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Influence of Several Methodological Factors on the Growth of *Clostridium perfringens* in Cooling Rate Challenge Studies

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ABSTRACT

Proper temperature control is essential in preventing *Clostridium perfringens* food poisoning. The U.S. Department of Agriculture Food Safety and Inspection Service cooling guidelines offer two options for the cooling of meat products: follow a standard time-temperature schedule or validate that alternative cooling regimens result in no more than a 1-log CFU/g increase of *C. perfringens* and no growth of *Clostridium botulinum.* The latter option requires laboratory challenge studies to validate the efficacy of a given cooling process. Accordingly, the objective of this study was to investigate the role of several methodological variables that might be encountered during typical *C. perfringens* challenge studies. Variables studied included plastic bag type (Whirlpak or Spiral Biotech), sealing method (Multivac or FoodSaver), initial spore inoculum size (1 to approximately 3 log CFU/g), and growth environment (ground beef or Trypticase–peptone–glucose–yeast extract [TPGY] broth). The major factors that affected growth were sample bag type and growth environment. Samples incubated in Whirlpak bags showed significantly less growth than those incubated in Spiral Biotech bags, which was likely due to the former bag's greater oxygen permeability. *C. perfringens* spores showed shorter germination, outgrowth, and lag times and *C. perfringens* cells showed faster growth rates in ground beef compared with TPGY broth. No significant difference was observed between two different sealing methods. Initial spore inoculum levels in the range studied had no significant effect on final *C. perfringens* cell concentration.

Clostridium perfringens continues to be a major food safety concern to the food industry. There were an estimated 654 outbreaks involving *C. perfringens*, resulting in 248,520 cases between 1983 and 1992 *(13).* This organism has been implicated as the cause of foodborne illness in roast beef, turkey, meat-containing Mexican foods, and other meat dishes *(4). C. perfringens* spores may survive cooking and receive sufficient heat activation to germinate and produce cells that may subsequently multiply in cooked foods if the rate and extent of cooling are not sufficient. *C. perfringens* generation time is as rapid as 7.4 min in autoclaved ground beef with an optimal growth range of 37 to 45°C, and growth has been reported at temperatures as low as 6°C *(20).*

The Food Safety and Inspection Service (FSIS) guidelines for the cooling of meat products specify that the internal product temperature should not remain between 130 and 80°F (54.4 and 26.7°C) for more than 1.5 h or between 80 and 40°F (26.7 and 4.4°C) for more than 5 h during cooling. If meat processors are unable to achieve this time-temperature schedule, they should prove that the alternative cooling regimen used will result in less than a 1-log CFU increase in *C. perfringens.* When processors deviate from these guidelines, it may be necessary to conduct challenge studies to determine if the performance standard has been met.

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Many studies have investigated the enumeration and plating methods of *C. perfringens* from ground beef and broth systems *(1, 2, 11, 16).* The effect of methodological differences typically encountered in challenge studies have yet to be described in the published literature, although studies with other pathogenic bacteria have shown that the growth medium and experimental conditions can have a large impact on growth *(3, 15).*

Preliminary experiments conducted at Rutgers, the State University of New Jersey, contradicted those published by the U.S. Department of Agriculture (USDA), Agricultural Research Service (ARS), Eastern Regional Research Center (ERRC). The preliminary Rutgers results showed no growth in cooked ground beef, whereas Juneja et al. *(9)* reported that cooling ground beef from 54.4 to 4.4°C in 15 h (or more) allowed greater than a 1-log CFU increase of growth. This discrepancy prompted a collaborative investigation into methodological differences that might have a large impact on the growth of *C. perfringens* during cooling. The objective of this study was to investigate several variables normally encountered when conducting *C. perfringens* challenge studies and to provide recommendations for future studies of this type.

MATERIALS AND METHODS

Sample preparation and inoculation of meat samples. Three strains of *C. perfringens*—NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and NCTC 10240 (Hobbs se-

rotype 13)—were obtained, maintained, and prepared according to procedures described by Juneja et al. *(8)*. Appropriate volumes of a spore cocktail (approximately 10^8 CFU/ml) were inoculated into 230 g of ground beef (25% fat), obtained at a retail store, to result in initial inoculum levels of between 10^2 and 10^4 concentrations of spores per ml. Different initial spore concentrations were used, since this variable has been shown to influence germination and growth of *Clostridium botulinum* at different salt and pH concentrations *(21)*. The ground beef and culture were blended for 4 min on high speed (level 6) in the sterilized bowl of a KitchenAid mixer (model no. K45SS, KitchenAid Inc., Greenville, Ohio). This mixing process ensured even dispersion of spores as evidenced by no significant differences observed in plate counts from a minimum of five samples removed from various locations within the mixing bowl. The mixing was halted once every minute to break apart large meat pieces and remove residual meat from the sides of the bowl and mixing paddle. Three grams of ground beef was aseptically weighed into either sterile WhirlPak sampling bags (4 oz, Nasco, Modesto, Calif.) or sterile stomacher filter bags (SFB-510, Spiral Biotech, Boston, Mass.). Bags were vacuum sealed at 12 mbar vacuum using a Multivac (model A300/16, Multivac Inc., Kansas City, Mo.) packaging machine or sealed using a retail vacuum packaging system (FoodSaver Vac 300, Tilia, Inc., San Francisco, Calif.) and frozen until used (no longer than 4 weeks). Initial spore concentrations were evaluated over the 4 weeks of frozen storage to ensure that levels did not decrease during storage. Pouches were thawed at 4.4°C overnight before heat activating spores at 75°C for 20 min in a circulating water bath.

Sample preparation and inoculation of broth samples. Growth of *C. perfringens* was also evaluated in Trypticase–peptone–glucose–yeast extract (TPGY) broth. TPGY broth is composed of 5% Trypticase (wt/vol), 0.5% peptone (wt/vol), 2% yeast extract (wt/vol), 0.1% cysteine hydrochloride (wt/vol), and 0.4% dextrose (wt/vol). TPGY broth (without cysteine hydrochloride) was sterilized by autoclaving. Filter sterilized cysteine hydrochloride was added aseptically, and the resultant mixture was dispensed in 9-ml quantities into sterile culture tubes. Each tube of broth was inoculated with heat-shocked spores to approximate the final target concentration of 10^2 and 10^4 concentrations of spores per ml and then subsequently flushed with sterile N_2 gas. Culture tubes of broth were closed with screw caps to maintain anaerobic conditions.

Dynamic temperature studies and sampling. Both static (unchanging) temperature experiments and dynamic (changing) temperature experiments were conducted as part of this study. Dynamic temperature experiments investigated cooling regimens where the product was cooled from 54.4 to 4.4°C over varying periods. Cooling has previously been shown to follow an exponential rate *(9)*, and a programmable water bath (model no. 1028P, Fisher Scientific, Pittsburgh, Pa.) was used to mimic this cooling behavior. Samples were removed at appropriate intervals throughout each cooling process. Static temperature samples were prepared as previously described, and growth was monitored at a constant temperature (42°C) in three different environments: (i) an incubator (Standard Isotemp Incubator, model large, Fisher Scientific), (ii) a circulating water bath (model 9101, Fisher Scientific), or (iii) separate Gas Pak containers (Baltimore Biological Laboratory, Cockeysville, Md.) inside an incubator (Standard Isotemp Incubator). Dilution, plating, and incubation of broth samples were as described by Labbe and Norris *(11)*. All experiments were performed in at least duplicate, with duplicate samples being analyzed at $t = 0$ h and $t = 18$ or 21 h. There were no changes to the appearance of any sample bags noticed throughout the studies, which would have indicated air leaks or loss of vacuum.

TABLE 1. *Comparisons of the growth of* C. perfringens *in ground beef during a 21-h cooling cycle using different sampling bags and spore stocks*

Sample bag	Spore stock	Log growth (CFU/g)
Spiral Biotech	A	5.29 ± 0.04
	B	5.01 ± 0.55
Whirlpak	A	2.05 ± 0.78
	B	0.81 ± 0.62

Other factors. To investigate variations attributed to personnel or facility, replicate experiments were performed with identical materials using the methods outlined above in a laboratory maintained either by the USDA ARS in Wyndmoor, Pa., or Rutgers University, Department of Food Science in New Brunswick, N.J. All personnel performing experiments were properly trained and identical techniques were used.

Analysis of growth curves. Estimates of germination, outgrowth, and lag (GOL) and exponential growth rate (EGR) from collected data were determined following Gompertz curve-fitting procedures *(14)* using Sigma Plot version 8.0 (SPSS Inc., Chicago, Ill.) as follows:

$$\log(L_t) = A + Ce^{-e^{[-B(t-M)]}}$$

where L_t is the log count of the number of *C. perfringens* at time t, A is the initial cell concentration, C is the maximum cell concentration, and e is the base of the natural logarithm. The parameter B is the relative growth rate at time point M, where M is the time at which the absolute growth rate is at a maximum. GOL is defined as $M - 1/B$ and EGR as $B \times C/e$.

Statistical analyses. All data were analyzed using analysis of variance techniques with SAS version 8.0 statistical software (SAS Institute, Raleigh, N.C.). Fisher's least significant difference procedure was used to determine significant difference ($P < 0.05$).

RESULTS

Influence of bag type, sealing method, and laboratory. More than a 5-log CFU increase in *C. perfringens* concentration was seen in ground beef contained in Spiral Biotech pouches compared with only a 0.81- to 2.05-log increase in samples in WhirlPak bags (Table 1). Little difference in net growth in ground beef was seen between two different spore stocks from the same cultures produced under identical conditions in different laboratories.

Table 2 shows the results of further investigations of growth during cooling over 18 and 21 h, with the inclusion of the sealing method as an additional variable. As in Table 1, the major factor that affected growth was the type of bag used. The mean log increase in *C. perfringens* concentration exceeded 3 log CFU/g for all replicates during 18- and 21-h cooling cycles when Spiral Biotech bags were used. An increase of 1 log CFU/g or less was observed when WhirlPak sampling bags were used. The sealing method used (industrial-scale Multivac or home-scale FoodSaver) showed no significant effect on the final log CFU/g increases observed. As expected, more growth (4.8 to 5.6 log

TABLE 2. *Comparison of growth of* C. perfringens *in ground beef as influenced by bag type, bag sealing method, and facility*

Bag type	Sealing method	Facility	Log growth (CFU/g)[a] 18-h cool	21-h cool
Spiral BioTech	Multivac	A	3.19 A[b]	5.15 A
	FoodSaver	A	3.99 A	5.27 A
	FoodSaver	B	5.22 B	5.83 A
Whirlpak	Multivac	A	0.86 C	1.42 B
	FoodSaver	A	0.63 C	0.84 BC
	FoodSaver	B	0.04 C	0.08 C

[a] All values are the means of at least three experiments.
[b] Means in the same column followed by different letters are significantly different ($P = 0.05$).

CFU/g) was observed during the cooling cycle over 21 h when compared with an 18-h cycle (2.28 to 4.84 log CFU/g) when the Spiral Biotech bags were used. No significant differences in final log CFU/g growth were observed, since initial inoculum levels ranged from 1.0 to 3.21 log CFU/g (data not shown).

Accurate replication of results between different research facilities using identical supplies and techniques is often difficult, as Table 2 shows. Growth during cooling cycles over 18 and 21 h between the two research facilities varied significantly. Facility B observed greater growth of *C. perfringens* in the Spiral Biotech bags over 18 h and significantly less growth in Whirlpak bags during a 21-h cooling period when compared with facility A (Table 2).

The results of additional studies undertaken to investigate possible explanations for the lack of growth observed in Whirlpak bags are depicted in Figure 1. Ground beef samples containing heat activated *C. perfringens* spores stored aerobically in an incubator at 42°C never showed growth (solid triangles); in fact, a 2-log CFU/g decrease was observed after 2 h followed by no change in cell concentration. *C. perfringens* in samples incubated in ground beef in WhirlPak bags in a water bath at 42°C (solid circles) experienced a decline similar to the aerobically incubated sample during the first 4 h of incubation. This was followed by exponential growth to a final level of approximately 10^7 CFU/g within 9 h. The *C. perfringens* cells in ground beef samples kept in an anaerobic environment at 42°C showed more than a 1-log CFU increase in concentration within 3 h and reached approximately 10^7 CFU/g within 6 h. These results indicate that oxygen transmission through the WhirlPak pouch material may be responsible for the slowed or inhibited growth of *C. perfringens.*

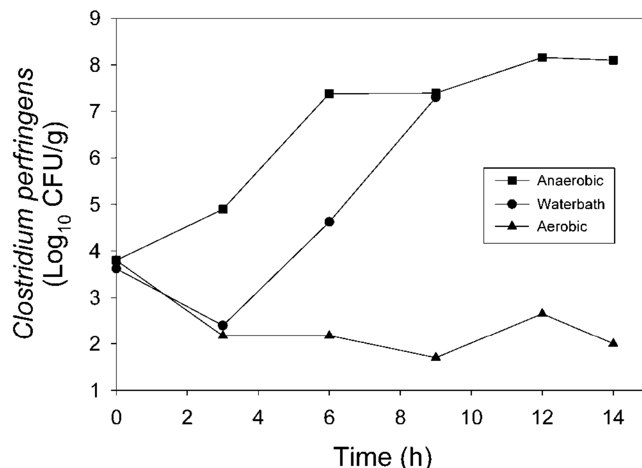

FIGURE 1. *Comparison of growth of* C. perfringens *in ground beef during anaerobic incubation (■), in a water bath (●), or during aerobic incubation (▲) at 42°C. One beef sample was removed from each incubation condition every 3 h over the incubation period.*

Influence of broth and meat media. Because of the inherent variability seen when working in real food systems, many predictive models have been developed in broth systems *(17).* Conventional predictive modeling wisdom dictates that because culture media are designed for growing microbes, models developed from data collected in broth culture are typically "fail-safe" or err by predicting shorter lag times and faster growth rates than seen in foods *(12).* A comparison of *C. perfringens* growth in TPGY broth and ground beef refutes such conventional wisdom (Fig. 2). The *C. perfringens* GOL time was shorter and the EGR faster in the ground beef system (open symbols) when compared with *C. perfringens* in broth (closed symbols) during cooling over 18 (triangles) or 21 h (squares). GOL times, as calculated by the modified Gompertz equation (solid lines), fit the growth data well and yielded values of 1.81 and 2.64 h during 18- and 21-h cooling times in ground beef, respectively. Calculated *C. perfringens* GOL times in TPGY broth were 4.88 and 4.13 h for 18 and 21 h of cooling, respectively. Modified Gompertz equation determinations of EGR were also significantly faster in ground beef versus beef broth: 1.40 and 1.67 log CFU/g/h during 18- and 21-h cooling times, respectively, in ground beef compared with 0.66 and 0.46 log CFU/g/h in broth

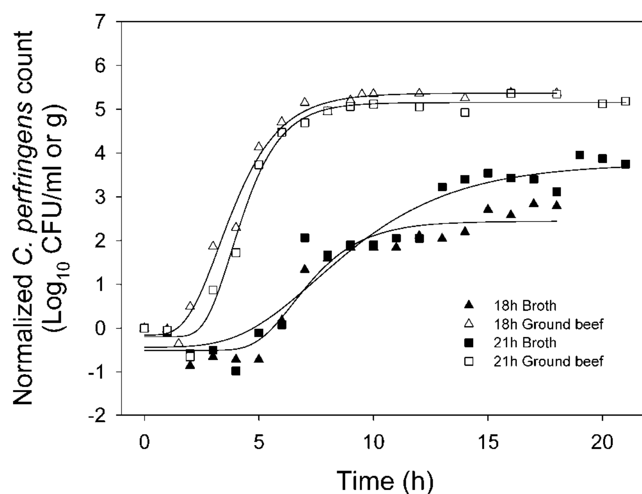

FIGURE 2. *Comparison of growth of* C. perfringens *in ground beef (open symbols) and TPGY extract broth (closed symbols) over 18-h (△, ▲) and 21-h (□, ■) cooling cycles. Solid lines represent a modified Gompertz function curve fitting to the growth data. Cooling cycles were performed in triplicate. A representative curve from each condition is shown.*

during 18- and 21-h cooling times, respectively. Shorter GOL and faster EGR in ground beef versus TPGY broth resulted in a greater net growth of *C. perfringens* by the end of the cooling cycle.

These results have important implications for those conducting challenge studies with *C. perfringens.* Since *C. perfringens* grows faster in some food products than it does in TPGY broth, this means that challenge studies must be performed with actual food products or with a medium containing those (as-yet-unidentified) food components that stimulate microbial growth. This also suggests that mathematical growth models for *C. perfringens* that have been developed in broth systems may have to be revised and/or based on new data collected in the most permissive growth environments (i.e., foods).

DISCUSSION

Results from laboratory challenge studies with *C. perfringens* can have a large impact on the decisions made by meat industry personnel concerning cooling schedule deviations. As shown herein, some challenge study design variables can have a large impact on the study results. The type of bag or pouch used in *C. perfringens* challenge studies can have a large impact on the rate and extent of *C. perfringens* growth. Bags that are permeable to oxygen may suppress or slow the growth of the facultative anaerobe *C. perfringens.* Although Whirlpak bags are cheap, sterile, and readily available in most food microbiology laboratories, their apparently high oxygen permeability renders them unsuitable for use in challenge studies. The current study provides evidence in support of this and also explains why more *C. perfringens* growth was observed in the current study than had been previously reported in an earlier study that also used Whirlpak bags *(9).* Sterile Spiral Biotech stomacher filter bags are more expensive, but the thick plastic design is apparently sufficient (compared with Whirlpak bags) to limit oxygen transmission and provide a suitable environment for *C. perfringens* growth, that is assuming oxygen is removed before the start of the experiment and the bags are properly sealed.

The effect of oxygen concentration on the growth of *C. perfringens* is well documented in the published literature. Significantly more growth of *C. perfringens* was observed in cooked turkey samples after 12 h at 28°C when the O_2 concentration was less than 20% *(7). C. perfringens* in ground beef grew greater than 7 log within 12 h at 28°C under anaerobic conditions compared with 6 log of growth after 36 h when incubated aerobically *(8).*

The medium chosen for challenge studies also had an effect on the growth of *C. perfringens* under nonisothermal cooling conditions. Data presented here showed shorter lag times, faster growth rates, and higher overall net growth over 18- and 21-h cooling cycles. These results were in agreement with the findings of Willardsen et al. *(20)*, who reported generation and lag times of 8.9 min and 1.2 h, respectively, in autoclaved ground beef versus 12.2 min and 1.9 h, respectively, in fluid thioglycollate medium during storage at 45°C. The same trend was observed under growth at temperatures increasing linearly with time from 25 to 60°C. The most rapid growth observed during rising temperatures of 7.5°C/h in autoclaved ground beef was 8.1 min compared with 11.0 min in fluid thioglycollate medium.

Two key variables did not affect challenge study results: sealing type and spore inoculum size. There was no additional benefit seen with the use of a commercial vacuum packaging system (Multivac) compared with an inexpensive home kitchen vacuum system (FoodSaver). These results suggest that laboratories performing *C. perfringens* challenge studies should be able to use an inexpensive system such as the FoodSaver and still achieve accurate results. It has been reported previously that *C. perfringens* is able to survive and proliferate at E_h values of less than 350 mV at pH 7.0 *(18).* This tolerance to low levels of oxygen may allow a less expensive vacuum and sealing system to perform in an equivalent manner to a more expensive industrial system.

Since no significant difference in *C. perfringens* behavior was noted with inoculum size varying between approximately 10^2 and 10^4 log CFU/ml, this further simplifies the cost and design of *C. perfringens* challenge studies, since only one inoculum level would need to be investigated, representing a worst case situation *(10).* Previous studies with spore-forming bacteria *C. botulinum (19, 21)* and *Bacillus megaterium (5)* showed significant inoculum size effects, and although no inoculum size effect was shown here, future studies investigating higher and lower inoculum sizes might reveal an effect. This study highlights the importance of considering key design variables in any challenge study *(6)*, but since *C. perfringens* grows so rapidly, even small methodological differences can have profound consequences, especially in situations where the growth of the organism is close to the USDA FSIS performance standard.

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