

## Effect of Curing Agents on the Growth and Survival of Food-Poisoning Strains of *Clostridium perfringens*

### SUMMARY

The effects of different combinations of curing salts (NaCl, NaNO<sub>3</sub>, NaNO<sub>2</sub>) and temperature on the growth, survival, and heat resistance of several strains of *Clostridium perfringens* were investigated. These strains were shown to survive and grow in concentrations of curing salts considerably higher than those used in normal curing operations. When used in conjunction with heat, the concentrations of curing salts required to affect cell survival were still well above those usually employed. Hams were pumped with curing brines inoculated with low concentrations of spores of a food-poisoning strain of *C. perfringens*, then cured and smoked according to routine procedures. *C. perfringens* could still be isolated from the hams after cooling.

A simple procedure for preparing reproducible inocula of aged spores is described. These aged spores showed much greater resistance to heat after storage in meat-brine mixtures than did spores from young sporulating cultures.

### INTRODUCTION

The role of *Clostridium perfringens* as a causative agent of food poisoning has received increasing attention in the last few years, particularly in the United States and England (Hobbs, 1962; Angelotti *et al.*, 1962). Hall *et al.* (1963), in a study of 83 strains of *C. perfringens* associated with cases of food poisoning in both countries, reported that the spores from the English strains were considerably more heat-resistant. It was suggested that cases of *C. perfringens* food poisoning in England came primarily from foods contaminated prior to cooking, and in the United States from food contaminated after cooking. Barnes *et al.* (1963) reported that there is a greater possibility of *C. perfringens* food poisoning if meat is contaminated prior to cooking.

Studies on the resistance of anaerobic bacteria to curing agents have indicated that *C. botulinum* and putrefactive anaerobes will survive and sometimes grow in the presence

of appreciable concentrations of curing agents (Silliker, 1959). An outbreak of food poisoning from salted beef contaminated with *C. perfringens* was reported by Hobbs (1962). She also reported that unpublished data from her laboratory had indicated that vegetative cells of *C. perfringens* would survive six days on raw meat immersed in 22% NaCl brine and that spores would germinate in 5% NaCl. No details of this work were presented, however, and no heat-resistance determinations were made. Studies by Strong *et al.* (1963) have shown a rather high incidence of contamination of fresh meats with *C. perfringens*. Hall and Angelotti (1965) have also shown a high incidence of fresh meat contamination with *C. perfringens*, but found only a small percentage of these to produce heat-resistant spores. These studies, however, do not give any data on the survival of *C. perfringens* in hams.

With increased interest in *C. perfringens* as a cause of food poisoning, the present investigation was undertaken to obtain more definitive data regarding the effects of curing salts on the growth and heat resistance of this microorganism as well as on its survival in a normal curing process.

### EXPERIMENTAL PROCEDURES

**Preparation of vegetable cell and spore suspensions.** When only vegetative cells of *C. perfringens* were required, a culture in the log phase of growth (3-4 hr) from fluid thioglycollate medium (Difco) was used. When a sporulating culture was needed, a 24-hr SEC culture, as described by Angelotti *et al.* (1962), was used. Although the freshly sporulated SEC cultures were adequate for the studies on heat resistance and survival in curing salts, a more stable preparation, primarily containing spores, was desired for inoculation into hams. Spore inocula prepared by heating a culture at 80°C for 5 min were unsatisfactory because this treatment decreased their heat resistance, probably through activation of the spores (Barnes *et al.*, 1963). Attempts failed to separate spores from vegetative cells by the procedures of Sacks

and Alderton (1961), Stewart and Halvorson (1953), and Long and Williams (1958). Strong and Canada (1964) used cultures stored on sterile soil for their spore inocula, and the procedure employed in our study was similar except that filter-paper discs were substituted for soil. Onto each sterile filter-paper disc (Schleicher and Schuell Co., No. 740-E) was placed 0.05 ml of a sporulating culture grown in SEC medium; these were dried over  $\text{CaSO}_4$  in a desiccator at room temperature. As indicated in Fig. 1, the unheated count (i.e., vegetative cells and any spores not requiring heat shock for germination) of the inoculated discs decreased rapidly during the first day after the culture was added to the disc, then slowly declined. The spore count (heated  $80^\circ\text{C}$ , 5 min), however, increased rapidly during the first day, decreased somewhat the second day, and declined very slowly thereafter. If all cells appearing in the unheated count are considered vegetative cells, this means that over 99% of the vegetative cells have lost their viability by the end of two days. Even if they are considered spores not requiring heat shock, they represent less than 5% of the total spore count. Therefore, use of a count on the spore discs after heat shock is a valid indication of total spore population. The discs afforded an easy way of obtaining spore inocula of approximately known concentrations without having to weigh the sample, as is necessary in using soil stocks. To use the discs, one or more were placed in a tube of sterile distilled water and agitated vigorously. The discs disintegrated completely, and the filter-paper pulp could be removed by filtration through sterile cheesecloth with no decrease in spore count.

**Plate counts and incubation.** Plate counts were made on SPS agar, on which strains of *C. perfringens* associated with food poisoning produce black colonies (Angelotti *et al.*, 1962). All

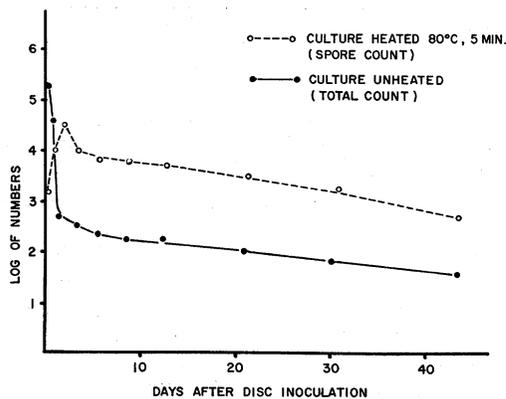


Fig. 1. Changes in total count and spore count of *C. perfringens* No. 15 on filter-paper discs.

plates and tubes (including thioglycollate tubes) were incubated at  $37^\circ\text{C}$  in an anaerobic incubator (National Appliance Co.) flushed four times with a mixture of 90%  $\text{N}_2$  and 10%  $\text{CO}_2$ . All plates were incubated for 48 hr.

**Effect of curing salts on growth of *C. perfringens* in an artificial medium.** Fluid thioglycollate medium was prepared containing various concentrations of curing salts. Castellani and Niven (1955) have shown that sterilization of glucose and nitrite together considerably reduced the apparent concentration of nitrite required to inhibit anaerobic growth of *Staphylococcus aureus*. Results were similar in our study with *C. perfringens*. Therefore, the nitrite solutions were sterilized by filtration through membrane filters and added aseptically to the fluid thioglycollate medium. Duplicate thioglycollate tubes containing each of the salt concentrations were inoculated with 0.1 ml of a vegetative cell culture. After 24 hr of incubation, the tubes were examined visually for extent of growth. Table 1 shows that 6% (w/v) NaCl, 10,000 ppm  $\text{NaNO}_3$ , or 400 ppm  $\text{NaNO}_2$  are required to inhibit the growth of *C. perfringens* significantly.

**Survival of *C. perfringens* in solutions of curing salts.** Four solutions were prepared containing the following range of concentrations of curing salts: from 7.5% (w/v) NaCl, 3,700 ppm  $\text{NaNO}_3$ , and 370 ppm  $\text{NaNO}_2$  to 17% NaCl, 23,000 ppm  $\text{NaNO}_3$ , and 2,300 ppm  $\text{NaNO}_2$ . They were sterilized by filtration, and 15 ml were placed in sterile screw-cap tubes ( $15 \times 150$  mm). One-half milliliter of a 24-hr SEC spore culture of each of five strains of *C. perfringens* was inoculated in triplicate into these solutions, and the inoculated solutions were stored at  $3^\circ\text{C}$ . (This approximates the temperature at which meats are held while being cured.) One-half-milliliter samples were withdrawn periodically from these tubes and inoculated into fluid thioglycollate medium. Four of the five strains tested survived at least 48 days in all of the stored brine solutions. In a subsequent trial, a spore disc culture of one of the most heat-resistant strains was used. Fifty percent of the spores (original inoculum 30/ml) remained viable for at least 35 days in a brine containing 21.5% NaCl, 1,800 ppm  $\text{NaNO}_3$ , and 1,200 ppm  $\text{NaNO}_2$ . This brine is similar in concentration to that pumped into hams in routine curing operations (Fields and Dunker, 1952).

Additional tests were carried out to determine the effect of curing salts on the heat resistance. In these tests the cultures survived  $100^\circ\text{C}$  for at least 30 min in the presence of 6% NaCl, 30,000 ppm  $\text{NaNO}_3$ , or 2,000 ppm  $\text{NaNO}_2$ , used individually. The cells also survived  $80^\circ\text{C}$  for at least

Table 1. Growth of 18 strains of *C. perfringens* in various concentrations of NaCl, NaNO<sub>3</sub>, and NaNO<sub>2</sub> contained in thioglycollate medium.

Extent of growth	NaCl (w/v %)			NaNO <sub>3</sub> (ppm)			NaNO <sub>2</sub> (ppm)		
	4	6	8	8,000	10,000	12,000	300	400	500
Good	14 <sup>a</sup>	1	0	18	0	0	18	0	0
Slight	4	8	1	0	11	0	0	13	0
None	0	9	17	0	7	18	0	5	18

<sup>a</sup> Number of strains.

6 hr in a combination of 10% NaCl, 3,000 ppm NaNO<sub>3</sub>, and 1,000 ppm NaNO<sub>2</sub>, a combination of heat and curing salts well above that normally encountered in cured meats.

**Survival of *C. perfringens* under simulated curing conditions in the laboratory.** Fresh meat was aseptically removed from hams and ground in a sterile grinder, and sterile concentrated solutions of the curing salts were added to give a final concentration of 3% NaCl, 500 ppm NaNO<sub>3</sub>, and 200 ppm NaNO<sub>2</sub>. These represent the maximum allowable concentration of NaNO<sub>2</sub> as well as the usual maximums of NaCl and NaNO<sub>3</sub> found in cured meats. A brine solution without meat was also prepared and placed in several tubes. Spore suspensions, treated as described in Table 2, were inoculated into these meat-brine and brine samples. The inoculated tubes were held at 3°C for 3 days and then heated at 80°C for 60 min. Plate counts were made, and the number of cells of *C. perfringens* type was determined after 48 hr of incubation. The uninoculated meat had an aerobic count of < 50 per gram; no anaerobic bacteria were encountered.

As seen in Table 2, both heated and unheated spores from SEC cultures survive in stored brine solutions better than in meat-brine mixtures, where nutrients are available. It is also evident that spores maintained on filter-paper discs have a much higher percentage of survival than unaged

spores from regular SEC cultures. Results were similar with four other strains.

#### Survival of *C. perfringens* in cured hams.

Whole hams were cured and smoked by a procedure similar to Type I of Fields and Dunker (1952). Hams were spray-pumped with a brine containing 17.9% (w/v) NaCl, 1,500 ppm NaNO<sub>3</sub>, and 980 ppm NaNO<sub>2</sub>. They were then cured for seven days at 3°C in a cover pickle containing 15.8% NaCl and 1,300 ppm each of NaNO<sub>3</sub> and NaNO<sub>2</sub>. The hams were smoked for 19–20 hr, during which time the internal temperature reached 61°C and remained there for about 1 hr. Twelve hams were pumped to 10% net weight increase with brine inoculated with a low concentration (20/ml) of *C. perfringens* spores prepared from paper discs. At various stages of the curing and smoking process, two hams were removed and a count was made of typical *C. perfringens*-type colonies. Samples were cut from three locations in the center and bone areas of the hams, and plate counts were made from the meat taken from these areas and from the fluid that drained into the cavity remaining after removal of the meat sample.

As indicated by Table 3, *C. perfringens* consistently survived both curing and smoking processes. The fluid probably contained exudate from the tissue as well as brine remaining in the intermuscular spaces. This fluid consistently had a higher count than the meat samples. In hams in which a heavier inoculum was used (40/ml brine), it was possible to isolate *C. perfringens* from the meat as well as from the fluid after smoking and cooling.

#### DISCUSSION

Barnes *et al.* (1963) showed that heat-shocking 48-hr spore cultures caused them to lose their heat resistance when subsequently cooled to 10°C within 3 hr in a meat medium. Curran and Pallansch (1963) have also demonstrated incipient germination of *Bacillus* spores at sub-minimal growth temperatures. Our investigation showed a similar loss of resistance on storage at 3°C for 3 days for the heat-shocked cells from both

Table 2. Survival of spores of *C. perfringens* in simulated curing procedures.

Inoculum <sup>a</sup>	Substrate <sup>b</sup>	Spore count <sup>c</sup>	% survival <sup>d</sup>
Unheated SEC	Meat + brine	4,500	<0.1
	Brine	2,280	30.0
Unheated disc	Meat + brine	70	39.0
	Brine	850	64.0
Heated SEC	Meat + brine	4,500	<0.1
	Brine	1,800	4.0
Heated disc	Meat + brine	850	0.4
	Brine	850	3.0

<sup>a</sup> Spore culture unheated or heated 80°C, 5 min.

<sup>b</sup> Final concentrations in the ground ham and brine without meat were 3% NaCl, 0.05% NaNO<sub>3</sub>, and 0.02% NaNO<sub>2</sub>.

<sup>c</sup> Number of spores added per gram of meat or ml of brine.

<sup>d</sup> After inoculation, held 3 days at 3°C, heated 60 min at 80°C.

Table 3. Survival of *C. perfringens* during routine curing procedures.

Phase of process <sup>a</sup>	Treatment of sample			
	Unheated		Heated <sup>b</sup>	
	Fluid <sup>c</sup>	Meat <sup>c</sup>	Fluid <sup>c</sup>	Meat <sup>c</sup>
After pumping	+	+	+	+
After curing	+	+	+	+
Smoked to 30°C <sup>d</sup>	+	-	+	-
Smoked to 45°C <sup>e</sup>	+	±	+	±
After smoking (not cooled)	+	+	+	+
After smoking and cooling	+	+	+	±
Uninoculated controls				
Fresh ham	-	-	-	-
After smoking and curing	-	-	-	-

<sup>a</sup> Two hams were removed for examination at each phase of the process.

<sup>b</sup> Samples heated 5 min at 80°C.

<sup>c</sup> Source of sample.

<sup>d</sup> Required 6 hr in smokehouse to reach 30°C.

<sup>e</sup> Required 13 hr in smokehouse to reach 45°C.

SEC cultures and spore disc cultures inoculated into a meat-brine substrate. However, for the spores that were stored 3 days at 3°C without prior heat shock < 0.1% of the spores from the SEC cultures retained their heat resistance whereas 39% of the spores from the discs were resistant. These data suggest that, while the young spores in the SEC culture had developed heat resistance, their immaturity permitted much more germination at sub-minimal growth temperatures than occurred in the mature spores. Since a mature spore is the type more likely to be encountered as a contaminant from the environment, it would appear that aged spores should be used in studies relating to the survival of *C. perfringens* in foods.

Although there are definite differences in the heat tolerance of different strains of *C. perfringens*, there was little difference in the effect of curing salts on the various strains even at levels well above that found in commercial curing operations. Not only will they survive, but they may actually grow if the suspending medium and temperature are favorable for growth. This ability to survive in concentrated brines indicates the need for proper sanitary precautions in storing commercial brines to be used for pumping hams and cover pickle.

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