Recovery of Heated _Clostridium perfringens_ Type A Spores on Selective Media

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Received for publication 1 August 1974

The enumeration of _Clostridium perfringens_ spores on sulfite-polymyxin-sulfadiazine agar (SPS), tryptone-sulfite-neomycin agar (TSN), Shahidi-Ferguson-perfringens agar (SFP), tryptone-sulfite-cycloserine agar (TSC), and TSN lacking antibiotics (BASE) was studied. The spores were heated at 105 to 120°C by the capillary-tube method. The media were about equally efficient for the enumeration of heat-activated spores. Efficiency of the media for the recovery of spores surviving heat treatments at ultrahigh temperatures varied as follows: TSC ≥ SFP > BASE > SPS > TSN. Greater recovery when survivors were enumerated on TSC or SFP was attributed to germination of injured spores by the lysozyme present in the egg yolk emulsion used in these media. Low recovery of survivors on TSN and SPS was due to both the absence of lysozyme and inhibition of injured spores by the selective agents of these media. Recovery of heated spores was reduced greatly by polymyxin, neomycin, and kanamycin, and slightly by sulfadiazine and d-cycloserine. The addition of lysozyme to SPS or TSN did not improve the percentage of heat-injured spores recovered because the selective agents of these media interfered with the action of lysozyme. The suitability of the selective media for the enumeration of survivors was greatly affected by the presence of certain foods.

In the past several years _Clostridium perfringens_ has been responsible for a significant number of outbreaks and cases of food poisoning (4, 5). The implicated foods usually had been cooked which should have killed all vegetative cells. This suggests that the cooked foods were recontaminated or that the high populations of _C. perfringens_ cells responsible for the food poisoning developed from spores which survived cooking.

Heat-resistant _C. perfringens_ spores could be important in commercially heat-processed foods. Thermal inactivation parameters for several strains of _C. perfringens_ spores heated at 80 to 135°C indicated that these spores are more heat resistant than spores of the other food poisoning spore formers, _C. botulinum_ and _Bacillus cereus_ (1, 14). Thermal processes are frequently based on the destruction of _Putrefactive Anaerobe_ 3679 spores, yet these spores also may be less heat resistant than the spores of certain _C. perfringens_ strains (1, 13).

Currently, there are no methods specifically recommended for the enumeration of _C. perfringens_ spores present in thermally processed foods. _C. perfringens_ spores may be sublethally damaged or injured during heating and require lysozyme for germination (9, 10). None of the media recommended for the enumeration of _C. perfringens_ from foods or for the general culture of anaerobes from canned foods (6) specifies the addition of lysozyme to the subculture medium. Inoculated pack studies or routine monitoring of the effectiveness of heat processes by incubating the canned product suffer from the same disadvantage.

The objectives of this study were to evaluate: (i) the suitability of currently available media for the enumeration of heated _C. perfringens_ spores, (ii) the effectiveness of adding lysozyme to these media to detect injured spores, and (iii) the influence of the selective agents in these media on the detection of injured spores.

**MATERIALS AND METHODS**

**Cultures.** _C. perfringens_ type A strains NCTC 8798, NCTC 10240, and ATCC 3624 were used. All but strain 3624 have been implicated in food poisoning outbreaks. All cultures were obtained from C. L. Duncan (University of Wisconsin, Madison). Maintenance of cultures and preparation of spore suspensions have been described (1).

**Media.** The plating media were (i) tryptone-sulfite-neomycin agar (TSN) (13), (ii) sulfite-polymyxin-
sulfadiazine agar (SPS) (3), (iii) Shahidi-Ferguson-perfringens agar (SFP) (19), (iv) tryptose-sulfite-cycloserine agar (TSC) (11) and (v) TSN agar without the selective agents (BASE).

The media were prepared from individual ingredients and were sterilized by autoclaving for 15 min at 121 C. Egg yolk emulsion, lysozyme (18,000 units/mg) (Sigma Chemical Co., St. Louis, Mo.), and antibiotics (Sigma) were added alone or in combination to the melted and cooled (50 C) media just before pouring the plates. The antibiotics were sterilized by filtration through a 0.45 μm membrane filter (Millipore Corp., Bedford, Mass.).

**Heat treatments.** Portions of aqueous stock spore suspensions were held at 75 C for 20 min to heat activate the spores. The spores were then heat inactivated or heat injured by the capillary-tube method (1, 8). Survivors were enumerated as described previously (1).

**Foods.** The influence of heated milk, meat broth, or tomato extract on the recovery of survivors was tested. Raw skim milk was held in flowing steam for 30 min. Meat broth was prepared by adding 125 g of cooked meat medium (BBL) to a liter of distilled water. The mixture was steamed for 30 min and filtered through sterile cheese cloth to remove meat particles. Tomatoes were washed, cut, and blended. The pulp was filtered through cheese cloth, and the filtered liquid was centrifuged at 6000 x g for 20 min. The supernatant was filtered through Whatman no. 4 filter paper and steamed for 30 min. One milliliter of the food was placed in each petri plate and mixed with 1 ml of spore suspension and 10 ml of enumeration medium. Control plates showed less than 1 organism per ml when each of these heated foods were plated on each medium.

**RESULTS AND DISCUSSION**

**Influence of media on the recovery of survivors.** Similar colony counts were obtained on BASE and the selective media for spore suspensions which had been heat activated but had not received an ultrahigh-temperature (UHT) treatment (Fig. 1). For enumerating spores which had been UHT treated, SFP was consistently superior to BASE and TSN was consistently inferior to BASE. Similar results were observed for spores of three other strains of C. perfringens and for heat treatments at 115 and 120 C. Since the differences between the media were observed only for the enumeration of UHT-treated spores, it appeared that during UHT treatment some of the spores had been injured, resulting in new cultural requirements.

**High recovery on SFP.** SFP contains egg yolk emulsion which Cassier and Sebald have reported improves recovery of heated strain 3624 spores (9). They attributed the greater recovery to germination of injured spores by lysozyme in the egg yolk emulsion. Lysozyme added to the plating medium has been shown to increase the number of heated C. perfringens spores capable of forming colonies (1, 9, 10), and its presence in SFP likely explains the high recovery of survivors on this medium.

When kanamycin and polymyxin, the selective agents of SFP, were added to BASE at the concentrations in which they occur in SFP, the recovery of UHT-treated strain 8798 spores was lowered by about 50%. Egg yolk emulsion added to BASE increased recovery by 1,000-fold, and lysozyme (1 μg/ml) had a similar effect. The influences of egg yolk emulsion and lysozyme also were similar for the enumeration of spores surviving various heat treatments (Fig. 2). Neither additive increased the recovery of spores surviving the early stages of heating, but both improved the recovery of spores surviving longer heat treatments. This is consistent with the assertion of Cassier and Sebald (9).

The source of lysozyme in the egg yolk emulsion was probably egg white. Egg white emulsion (10% egg white in 0.85% NaCl) added to

![Fig. 1. Recovery of non-UHT-treated (open bar) and UHT-treated (solid bar) C. perfringens spores on selective and nonselective media.](image-url)

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BASE increased the number of colonies formed by UHT-treated spores. Duncan et al. (10) reported that as little as 0.01 μg of lysozyme per ml of plating medium increased the number of heated C. perfringens spores that formed colonies. In the present study, when whole egg yolks were washed free of adhering egg white, and the yolk membrane was excluded from the egg yolk emulsion, the effectiveness of the egg yolk emulsion for enhancing the recovery of heated spores was not diminished. This emphasized that such low levels of lysozyme are required that foods containing injured C. perfringens spores could be sources of food poisoning if mixed with even small amounts of whole egg, egg white, or egg yolk.

**Low recovery on TSN and SPS.** TSN and SPS contain selective agents, whereas BASE is a nonselective medium. This suggests that the inability of some survivors to form colonies on TSN or SPS may be due to inhibition of injured spores by the selective agents. Heated spores are often more sensitive to inhibitors than are unheated spores (15). Incorporation of the selective agents of SPS, TSN, or SFP, alone or in combination into BASE, did not greatly influence the colony counts of heat-activated spores (Table 1). The recovery of spores that had also received a UHT treatment was lower on TSN and SPS than on BASE. In the absence of egg yolk emulsion, recovery of spores on SFP was also low. Recovery of UHT-treated spores on BASE plus neomycin was 26% of the recovery on BASE. Kanamycin and polymyxin also prevented colony formation by some survivors; sulfadiazine did so to a lesser extent. For spores receiving a UHT treatment, colony counts on BASE plus combinations of selective agents were lower than on TSN, suggesting that some injured spores were able to grow on TSN. Inhibition of the outgrowth of heat-injured C. perfringens spores by nitrite and NaCl also has been reported (12, 16).

The addition of lysozyme to TSN or SPS did not permit the recovery of all injured spores that required lysozyme (Table 1). Lysozyme increased the recovery of survivors on BASE by more than 10-fold, but in the presence of polymyxin, kanamycin, or neomycin much less improvement was observed. These selective agents apparently prevented germination of the spores by lysozyme. Most of the unheated spores which had been treated with ethylenediaminetetraacetic acid to sensitize them to lysozyme (2) germinated (65% decrease in optical
density equals essentially 100% germination) in a lysozyme-NaCl mixture (Fig. 3). In the presence of neomycin and lysozyme, however, germination was no greater than in the control medium which lacked lysozyme. Thus, the addition of lysozyme to SPS or TSN does not enhance the suitability of these media for enumeration of injured C. perfringens spores.

Recovery of heated spores on TSC. The previous results indicated that if selective conditions were desired in the enumeration of heated C. perfringens spores, the selective agents should not be inhibitory to injured spores and should not interfere with the action of lysozyme. In the course of this study, Harmon et al. (11) introduced a modified SFP medium in which D-cycloserine was substituted for kanamycin and polymyxin. D-cycloserine, added to BASE at the concentration reported to inhibit unwanted organisms, reduced the colony counts of spores surviving UHT treatment by 50% (Table 2). This reduction was similar to that observed for heat-activated spores, indicating that injured spores were not unusually sensitive to D-cycloserine. TSC contains egg yolk emulsion which acted as a source of lysozyme. When D-cycloserine was added to BASE with lysozyme, the decrease in recovery was no greater than when it was added to BASE alone. This indicated that D-cycloserine did not interfere with the action of lysozyme on injured spores. Thus, TSC appears to be superior to SPS, TSN, and possibly SFP for the enumeration of heated C. perfringens NCTC 8798 spores.

The influence of foods and food extracts on the recovery of C. perfringens spores. If the number of survivors is low, the food containing the spores may be added to the recovery medium in relatively large amounts. Components of the food may be inhibitory to the spores (7, 17), especially to injured spores, and prevent accurate enumeration of survivors. Milk and meat broth had little effect on the enumeration of spores not receiving a UHT treatment (Table 3). Tomato extract suppressed recovery on BASE by 19% and on SPS, SFP, and TSC by 27 to 40%. The influence of tomato extract did not

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**TABLE 2. Enumeration of UHT-treated* spores of C. perfringens strain 8798 on media containing D-cycloserine**

| Medium           | Colony counts/ml |
|------------------|------------------|
| BASE             | 2.4 x 10⁴        |
| BASE + D-cycloserine* | 1.2 x 10⁴      |
| BASE + lysozyme*  | 1.7 x 10⁴        |
| BASE + D-cycloserine* and lysozyme* | 9.3 x 10⁴ |

* Treated at 105 C for 8.5 min.

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**TABLE 3. Effect of foods on the enumeration of spores of C. perfringens strain 8798**

| Food added      | UHT treatment None | For 8.5 min at 105 C |
|-----------------|---------------------|----------------------|
|                 | BASE | TSN | SPS | SFP | TSC | BASE | TSN | SPS | SFP | TSC |
| None            | 5.3* | 4.9 | 5.5 | 5.2 | 4.8 | 13*  | 0.60| 6.4 | 21  | 34  |
| Milk            | 4.7  | 5.3 | 5.0 | 5.4 | 4.8 | 10   | 0.79| 9.6 | 11  | 21  |
| Meat broth      | 5.3  | 4.8 | 5.7 | 5.1 | 5.5 | 12   | 0.89| 6.3 | 17  | 29  |
| Tomato extract  | 4.3  | 5.1 | 4.0 | 3.4 | 2.9 | 11   | 0.86| 8.0 | 3.9 | 7.4 |

* Colony counts x 10⁴/ml.

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**FIG. 3. Germination of ethylenediaminetetraacetate-sensitised spores of C. perfringens strain 8798 in 200 mM NaCl, triangle; 1 μg of lysozyme per ml plus 200 mM NaCl, circle; or 1 μg of lysozyme per ml plus 200 mM NaCl plus 50 μg of neomycin per ml, square.**
appear to be due to a decrease in the pH of the media.

Recovery of spores receiving a UHT treatment on BASE was suppressed by milk. Milk reduced recovery on SFP and TSC to an even greater degree but enhanced recovery on SPS and TSN. The effect of meat broth was similar to that of milk. Tomato extract decreased recovery of survivors on TSN, SPS, SFP, and TSC by 40 to 88% compared to less than a 40% decrease for non-UHT-treated spores. On BASE, the UHT-treated spores were no more sensitive to tomato extract than were non-UHT-treated spores. The recovery of survivors was actually greater on BASE than on TSC or SFP.

The data suggest interactions between the influences of UHT treatment, medium components, and food components on the spores, and emphasize the complexity of enumerating heat-injured spores from foods. The suitability of any medium for enumeration of heat-injured spores may depend greatly on the food in which the spores are suspended.

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