

Potential for Growth from Spores of *Bacillus cereus* and *Clostridium botulinum* and Vegetative Cells of *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella* Serotypes in Cooked Ground Beef during Cooling[†]

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ABSTRACT

The ability of 16 foodborne pathogens, representative of 5 different species, to grow during cooling of previously sterilized cooked beef was studied to determine a safe cooling rate. Autoclaved ground beef samples (3 g) were inoculated with heat-shocked spores of *Bacillus cereus* (strain BH 86) or *Clostridium botulinum* (nonproteolytic type B strains CBW 25, 17B, and KAP B5 and type E strains Whitefish, Saratoga, and Alaska) or vegetative cells of *Listeria monocytogenes* (strains HO-VJ-S, V-7, and Scott A), *Staphylococcus aureus* (strains 196E, B121, and B124), or *Salmonella* serotypes (*S. dublin*, *S. enteritidis*, and *S. typhimurium*), vacuum-packaged, and cooked in a stirred water bath to an internal temperature of 60°C in 1 h. In some experiments combinations of *C. botulinum* and *B. cereus* spores or *S. aureus* and salmonellae vegetative cells were used. Heated samples were cooled through the temperature range of 54.4 to 7.2°C at rates varying from 6 to 21 h. Samples were removed at various times during cooling to determine if growth of the pathogens had occurred. No growth was observed with cooling periods of up to 21 h. This study with the model meat system (3 g autoclaved ground beef inoculated with selected pathogens and then pasteurized) indicated that cooling from 52.4 to 7.2°C in up to 21 h would not pose a food safety hazard from growth of these pathogens.

Key words: Spores, vegetative cells, cooked ground beef, cooling rate

Minimally processed, ready-to-eat, refrigerated, extended shelf life foods are being manufactured on an increasing scale in response to consumer demands for fresh, preservative-free, convenience foods. The mild heat treatment given to such food is aimed at killing the vegetative cells of spoilage and pathogenic bacteria. Since heat-

resistant spore-forming foodborne pathogens such as the genera of *Bacillus* and *Clostridium* can be present, pasteurization exerts a selective effect on the type of surviving bacteria which remain in such foods. An inadequate cooling rate and duration can provide time-temperature conditions suitable to promote germination of surviving spores, with subsequent outgrowth leading to toxin elaboration.

Roast beef has been frequently identified as a vehicle associated with outbreaks of foodborne diseases (1). Roasts may be inadequately cooked, contaminated during slicing/portioning operations or packaging, cooled too slowly, or improperly stored (3). Recontamination of cooked foods by pathogens, such as staphylococci or salmonellae, from the hands of workers or from equipment or utensils is well documented (3, 4). Additionally, surveys in supermarkets of ready-to-eat meat products provide evidence for the occurrence of *Listeria monocytogenes* (8, 11). Roberts (13) indicated that in most food poisoning incidents some form of temperature abuse occurred during food preparation or storage. Bryan (2) identified inadequate cooling and lapse of a day or more between food preparation and service as primary factors contributing to outbreaks of foodborne disease. Improper storage or holding temperature was the factor most often reported to contribute in *Bacillus cereus* (94%), *Clostridium botulinum* (34%), *Clostridium perfringens* (97%), *Salmonella* (83%), or *Staphylococcus aureus* (98%) outbreaks (1).

In food service operations foods are only pasteurized by temperatures and times used to cook or prepare food, and heat-resistant spores and vegetative cells of foodborne pathogens may survive and multiply in cooked meat and poultry if the rate and extent of cooling is not sufficient. This clearly implies that fast cooling of foods is critical. The current regulations for cooked beef products and cooked meat patties require that these cooked products be quickly cooled following cooking in order to inhibit the growth of vegetative, spore-forming bacteria (15, 16). The United States Department of Agriculture (USDA) safe cooling

standard (15) for cooked beef, roast beef, and cooked corned beef requires that establishments begin chilling cooked beef products within 90 min of heat processing; the products must be chilled from 48.8 to 12.8°C in no more than 6 h. The regulations for cooked meat patties (16) require that these cooked products be cooled to an internal temperature of 4.4°C or below within 2 h of heat processing. The time/temperature guidelines for cooling heated products recommends that the maximum internal temperature should not remain between 54.4 and 26.7°C for more than 1.5 h nor between 26.7 and 4.4°C for more than 5 h (17). The U.S. Food and Drug Administration (FDA) Division of Retail Food Protection recognized that inadequate cooling was a major food safety problem (7) and established a recommendation (Food Code, 1993) that all food should be cooled from 60 to 5°C in 6 h or less.

Utilizing *Clostridium perfringens* in cooked beef with cooling from 54.4 to 7.2°C at rates varying from 6 to 18 h, Juneja et al. (10) found that a cooling period of 15 h or less prevented growth from a spore inoculum. While the study by Juneja et al. (10) determined the safe cooling rate for cooked beef with respect to *C. perfringens*, there appears to be no work available on the impact of cooling rates on growth from spores of *B. cereus* and *C. botulinum*, and vegetative cells of *L. monocytogenes*, *S. aureus*, and *Salmonella* serotypes in cooked ground beef. Accordingly, the study was designed to determine if spores of *B. cereus* and *C. botulinum* and vegetative cells of *L. monocytogenes*, *S. aureus*, and salmonellae would pose a food safety hazard in pasteurized cooked ground beef within a cooling period of 15 h.

MATERIALS AND METHODS

Strains and spore suspension

One strain of *B. cereus* (BH 86), six strains of *C. botulinum* (nonproteolytic type B strains CBW 25, 17B, and KAP B5 and type E strains Whitefish, Saratoga, and Alaska), three strains of *L. monocytogenes* (HO-VJ-S, V-7, and Scott A), three strains of *S. aureus* (196E, B121, and B124), three serotypes of *Salmonella* (*S. dublin*, *S. enteritidis*, and *S. typhimurium*) from the laboratory culture collection were included in the study. The spore suspension of *B. cereus* was prepared according to the method of Dingman and Stahly (6). Nonproteolytic *C. botulinum* type B and type E spores were prepared in Trypticase-peptone-glucose-yeast extract broth by the procedure described previously (9). Final spore pellets were stored in sterile distilled water at 2°C. Spore mixtures containing all six strains of *C. botulinum* were prepared immediately prior to experiments by mixing equivalent numbers of spores from each suspension. This composite mixture of spore strains (7 log spores/ml) was heat-shocked for 10 min at 60°C and the *B. cereus* spore suspension was heat-shocked at 70°C for 15 min prior to use. Spores were then serially diluted in 0.1% (wt/vol) peptone water to appropriate concentrations for sample inoculation. *L. monocytogenes* strains were grown for 18 h at 37°C in brain heart infusion broth (Difco, Detroit, MI). Equal volumes of the cultures were pooled to obtain a mixture of *L. monocytogenes* strains. The cells were harvested by centrifugation at room temperature for 10 min at 7,700 × g, the cell pellet washed twice in sterile 0.1% (wt/vol) peptone water, resuspended, and serially diluted in peptone water, and a 10⁻⁴ dilution was used for sample inoculation. Similar

TABLE 1. *Plating media and growth conditions used for pathogens*

Pathogens	Plating medium ^a	Incubation conditions
<i>Staphylococcus aureus</i>	Tryptic soy agar	Aerobic; 37°C/48 h
<i>Salmonella</i>	Tryptic soy agar	Aerobic; 37°C/48 h
<i>Listeria monocytogenes</i>	Tryptic soy agar	Aerobic; 30°C/72 h
<i>Bacillus cereus</i>	Tryptic soy agar	Aerobic; 37°C/48 h
<i>Clostridium botulinum</i>	Tryptic soy agar	Anaerobic; 37°C/48 h
<i>B. cereus</i> and <i>C. botulinum</i>	Tryptic soy agar with and without lysozyme ^b	Aerobic and anaerobic; 37°C and 28°C/48 h
<i>S. aureus</i> and <i>Salmonella</i>	Baird-Parker and xylose lysine deoxycholate (XLD) agar	Aerobic; 37°C/48 h

^a All media were obtained from Difco, Detroit, MI.

^b Lysozyme concentration (10 µg/ml; Sigma, 41,000 U/mg).

^c Anaerobic conditions were obtained in a GasPak system (Baltimore Biological Laboratory, Cockeysville, MD).

procedures were used to prepare separate vegetative cell mixtures of *S. aureus* strains and salmonellae.

Sample preparation and inoculation

Ground beef (90% lean), obtained from a local retail market, was placed in a thin layer (0.5 in. [ca. 12 mm]) on plastic trays and sterilized at 121°C for 15 min. The fat was aseptically drained while the beef was hot and then the ground beef cooled at 4°C to an internal temperature of 25°C. The pH of the cooked ground beef was determined using a combination electrode (Sensorex, semi-micro, A. H. Thomas, Philadelphia, PA) attached to an Orion model 601A pH meter. Duplicate 3-g ground beef samples were aseptically weighed into sterile Whirl-Pak sampling bags (15 by 22.9 cm, Model B736, NASCO, Modesto, CA). Beef samples were inoculated (0.1 ml of culture) with about 3 log CFU/g heat-shocked spores of *B. cereus* or *C. botulinum* or vegetative cells of *S. aureus*, *Salmonella* serotypes, or *L. monocytogenes*. In addition, beef samples were co-inoculated (0.1 ml of each culture) with spores of *B. cereus* and *C. botulinum* or with vegetative cells of *S. aureus* and *Salmonella* serotypes. The inoculated bags were manually mixed to ensure even distribution of the organisms in the meat sample. The bags were evacuated to a negative gauge pressure of 1 bar (10⁵ Pa) and heat-sealed using a Multivac Model A300/16 packaging machine (Germany).

Cooking and cooling procedures

Prior to cooking, two bags were opened, sterile copper-constantan thermocouples were placed at the center of each of the ground beef samples, and the bags were resealed. Racks holding the ground beef samples were fully submerged in 4.4°C water in a water circulating bath (Exacal, Model Ex-251HT, Neslab Instruments, Inc., Newington, NH). To initiate cooking, the bath temperature was raised in a linear fashion to 60°C within 1 h. The cooling study was performed through the temperature range of 54.4 to 7.2°C by adding ice to the stirred water bath at varying rates in order to simulate the desired cooling rate. The internal temperature of the samples was constantly monitored by the thermocouples. A Keithly-Metrabyte data logger Model DDL 4100 (Taunton, MA) connected to a microcomputer was used to record thermocouple readings. The thermocouple signal was sampled every second, and

the two readings were averaged to determine the sample temperature. The time-temperature profile for cooling was monitored or controlled by adding ice at approximately each 5-min interval to reduce the temperature according to the desired cooling schedule. Two replications were performed for the cooking and subsequent time-temperature intervals for cooling.

Enumeration procedure

Samples were removed at the appropriate time intervals based on 6-, 9-, 12-, 15-, 18-, or 21-h cooling periods to achieve cooling from 54.4 to 7.2°C. Sterile 0.1% peptone water (3 ml) was added to each bag to give 1:1 (wt/vol) slurry and homogenized for 1 min in a Lab-blender stomacher (Model 400, Spiral Systems, Inc., Cincinnati, OH). Bacterial populations were enumerated by spiral plating (Spiral Systems Model D) of dilutions of the slurry in duplicate onto various agars (Table 1). In addition, a 0.1-ml portion of the slurry was spread over the surface of appropriate agars (Table 1).

RESULTS AND DISCUSSION

This study investigated the potential for outgrowth of spores of *B. cereus* or *C. botulinum* or vegetative cells of *L. monocytogenes*, *S. aureus*, or *Salmonella* serotypes in cooked ground beef (previously autoclaved) cooled from 54.4 to 7.2°C using cooling times varying from 6 to 21 h. When hot cooked food is allowed to cool, the temperature must pass through a range that is favorable for pathogenic spore germination and multiplication of the vegetative cells.

The pH of the cooked ground beef used in the study was 6.24. When spores of *B. cereus* or *C. botulinum* or vegetative cells of *L. monocytogenes*, *S. aureus*, or *Salmonella* serotypes were inoculated separately in cooked ground beef cooled from 54.4 to 7.2°C in 6, 9, 12, 15, 18, or 21 h, population increases were not observed in the case of all organisms except *C. botulinum*; spores of *C. botulinum* germinated and grew, and the population densities increased by about 1 log unit (data not shown). Vegetative cells of *S. aureus* and *Salmonella* inoculated together in cooked ground beef did not grow in a cooling period of 18 h (Table 2) or 21 h. The constituents of Baird-Parker agar effectively inhibited growth of *Salmonella* while allowing development of *S. aureus*. Thus, when *S. aureus* and *Salmonella* were inoculated together, both could be enumerated separately. Injury was not observed since plate counts on selective agars did not differ from those on nonselective medium (tryptic soy agar). Experiments conducted on the mixed culture growth of *S. aureus* and *Salmonella* or *B. cereus* and *C. botulinum* spores were performed to assess competitive interactions between the vegetative cells or spores of foodborne pathogens. While no growth from spores of *B. cereus* occurred in 21 h, the *C. botulinum* population did increase by about 1 log unit (3.55 log CFU/g to 4.58 log CFU/g; Table 3). *B. cereus* and *C. botulinum* numbers did not differ regardless of the presence or absence of lysozyme in the plating media (data not shown). Lysozyme was added in the plating medium because of its role in increasing the recovery of heat-injured spores; lysozyme replaces the thermally inactivated spore germination enzymes (14).

To simulate exponential cooling of cooked foods, the formulas to calculate the times and temperatures of the hot

TABLE 2. Influence of cooling rate of cooked beef on growth of an inoculum mixture containing *Staphylococcus aureus* (four strains) and *Salmonella* (three strains)^a

Elapsed time (hours)	Temperature (°C)	Log CFU/g	
		<i>S. aureus</i> ^b	<i>Salmonella</i> ^c
0	54.4	1.85 ± 0.06	1.32 ± 0.08
2	41.7	1.93 ± 0.13	1.47 ± 0.34
3	36.6	1.84 ± 0.22	1.29 ± 0.50
4	32.2	1.49 ± 0.08	1.21 ± 0.01
5	28.8	1.65 ± 0.32	1.56 ± 0.07
6	25.0	1.55 ± 0.15	1.15 ± 0.42
7	22.1	1.68 ± 0.07	1.33 ± 0.37
8	19.6	1.30 ± 0.15	1.52 ± 0.51
9	17.4	1.25 ± 0.42	1.17 ± 0.33
10	15.6	1.51 ± 0.28	1.23 ± 0.09
11	13.8	1.57 ± 0.06	1.47 ± 0.07
12	12.5	1.62 ± 0.06	1.13 ± 0.07
13	11.3	1.25 ± 0.17	1.31 ± 0.13
14	10.2	1.42 ± 0.42	1.28 ± 0.41
15	9.3	1.51 ± 0.36	1.08 ± 0.01
16	8.5	1.38 ± 0.07	0.93 ± 0.06
17	7.8	1.32 ± 0.06	1.24 ± 0.33
18	7.2	1.29 ± 0.04	1.06 ± 0.21

^a Data represent mean ± standard deviation of two replications.

^b Plating medium: Baird-Parker agar.

^c Plating medium: XLD agar.

TABLE 3. Influence of cooling rate of cooked beef on growth of an inoculum mixture of spores of *Bacillus cereus* and *Clostridium botulinum*^{a,b}

Elapsed time (hours)	Temperature (°C)	Log CFU/g	
		<i>Bacillus cereus</i>	<i>Clostridium botulinum</i>
0	54.4	2.41 ± 0.17	3.55 ± 0.53
1	48.5	2.29 ± 0.13	3.43 ± 0.47
2	43.3	2.37 ± 0.54	3.62 ± 0.51
3	38.7	2.32 ± 0.33	3.48 ± 0.07
4	34.6	2.62 ± 0.17	3.53 ± 0.46
5	31.0	2.51 ± 0.43	3.46 ± 0.35
6	27.8	2.50 ± 0.26	3.73 ± 0.02
7	25.0	2.49 ± 0.24	3.75 ± 0.51
8	22.5	2.72 ± 0.15	4.03 ± 0.43
9	20.3	2.73 ± 0.43	4.05 ± 0.28
10	18.3	2.74 ± 0.31	3.98 ± 0.37
11	16.6	3.02 ± 0.04	3.96 ± 0.23
12	15.1	2.95 ± 0.53	4.15 ± 0.35
13	13.7	3.08 ± 0.29	4.16 ± 0.31
14	12.5	3.01 ± 0.31	4.08 ± 0.07
15	11.4	2.12 ± 0.16	4.25 ± 0.24
16	10.5	2.49 ± 0.45	4.24 ± 0.56
17	9.7	2.57 ± 0.13	4.30 ± 0.13
18	8.9	2.33 ± 0.07	4.58 ± 0.39
19	8.3	2.24 ± 0.39	4.26 ± 0.28
20	7.7	2.16 ± 0.26	4.15 ± 0.16
21	7.2	2.26 ± 0.31	4.35 ± 0.13

^a Data represent mean ± standard deviation of two replications.

^b Plating medium: tryptic soy agar.

samples were based on the work of Dickerson and Read (5) and Pflug and Blaisdell (12). The temperatures computed for 15-, 18-, and 21-h cooling periods may be calculated by the equations given by Juneja et al. (10) and are given in Tables 2 and 3.

A previously sterilized 3-g sample of pasteurized cooked ground beef can be cooled to 7.2°C in up to 21 h without food safety hazard from the pathogens utilized in this study. However, *C. perfringens* would grow to potentially hazardous infective dose levels of >6 log CFU/g if the cooling period is extended beyond 15 h (10). Therefore, it is concluded from the data presented here that if pasteurized cooked beef is cooled to 7.2°C in 15 h or less then the likelihood of a foodborne disease outbreak can be minimized. However, excessive time in cooling of heated products may occur due to equipment malfunction or electrical outage. Such cooling deviations or discontinuous cooling scenarios when product cooling proceeds downward through a temperature range, followed by a rise in temperature with subsequent continuation of cooling, need to be evaluated to determine if the product remains safe.

REFERENCES

1. Bean, N. H., and P. M. Griffin. 1990. Foodborne disease outbreaks in the United States, 1973-1987: pathogens, vehicles, and trends. *J. Food Prot.* 53:804-817.
2. Bryan, F. L. 1978. Factors that contribute to outbreaks of foodborne disease. *J. Food Prot.* 41:816.
3. Bryan, F. L. 1988. Risks associated with vehicles of foodborne pathogens and toxins. *J. Food Prot.* 51:498-508.
4. Cremer, M. L., and J. R. Chipley. 1980. Time and temperature, microbiological and sensory assessment of roast beef in a hospital food service system. *J. Food Sci.* 45:1472-1477.
5. Dickerson, R. W., Jr., and R. B. Read, Jr. 1973. Cooling rates of foods. *J. Milk Food Technol.* 36:167-171.
6. Dingman, D. W., and D. P. Stahly. 1983. Medium promoting sporulation of *Bacillus larvae* and metabolism of medium components. *Appl. Environ. Microbiol.* 46:860-869.
7. FDA Division of Retail Food Protection. 1993. Food Code, p. 55. U.S. Department of Health and Human Services, Public Health Service. Food and Drug Administration, Washington, D.C.
8. Gilbert, R. J., K. L. Miller, and D. Roberts. 1989. *Listeria monocytogenes* and chilled foods. *Lancet* i:383-384.
9. Juneja, V. K., B. S. Eblen, B. S. Marmer, A. C. Williams, S. A. Palumbo, and A. J. Miller. 1994. Thermal resistance of non-proteolytic type B and type E *Clostridium botulinum* spores in phosphate buffer and turkey slurry. *J. Food Prot.* 58:758-763.
10. Juneja, V. K., O. P. Snyder, and M. Cygnarowicz-Provost. 1994. Influence of cooling rate on outgrowth of *Clostridium perfringens* spores in cooked ground beef. *J. Food Prot.* 57:1063-1067.
11. Kerr, K. J., S. F. Dealler, and R. W. Lacey. 1988. *Listeria* in cook-chill food. *Lancet* ii:37-38.
12. Pflug, I. J., and J. L. Blaisdell. 1963. Methods of analysis of precooling data. *ASHRAE J.* 5:33-40, 49.
13. Roberts, D. 1988. Trends in food poisoning. *Food Sci. Technol. Today* 2:28-34, 36.
14. Scott, V. N., and D. T. Bernard. 1985. The effect of lysozyme on the apparent heat resistance of nonproteolytic type B *Clostridium botulinum*. *J. Food Safety* 7:145-154.
15. U.S. Department of Agriculture. 1994. 9CFR 318.17. Requirements for the production of cooked beef, roast beef, and cooked corned beef. Office of Federal Register, National Archives and Records Administration, Washington, D.C.
16. U.S. Department of Agriculture. 1994. 9CFR 318.23. Heat processing procedures, cooking instructions, and cooling, handling and storage requirements for uncured meat patties. Office of Federal Register, National Archives and Records Administration, Washington, D.C.
17. U.S. Department of Agriculture, Food Safety and Inspection Service. 1989. Time/temperature guidelines for cooling heated products. FSIS Directive 7110.3 Rev. 1.