Thermal Resistance of *Staphylococcus aureus* in Milk, Whey, and Phosphate Buffer

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**ABSTRACT**

WALKER, G. C. (Michigan State University, East Lansing), and L. G. HARMON. Thermal resistance of *Staphylococcus aureus* in milk, whey, and phosphate buffer. Appl. Microbiol. 14:584–590. 1966.—The thermal resistance of four strains of coagulase-positive *Staphylococcus aureus* was determined in phosphate buffer, whole milk, skim milk, and Cheddar cheese whey. The logarithmic order of death prevailed until about 99.99 to 99.999% of the organisms were destroyed, after which there was a decline in the rate of destruction. The organisms were more resistant in skim milk and Cheddar cheese whey than in phosphate buffer and whole milk. Thermal resistance varied among strains of *S. aureus* but was consistent with individual strains. As the age of cultures of strain B-120 increased from 12 to 228 hr, the *D*~50~ values increased from 0.95 to 3.0. The thermal resistance of cultures obtained from survivors to partial thermal destruction was similar to that of the parent cultures.

The direct relationship between staphylococci and food poisoning was first suspected by Denys (4) and has been confirmed by numerous investigators including Barber (1) and Dack et al. (3). In recent years, the incidence of staphylococci in bovine milk has increased, presumably due to the resistance of these organisms to some of the antibiotics used in mastitis therapy. Manufacturers of several varieties of cheese prefer to use raw milk or milk subjected to low heat treatment to preserve the native enzymes allegedly important in cheese ripening. Food-poisoning outbreaks involving coagulase-positive *Staphylococcus aureus* have incrimented cheese made from raw milk, milk subjected to subpasteurization time-temperature treatments, and milk exposed to postpasteurization contamination. A knowledge of the thermal resistance of *S. aureus* in milk and whey would aid in recommending appropriate temperature combinations to use with milk intended for cheese.

Certain aspects of the thermal resistance and recovery of sublethally heated staphylococci in milk have been studied by other investigators. Bhatt and Bennett (Abstr. J. Dairy Sci. 47:666, 1964) heated “mixtures of 171 strains” of *S. aureus* in whole milk and reported “survival rates” of 1.5% after 30 min at 143 F (61.7 C) and 0.38% after 15 sec at 161 F (71.7 C). No survivors were detected after 45 min at 143 F or 35 sec at 161 F. They reported *D* values of 14.5 min at 143 F, and 0.128 min at 161 F. Zottola and Jezeski (Abstr. J. Dairy Sci. 46:600, 1963) reported “complete destruction” of staphylococci at 147 F (63.9 C) for 21 sec or 150 F (65.6 C) for 16 sec. Temperature-time combinations providing less heat treatment resulted in survivors. Busta and Jezeski (2) found that 29 min at 60 C was required to destroy *S. aureus* 196-E when survivors were determined in 5-m1 quantities of the heated substrate in Plate Count Agar. These authors reported more efficient recovery of heat-shocked *S. aureus* strain 196-E on Plate Count Agar than on Staphylococcus Medium No. 110. They attributed this to the inhibitory effect of the NaCl in the latter medium and pointed out the fallacy of using inhibitory media for measuring survivors in thermal death rate studies.

**MATERIALS AND METHODS**

Several strains of coagulase-positive *S. aureus* were subjected to preliminary examination for resistance to heat. From this group, the following four representative strains were selected for more intensive study of thermal resistance. Strain 161-C was obtained from the Robert A. Taft Sanitary Engineering Center,
Cincinnati, Ohio. Strain B-120 was secured from the Northern Regional Research Laboratory, Peoria, Ill. Both of these strains have been identified with food-poisoning outbreaks. Strains designated as S-1 and S-18 were isolated from milk from cows with subclinical mastitis. The strains were classified according to the morphological and biochemical characteristics listed in Bergey's Manual of Determinative Bacteriology.

The organisms were transferred daily in a broth composed of 3.7% Brain Heart Infusion, 2% mannitol, and 1% yeast extract. To prepare a solid medium for plate counts, 2% agar was added. After preliminary experimentation with different media and combinations of ingredients, including Plate Count Agar, Veal Infusion Agar, Beef Lactose Agar, Dextrose Tryptone Agar, and the several media recommended for growing the staphylococci, the above medium was selected as the best for propagation of cultures and recovery of sublithically heat-treated cells.

Preparation of heating media. Reconstituted skim milk containing 10% solids-not-fat was prepared from nonfat dry milk and distilled water. Reconstituted whole milk containing 3.6% fat and 9.4% solids-not-fat was prepared from nonfat dry milk, sweet cream, and distilled water, and was homogenized at 2,000 psi. The pH of the milks was adjusted to 6.65. Cheddar cheese whey containing 0.4% milk fat and with a pH of 6.5 was obtained immediately after the curd was cut during manufacture of Cheddar cheese. Phosphate buffer (0.067 M, pH 7.0) was prepared. The above media and buffer were dispensed in 200-ml quantities in 8-oz glass jars, sterilized at 121°C for 15 min, and stored at 0°C until used.

Preparation of organisms for thermal destruction trials. Active cultures were inoculated into 6-oz bottles containing 50 ml of broth and glass beads and incubated for 12 hr at 37°C. In cultures of S. aureus, the cells tend to clump. Satisfactory dispersion of these clumps, as determined by microscopic examination, was accomplished by agitating the cultures on a mechanical shaker for 5 min at 350 gyrations per min immediately prior to inoculation into the heating medium. This procedure facilitated determination of the number of cells inoculated.

Thermal destruction. Thermal death time studies were made in the heating media previously described. Two strains of S. aureus (161-C and S-1) were tested in all four media. Strains B-120 and S-18 were tested only in phosphate buffer and whole milk. Strain B-120 was used to determine the effect of age on the thermal resistance of cells.

The apparatus used for heating the inoculated medium was similar to the unit designed by Kaufmann and Andrews (5). The 200 ml of heating medium, maintained under continuous uniform agitation and adjusted to the test temperature, was inoculated with 1 ml of the broth culture of S. aureus injected with a syringe and needle. The decrease in temperature caused by the inoculum was less than 0.25°F (0.14°C) as measured on a recording potentiometer.

At zero-time, the number of cells in the inoculated heating medium ranged from $6.2 \times 10^4$ to $19 \times 10^4$ per milliliter. Approximately 5 ml of the medium was withdrawn at 1- or 2-min intervals with a syringe and needle. The samples were promptly placed in sterile chilled test tubes and agitated in an ice bath for 20 sec to prevent further destruction or multiplication of the surviving cells. The survivors were enumerated on the agar medium previously described by use of the quantitative surface inoculation technique of Punch and Olson (Abstr. J. Dairy Sci. 44:1160, 1961) with 1 ml spread on five plates, or approximately 0.2 ml per plate. The plates were incubated for 48 hr at 37°C. The range and the average of the results of two to four replicate trials are indicated by the range bars on the curves in Fig. 1 to 4.

RESULTS AND DISCUSSION

Thermal destruction of S. aureus. Figures 1 and 2 show the percentage destruction of S. aureus strains 161-C and S-1, respectively, when heated in neutral 0.067 M phosphate buffer, whole milk, skim milk, and Cheddar cheese whey. The cell count at the beginning of heating varied from $6.5 \times 10^6$ to $16 \times 10^6$ with strain 161-C, and from $7.5 \times 10^6$ to $19 \times 10^6$ with strain S-1.

Figures 3 and 4 show the percentage destruction of strains B-120 and S-18 when heated in phosphate buffer and whole milk. The initial populations in the inoculated substrates varied from $6.2 \times 10^6$ to $9.5 \times 10^6$ with strain B-120 and from $10 \times 10^6$ to $15 \times 10^6$ with strain S-18.

The data show that these strains of S. aureus exhibited a logarithmic order of death through 99.99% to 99.999% destruction, at which time the surviving population numbered about 100 to 1,000 per ml. Because of the decreased rate of destruction of the last 0.01 to 0.001% of the cells, determination of $D$ values was restricted to the portion of the curve mentioned above. The decrease in destruction during the latter moments of the heating interval occurred to some extent at all heating temperatures, but was less apparent at the higher temperatures where greater lethality during the few seconds required for withdrawal and cooling of samples may have been a factor. $D$ values measured from the portion of the curves representing 99.99% destruction are shown in Table 1. The data indicate that S. aureus strains 161-C and S-1 were usually more resistant to thermal destruction in skim milk and Cheddar cheese whey than in phosphate buffer and whole milk. The data also show little difference in resistance of cells between phosphate buffer and whole milk, or between skim milk and Cheddar cheese whey. The thermal resistance varies among coagulase-positive strains of S. aureus, with a distinct difference existing between strains 161-C and B-120 identified with food poisoning outbreaks, and between strains S-1 and S-18 isolated from milk.

An experiment was devised to determine
whether the resistant cells responsible for the decrease in the rate of destruction toward the end of the heating interval were either mutants of the parent strain or members of different strains. Three colonies, respectively designated A, B, and C, were picked from plates representing samples secured during the early (2 to 6 min), middle (8 min), and late (12 to 16 min) periods of heating. Each of these colonies was subcultured in broth for 12 hr at 37°C, and these

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**FIG. 1.** Percentage destruction of Staphylococcus aureus strain 161-C in (A) neutral phosphate buffer, (B) whole milk, (C) skim milk, and (D) Cheddar cheese whey after growth in broth for 12 hr at 37°C.
cultures, as well as the parent culture, were heated separately in 0.067 M phosphate buffer. The number of cells inoculated per milliliter of heating medium varied from $3.2 \times 10^4$ to $7.7 \times 10^8$, with the greater number in the parent culture. The procedure was repeated several times to
FIG. 3. Percentage destruction of Staphylococcus aureus strain B-120 in (A) neutral phosphate buffer and (B) whole milk after growth in broth for 12 hr at 37°C.

FIG. 4. Percentage destruction of Staphylococcus aureus strain S-18 in (A) neutral phosphate buffer and (B) whole milk after growth in broth for 12 hr at 37°C.
TABLE 1. *D* values determined from the portion of the curves representing 99.99% destruction of *Staphylococcus aureus*

<table>
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<tr>
<th>Strain</th>
<th>Temp of heating</th>
<th><em>D</em> value (min)<em>a</em> when cells were heated in</th>
<th>Phosphate buffer</th>
<th>Whole milk</th>
<th>Skim milk</th>
<th>Cheddar cheese whey</th>
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<tr>
<td>161-C</td>
<td>58 C</td>
<td>1.80</td>
<td>1.85</td>
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<td></td>
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<tr>
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*a* Dash indicates not determined.

eliminate the remote mathematical possibility of adventitious results obtained by picking a resistant strain during the early phase of the heating interval.

In all trials, the destruction curves of the parent culture and the cultures of the three separate colonies were similar. Figure 5 shows typical data obtained with strain B-120. *D* values derived from the portions of the four curves representing 99.99% destruction are as follows: parent culture, 1.52; colony A, 1.35; colony B, 1.52; and colony C, 1.60 min. These results indicate that neither mutants nor mixed strains were responsible for the greater resistance of the surviving cells. These results also suggest that in a thermal process the coagulase-positive strains of *S. aureus* do not follow the logarithmic order of death to infinity.

**Effect of age of culture on thermal resistance of cells.** Many investigators have shown that young cells are less resistant to a deleterious environment than are mature cells, but no information was found on the effect of age on the thermal resistance of *S. aureus*. Tests were performed to determine the thermal resistance of *S. aureus* strain B-120 after continuous incubation in

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**Fig. 5.** Thermal destruction curves for cultures of *Staphylococcus aureus* strain B-120 prepared from colonies surviving 53 C in phosphate buffer for 4 min (colony A), 8 min (colony B), and 16 min (colony C).

**Fig. 6.** Curves showing percentage destruction of cultures of B-120 incubated in broth at 37 C for various lengths of time and heated in phosphate buffer at 55 C.
broth at 37 C for intervals ranging from 12 to 228 hr. The data in Fig. 6 show typical thermal destruction curves obtained from heating cultures which had been incubated at 37 C for 12, 60, and 228 hr. The initial numbers of cells in the heating medium were 13 × 10^4 for the 12-hr culture, 2.1 × 10^4 for the 60-hr culture, and 3.5 × 10^4 for the 228-hr culture. The D_55 values measured from the portion of the curves representing 99.99% destruction were approximately 0.95, 2.7, and 3.0 min for the 12-, 60-, and 228-hr cultures, respectively. Approximately equal numbers of the cells of the various ages were obtained by centrifuging the cells out of 1 volume of the 12-hr culture, 5 volumes of the 60-hr culture, and 25 volumes of the 228-hr cultures, and resuspending each portion of the cells in broth. The results obtained from heating the resuspended cells in buffer at 55 C were similar to those shown in Fig. 6.

Investigators have tended to use cultures in the logarithmic or early portion of the stationary phase of growth in determining susceptibility of cells to deleterious agents. The greater resistance of aged cells is an important factor in calculating a thermal process. The data in Fig. 6 indicate that the D value of staphylococci increases at least threefold as the age of the cells increases from 12 to 60 hr or more. S. aureus 161-C was the most resistant organism tested. The data in Fig. 1 show that the time required to destroy 99.999% of a 12-hr culture of this strain heated at 60 C was approximately 4 min in whole milk, 7 min in skim milk, and 6.5 min in whey. If aged cultures are three times as resistant as young cultures and 99.999% destruction is desired, then whole and skim milk should be heated at 60 C for 12 and 21 min, respectively, or a temperature combination of equivalent lethality. This heat treatment is much more severe than the "flash" treatments of approximately 55 C for 20 to 30 sec commonly used in making raw milk cheese. In the manufacture of cheese, it is necessary to accomplish destruction of staphylococci in the milk, because the time-temperature combinations used in so-called cooking of most varieties of cheese will not destroy the organism. In fact, substantial increases in numbers of staphylococci have occurred in the curd and whey during cooking (6).

ACKNOWLEDGMENT

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LITERATURE CITED