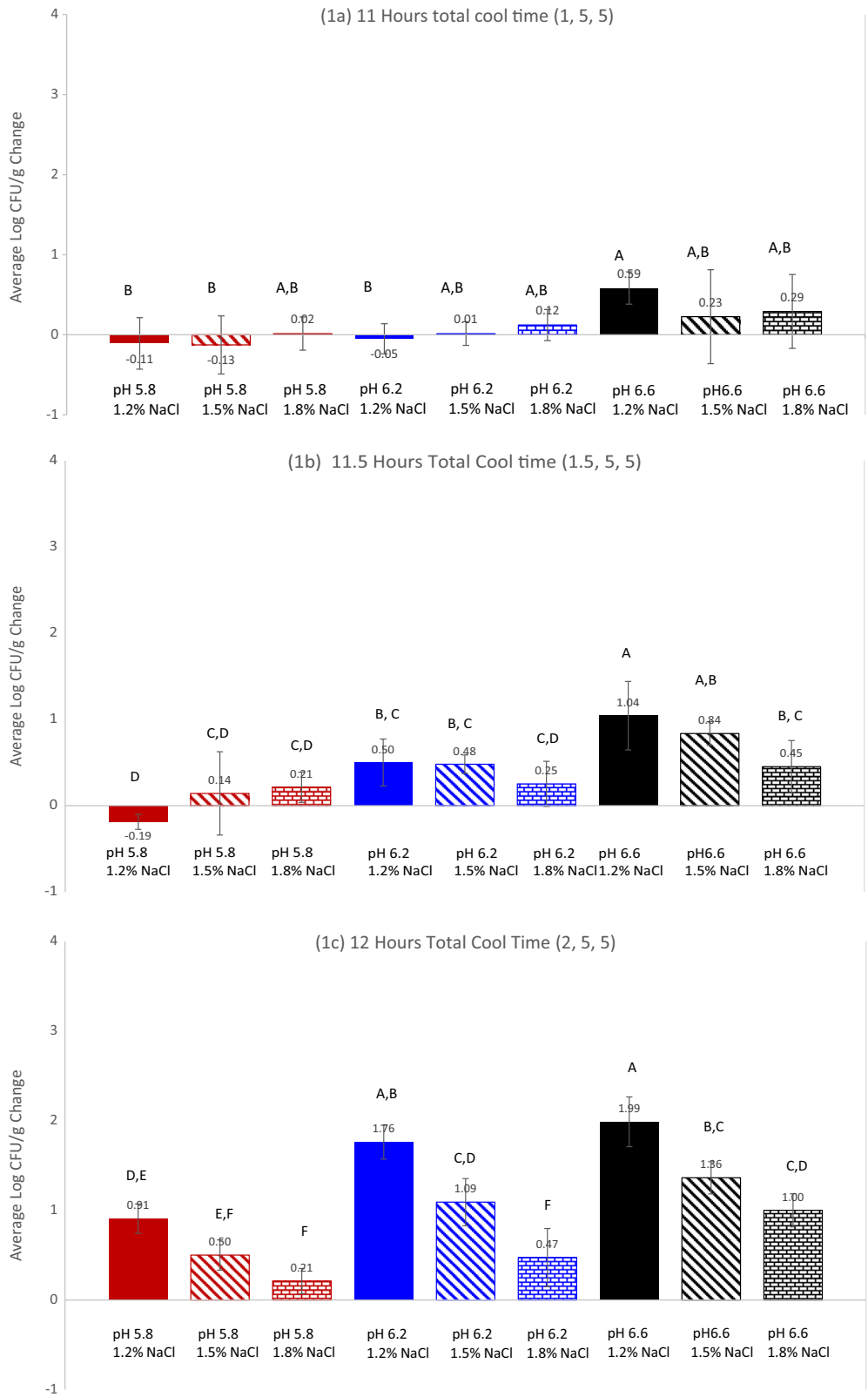

Figure 1. Changes in populations of *Clostridium perfringens* in uncured turkey formulations (75% moisture) during extended cooling from 48.9 °C to 26.7 °C (phase 1) in 1, 1.5, 2, 2.5, or 3 h (Figures 1a, 1b, 1c, 1d, and 1e, respectively); from 26.7 °C to 12.8 °C (phase 2) in 5 h; and from 12.8 °C to 4.4 °C (phase 3) in 5 h (total cooling 11, 11.5, 12, 12.5, and 13 h). Data reported are the difference between 0-time and final populations at the end of phase 3; testing for end of phase 1 and phase 2 is not reported. Levels sharing same letter are not significantly different (Tukey HSD).

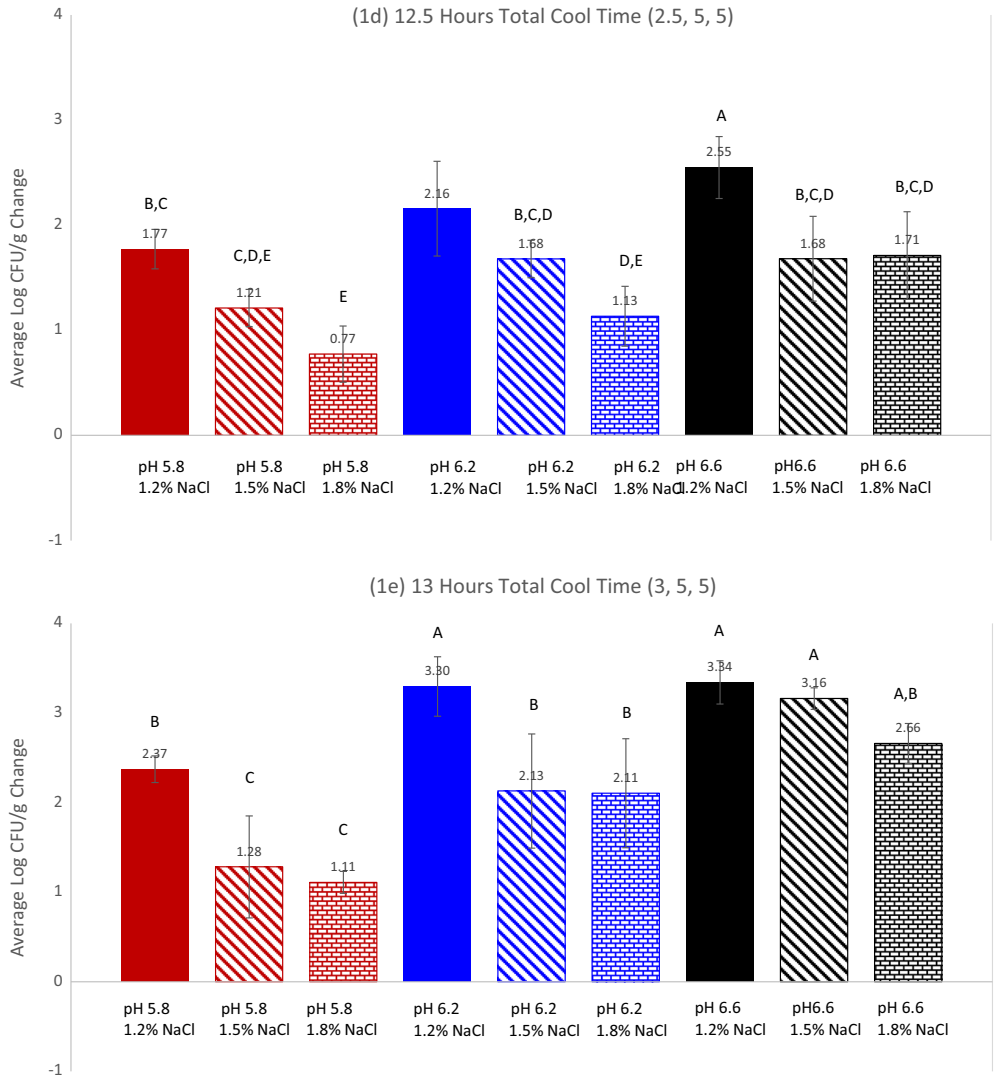

Figure 1. Continued

C. botulinum were 0.3 log higher than average 0-time inoculated populations in a total of 88 samples among the 9 formulations; 14 of these samples were observed in the 1-h phase 1 cool (current USDA-FSIS Option 2 guidelines for phase 1 cooling), whereas only 7 samples were greater than 0.3 log difference in the 3-h extended cool. The greatest difference in populations of *C. botulinum* was observed in a single sample of treatment 7 (1.2% salt, pH 6.6) at the end point of cooling (4.4 °C) for the 2.5 h extended cool (0.82 log higher than inoculated levels). No botulinum toxin was detected in any of the samples assayed at the end of any cooling profile for any formulation tested nor in any of the intermediate samples with 0.3 log higher than inoculated levels. The observed changes in populations in this study may represent growth or normal variation in sampling, diluting, and enumeration. Although FSIS standards are to limit *C. botulinum* growth to 0.3 log to prevent toxin production, other data suggest that the growth required for detectable toxin by proteolytic *C. botulinum* likely exceeds 1-log (Ter Steeg et al., 1995; Ward et al., 2023). Therefore, detecting a 0.3-log difference of *C. botulinum* from initial inoculum may not represent unsafe conditions. Furthermore, because *C. botulinum* did not grow under the conditions tested in this study (pH, salt, phase 1 cooling), no statistically significant differences were observed for inhibition based on formulation or cooling.

Bacillus cereus

B. cereus did not grow (<1-log increase) in any treatment when the phase 1 cooling profile was <2 h (total 12 h cooling) (Figure 4). When the phase 1 cool was extended to 2.5 h, one sample from

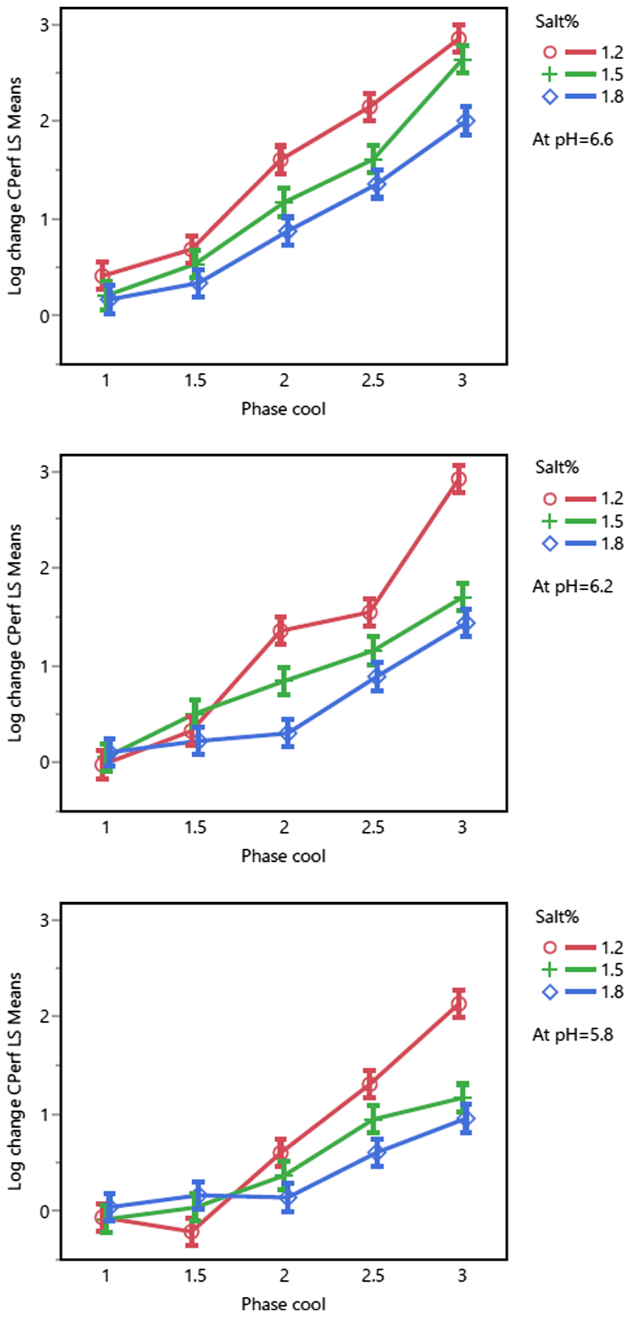

Figure 2. Response Log change *Clostridium perfringens* least-squares means plot hours phase 1 cool (1, 1.5, 2, 2.5, 3 [total cooling 11, 11.5, 12, 12.5, or 13 h] * pH *5.8, 6.2, 6.6) * % salt (1.2, 1.5, 1.8%)

Table 2. Conservative times for phase 1 cooling (48.8–26.7 °C; 120–80 °F) for various salt-pH combinations in uncured poultry with 75% moisture to consistently limit growth of *Clostridium perfringens* to ≤1-log.

	≥1.2% Salt	≥1.5 % Salt	≥1.8% Salt
pH ≤5.8	2.0 h	2.0 h	2.5 h
pH ≤6.2	1.5 h	1.5 h	2.0 h
pH ≤6.6	1.0 h	1.5 h	1.5 h

treatment 7 (1.2% salt, pH 6.6) supported a 1.07 log increase at the end point of cooling (4.4 °C) (total 12.5 h cooling) (data not shown). When phase 1 cooling was extended to 3 h, average populations of *B. cereus* increased by 1.12, 0.93, and 0.87 log in pH 6.6 treatments with 1.2%, 1.5%, and 1.8% salt concentrations, respectively; individual samples in the 1.5% and 1.8% salt treatments also supported >1 log increase even when the average increase was <1 log. In contrast to *C. perfringens*, salt level (1.2% to 1.8%) was not significant ($P > 0.05$) for inhibition of *B. cereus*, but pH and phase 1 cooling extended to 3 h were significant.

With the exception of meats with pH 6.6, with phase 1 cooling extended to 2.5 h or longer, none of the other treatments supported more than a 1-log increase. The maximum log increase for any sample tested for the 13-h total cool was 1.59 log. Given that populations of *B. cereus* need to exceed 10^6 CFU in order to produce sufficient enterotoxin to cause illness (U.S. Food and Drug Administration, 2012) and starting populations in raw meats are generally lower than 10^2 CFU/g (Konuma et al., 1988), the final populations will be less than what is needed to be toxigenic. Although *B. cereus* has been identified as the causative agent for outbreaks associated with the cooked meats and poultry in restaurants and institutions, no illnesses have been associated with commercially prepared product (Centers for Disease Control and Prevention, 2024). Furthermore, this is the reason that the FSIS removed reference to control *B. cereus* during extended cooling from the 2021 revised guidelines because cooling/formulation limits sufficient to inhibit growth of *C. perfringens* are considered appropriate for control of *B. cereus* (U.S. Department of Agriculture Food Safety and Inspection Service, 2017; U.S. Department of Agriculture Food Safety and Inspection Service, 2021).

Comparison with predictive models

Data were compared to output from several available models. ComBase Perfringens Predictor (ComBase, 2023) accurately predicted growth (±0.5 log) or was fail safe for all treatments when phase 1 cooling was extended to 2 h (Table 3). At 2.5 and 3.0 h phase 1 cooling, ComBase model underpredicted average growth for treatments with the lowest salt value of 1.2% (−0.81 and −0.98 log difference for pH 5.8 treatments at the 2 extended cooling profiles, respectively; −0.62 log difference for pH 6.2 at 3 h and −0.50 log for pH 6.6 at 3 h) but was accurate for other treatments.

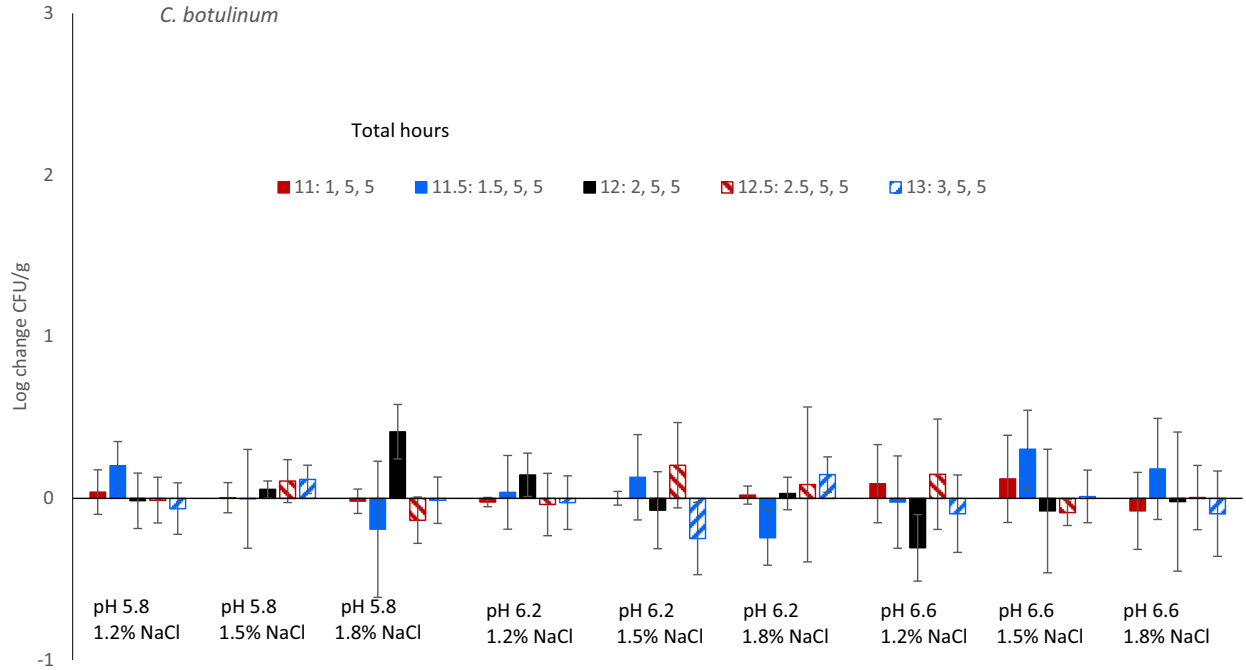

Figure 3. Changes in populations of *Clostridium botulinum* in uncured turkey formulations (75% moisture) during extended cooling from 48.9 °C to 26.7 °C (phase 1) in 1, 1.5, 2, 2.5, or 3 h; from 26.7 °C to 12.8 °C (phase 2) in 5 h; and from 12.8 °C to 4.4° C (phase 3) in 5 h (total cooling 11, 11.5, 12, 12.5, and 13 h). Data reported are the difference between 0-time and final populations at the end of phase 3; testing for end of phase 1 and phase 2 is not reported.

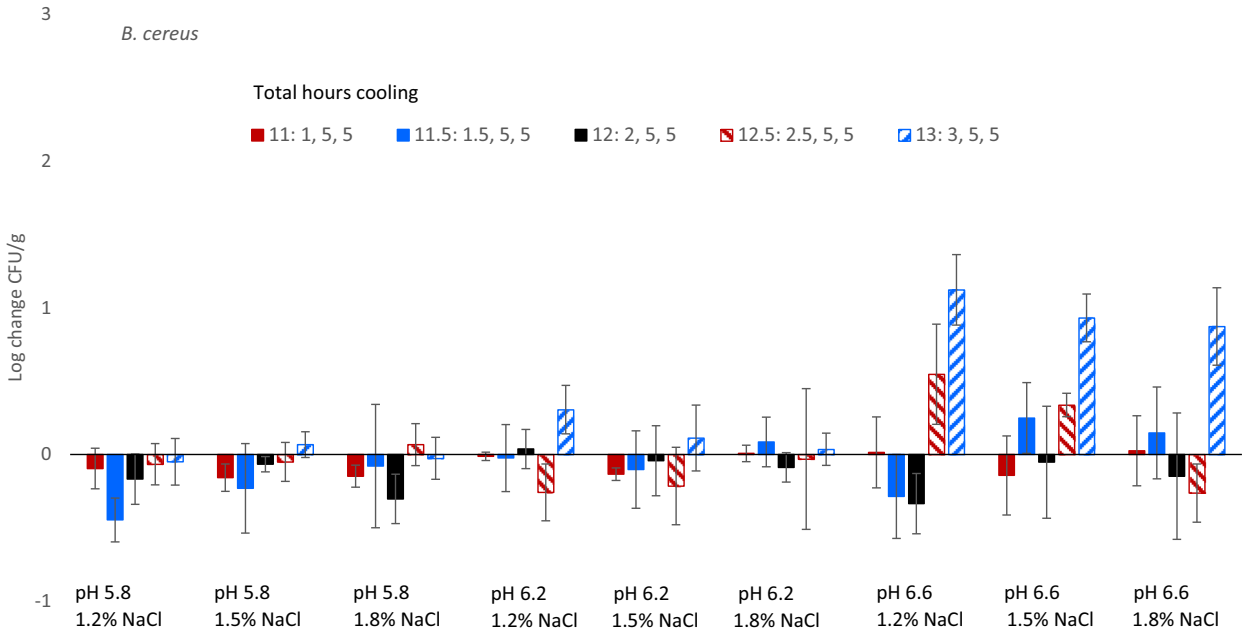

Figure 4. Changes in populations of *Bacillus cereus* in uncured turkey formulations (75% moisture) during extended cooling from 48.9 °C to 26.7 °C (phase 1) in 1, 1.5, 2, 2.5, or 3 h; from 26.7 °C to 12.8 °C in 5 h; and from 12.8 °C to 4.4 °C in 5 h (total cooling 11, 11.5, 12, 12.5, and 13 h). Data reported are the difference between 0-time and final populations at the end of phase 3; testing for end of phase 1 and phase 2 is not reported.

The data underlying Perfringens Predictor were collected in minced beef, pork, or turkey homogenized in Reinforced Clostridium media (10% meat). The pH and NaCl concentrations were adjusted for specific treatments, but moisture was not reported (Le Marc et al., 2008). Therefore, it is unclear how the salt-in-moisture phase for the model compares with the current data. Furthermore, strain differences and handling of the inoculated meat samples may also be responsible for the slight differences in growth.

Table 3. Log increase for *Clostridium perfringens* in model uncured turkey (75% moisture; current study data) compared with ComBase Perfringens Predictor model output covering cooling temperatures from 48.8 °C to 4 °C

	Study data					Model output				
	11.0	11.5	12.0	12.5	13.0	11.0	11.5	12.0	12.5	13.0
Formulation										
pH 5.8 1.2% NaCl	−0.11	−0.19	0.91	1.77	2.37	0.00	0.31	0.59	0.96	1.39
pH 5.8 1.5% NaCl	−0.13	0.14	0.50	1.21	1.28	0.00	0.27	0.51	0.85	1.24
pH 5.8 1.8% NaCl	0.02	0.21	0.21	0.77	1.11	0.00	0.21	0.42	0.72	1.07
pH 6.2 1.2% NaCl	−0.05	0.50	1.76	2.16	3.30	0.39	0.85	1.43	2.05	2.68
pH 6.2 1.5% NaCl	0.01	0.48	1.09	1.68	2.13	0.33	0.75	1.29	1.87	2.47
pH 6.2 1.8% NaCl	0.12	0.25	0.47	1.13	2.11	0.27	0.63	1.11	1.65	2.21
pH 6.6 1.2% NaCl	0.59	1.04	1.99	2.55	3.34	0.44	0.94	1.55	2.19	2.84
pH 6.6 1.5% NaCl	0.23	0.84	1.36	1.68	3.16	0.37	0.83	1.40	2.01	2.63
pH 6.6 1.8% NaCl	0.29	0.45	1.00	1.71	2.66	0.31	0.70	1.21	1.78	2.36

Total hours cooling 11.0, 11.5, 12.0, 12.5, and 13.0 corresponding to 1.0, 1.5, 2.0, 2.5, and 3.0 phase 1 cooling; 5 h phase 2 and 5 h phase 3 cooling.

Table 4. Comparison of log increase for *Clostridium botulinum* in model uncured turkey (75% moisture) with ARS Pathogen Modeling Program (chicken, pork, beef) covering cooling temperatures from 48.8 °C to 4 °C

	Total hours cooling				
	11	11.5	12	12.5	13
Formulation					
pH 5.8 1.2% NaCl	0.04	0.20	−0.01	−0.01	−0.06
pH 5.8 1.5% NaCl	0.00	0.00	0.05	0.11	0.12
pH 5.8 1.8% NaCl	−0.02	−0.19	0.41	−0.14	−0.01
pH 6.2 1.2% NaCl	−0.02	0.04	0.15	−0.04	−0.03
pH 6.2 1.5% NaCl	0.00	0.13	−0.07	0.21	−0.25
pH 6.2 1.8% NaCl	0.02	−0.25	0.03	0.09	0.15
pH 6.6 1.2% NaCl	0.09	−0.02	−0.31	0.15	−0.09
pH 6.6 1.5% NaCl	0.12	0.30	−0.08	−0.09	0.01
pH 6.6 1.8% NaCl	−0.08	0.18	−0.02	0.01	−0.10
Maximum average log increase for any formulation	0.12	0.30	0.41	0.21	0.15
ARS PMP output					
Chicken	0.01	0.01	0.02	0.02	0.03
Pork	0.21	0.27	0.33	0.39	0.46
Beef	0.09	0.12	0.16	0.21	0.26

Total hours cooling 11.0, 11.5, 12.0, 12.5, and 13.0 corresponding to 1.0, 1.5, 2.0, 2.5, and 3.0 phase 1 cooling; 5 h phase 2 and 5 h phase 3 cooling.

ARS, United States Department of Agriculture Agricultural Research Service; PMP, Pathogen Modeling Program.

Table 5. Comparison of log increase for *Bacillus cereus* in model uncured turkey (75% moisture) with ARS Pathogen Modeling Program (beans, rice, pasta) covering cooling temperatures from 48.8 °C to 4 °C

	Total hours cooling				
	11	11.5	12	12.5	13
Formulation					
pH 5.8 1.2% NaCl	−0.10	−0.45	−0.17	−0.07	−0.05
pH 5.8 1.5% NaCl	−0.16	−0.23	−0.07	−0.05	0.07
pH 5.8 1.8% NaCl	−0.15	−0.08	−0.30	0.07	−0.03
pH 6.2 1.2% NaCl	−0.01	−0.03	0.04	−0.26	0.31
pH 6.2 1.5% NaCl	−0.14	−0.10	−0.04	−0.22	0.11
pH 6.2 1.8% NaCl	0.01	0.09	−0.09	−0.03	0.03
pH 6.6 1.2% NaCl	0.01	−0.29	−0.34	0.55	1.12
pH 6.6 1.5% NaCl	−0.14	0.25	−0.05	0.34	0.93
pH 6.6 1.8% NaCl	0.03	0.15	−0.15	−0.26	0.87
Maximum average log increase for any formulation	0.03	0.25	0.04	0.55	1.12
ARS PMP output					
Beans	0.27	0.47	0.74	1.05	1.38
Rice	0.39	0.64	0.95	1.30	1.65
Pasta	0.61	0.93	1.29	1.67	2.06

Total hours cooling 11.0, 11.5, 12.0, 12.5, and 13.0 corresponding to 1.0, 1.5, 2.0, 2.5, and 3.0 phase 1 cooling; 5 h phase 2 and 5 h phase 3 cooling.

ARS, United States Department of Agriculture Agricultural Research Service; PMP, Pathogen Modeling Program.

Findings for *C. botulinum* are consistent with cooling models for uncured beef, chicken, and pork without salt or pH adjustment, which predict less than a 0.5 log increase under similar cooling conditions (Juneja et al., 2021a; Juneja et al., 2021b; Juneja et al., 2022; U.S. Department of Agriculture Agricultural Research Service, 2023) (Table 4). The *B. cereus* USDA Agricultural Research Service (ARS) models for cooling of beans, rice, and pasta, which also do not allow adjustment of salt or pH, were similarly fail safe (predicted growth at higher rates than actual growth of *B. cereus* in the meat products tested in this study; Table 5). The pH and water activity of the matrices tested for the ARS *B. cereus* model were not reported in the manuscripts used to generate the model (Juneja et al., 2018; Juneja et al., 2019a; Juneja et al., 2019b). However, the typical pH of white

rice is 6 to 6.5, black beans 5.8 to 6.0, and pasta 5.7 to 6.7 (Del Torre et al., 1998; U.S. Food and Drug Administration, 2004), which are similar to that found in processed meats.

Conclusions

The results from this study confirmed that the revised 2021 USDA-FSIS Option 2 guidelines will inhibit growth of pathogenic sporeformer regardless of formulation (≤75% moisture, ≥1.2% salt, and pH ≤6.6), and growth rates are similar to those identified with ComBase Perfringens Predictor for uncured meat and the ARS Pathogen Modeling Program for cooling of meats, beans, pasta, and rice. Data from this study suggest the initial cooling phase can be extended up to 2.5 h depending on the formulation's final analyzed salt and pH combinations. This study confirms our hypothesis that conditions inhibiting growth of *Clostridium perfringens* to less than 1-log will also provide sufficient margin of safety to effectively inhibit the growth of *Bacillus cereus* and growth/toxin production of *Clostridium botulinum*.

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