Repair and Enumeration of Injured Coliforms in Frozen Foods

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Two strains of Escherichia coli manifested death and repairable injury after being frozen in water or sterile foods at −20 C. The injured survivors were inhibited from forming colonies on violet red bile agar (VRBA) or deoxycholate lactose agar; this inhibition was greater when enumeration was done by the pour plate method instead of the surface or surface-overlay method. Injured cells repaired rapidly in Trypticase soy broth (TSB), and the repair was about maximum after 1 h at 25 C. When the injured cells were added to different foods and incubated at 25 C, repair also occurred; however, recovery was better and more uniform when the samples were mixed with TSB and incubated 1 h at 25 C. Cell multiplication was not evident until after 90 to 120 min at 25 C. The enumeration of coliforms from commercially frozen foods was increased when the thawed samples were mixed with TSB and the cells were allowed to repair 1 h at 25 C. In some samples, the repair permitted at least a 20-fold increase in the coliform count. The associated flora in the commercially frozen foods gave no evidence of impairing the repair of coliforms, nor did they start multiplication prior to 90 min after being incubated in TSB at 25 C. Generally, the plating gave more reproducible recovery of coliforms than did the most probable number method. Also, a higher number of coliforms were obtained by the surface-overlay method of plating using VRBA.

There is increasing evidence that Escherichia coli and other coliforms present in frozen samples may remain undetected by procedures normally used for their enumeration (1−3, 9, 11). Bile salts, deoxycholate, and lauryl sulfate present in the selective media are inhibitory for the repair and subsequent multiplication of the freeze-injured coliforms. When injured cells have repaired they regain their resistance to these compounds; it would appear therefore, that repair of damage is essential if enumeration of all cells by the selective media is to be accomplished. Previously, we reported that the detection of unfrozen E. coli also depends upon methods and media used for plating (10). Included in this study are observations concerning the enumeration of coliforms from naturally contaminated frozen foods. Some of these results have been presented previously (Inst. Food Technol. Annu. Meet., 1973).

MATERIALS AND METHODS

Freezing, thawing, and injury repair in laboratory strains of E. coli cells. E. coli strains NCSM and K-12, from the department culture collection, were grown in Trypticase soy broth (TSB) for 16 to 20 h at 35 C, harvested by centrifugation, and washed with sterile water. They were suspended either in sterile glass distilled water or in selected sterile foods to a population of about 2 × 10^8 cells/ml. The samples were frozen in 10-ml portions at −20 C in an ethylene glycol bath; after storage for 20 to 24 h, they were thawed at 10 C in a circulating water bath. Sterile foods used for suspending E. coli were 10% reconstituted nonfat dry milk, veal infusion (5% solids), crab meat blended 1:10 in water, and liquid whole egg. Details of the procedures, including preparation of the sterile foods, have been described elsewhere (9−11).

To study repair of the injured cells frozen in water, 1 ml of the thawed sample was transferred to 9 ml each of sterile TSB and water, incubated at 25 C, and enumerated at intervals on several plating media. When cells were frozen in sterile foods, 1 ml of the thawed sample was transferred to 9 ml each of TSB and the respective sterile foods, incubated, and enumerated as before.

Enumeration of frozen E. coli. Samples were serially diluted in sterile distilled water and plated in 0.1-ml portions in triplicate on each of the following: (i) Trypticase soy agar (TSA); (ii) violet red bile agar (VRBA); and (iii) deoxycholate lactose agar (DLA). Samples were plated in each medium by the pour, surface, and surface-overlay methods (10, 11). The pour method was the conventional plating procedure. In the surface-overlay method, 0.1 ml of the sample
was spread over the dried surface of a prepped plate, and after about 10 to 15 min at room temperature another 4 ml of the same media (45 C) was layered over the surface. The surface method was done in the same manner except that the overlay was omitted. The plates were incubated at 35 C for 20 to 24 h, and the colonies were counted. TSA poured plates were incubated for 48 h.

**Enumeration of coliforms from naturally contaminated frozen foods.** Samples of commercially produced frozen foods were obtained from the Food Microbiology Laboratory of the North Carolina Department of Agriculture and from local supermarkets. Unprocessed, processed, and ready-to-serve products from different categories of foods were randomly selected. The frozen food samples were first subjected to screening, and those containing a higher number of coliforms were used for actual tests. For screening, a 10-g portion of the sample was blended for 2 min with 40 ml of sterile water (1:5 dilution), then 10 ml of the blended material was added to 10 ml of single-strength TSB (1:10 dilution) and incubated at 25 C for 1 h. Samples in 1.0-ml and 0.1-ml portions were then plated on VRBA by the pour plate method. After 24 h at 35 C the coliform colonies were counted.

Foods found to contain a relatively large number of coliforms (100/g or more) were sampled randomly from four to five areas. A 25-g sample was then blended for 2 min in 100 ml of sterile water (1:5); 10 ml was then mixed with 10 ml each of single-strength TSB and water (1:10) and incubated at 25 C. At intervals, samples were plated (0.1 ml/plate) in triplicate by the pour and surface-overlay methods on VRBA and DLA. In tests where the total counts were determined, samples were also plated on TSA. The food sample suspended in water and made to the 1:10 dilution was used for the 0-h enumeration within 2 to 3 min after blending.

Food samples also were enumerated for coliforms by the most probable number (MPN) procedure (three tubes, three dilutions) using brilliant green bile broth (BGB) and lauryl sulfate broth (LSB). Samples from TSB and water (1:10 dilution) were used as the first dilution. The tubes were incubated at 35 C for 48 h and checked for gas formation. Samples from all LSB tubes showing the presence of gas were reinculuated into BGB tubes; these were incubated for another 48 h at 35 C and examined for gas formation. MPN estimates were then determined on the basis of BGB tubes showing gas production (1, 2).

In a number of the samples that contained higher levels of coliforms, presumptive tests were performed. Blended samples suspended in water and TSB were plated on VRBA by the pour and surface-overlay methods. The plates were incubated for 24 h at 35 C, and all red colonies were counted as coliforms. Depending upon the number on the plate, 10 to 100% of the colonies were inoculated into tubes containing 9 ml of BGB and incubated at 35 C for 48 h. Tubes showing gas production were considered coliforms.

For the determination of cell death, injury, and repair, conventional methods were used (9, 11).

**RESULTS**

**Effect of plating methods on the detection of frozen E. coli.** Previous studies in this laboratory have indicated that recovery of unfrozen and frozen cells of E. coli on selective agar media could be influenced by plating methods (10, 11). The results of the present study with two E. coli strains were in agreement with the previous findings (Table 1). Of the two test strains, NCSM appeared to be much more resistant than K-12 to freezing and to pour plating with selective media. In both strains a large number of survivors were unable to form colonies on VRBA and DLA (i.e., the cells were injured). Pour plating on DLA was more inhibitory to the survivors of both strains. Unfrozen cells, especially of strain K-12, were highly susceptible to pour plating on DLA.

**Repair of freeze-injured E. coli.** Rapid repair occurred when thawed cells of E. coli were suspended in TSB and incubated at 25 C (Fig. 1). This was evidenced by the increase in VRBA counts; both the initial rate of repair and the extent of repair at 60 min were much higher in strain NCSM than in K-12. The lower repair in strain K-12 could be related to the greater inherent sensitivity of this strain to selective media. Cells suspended in water did not repair; rather strain K-12 showed progressive death and injury (reduction in TSA and VRBA counts, respectively). In TSB, both strains started cell multiplication after 90 to 120 min of incubation.

*E. coli* K-12 was frozen in sterile foods; after thawing, a sample was transferred to TSB or to the respective sterile foods and the mixtures were incubated for 3 h at 25 C. At intervals, samples were plated by the surface-overlay method.
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method on TSA and VRBA. Results (Fig. 2) showed that in crab meat and veal infusion the injured cells repaired, but in veal infusion more cells repaired when they were in the TSB. Cell multiplication did not start before 2 h in any sample. Since both foods had the same number of cells before freezing, cell death appeared to be higher in veal infusion than in crab meat. Among two other foods tested, the least death and injury and maximum repair occurred in milk. Cell death was maximum in liquid egg which probably was due to the sensitivity of the injured cells to egg white lysozyme (8) (data not presented).

Repair and multiplication of coliforms present in naturally contaminated frozen foods. A frozen sample of breaded oyster after blending was suspended in TSB and sterile water (1:10 dilution) and incubated at 4 h at 25 C. At intervals, samples were plated by the pour and surface-overlap methods on VRBA; because of the presence of associated organisms surface plating was not used for testing commercial frozen products. Initially, and up to 60 min, more coliforms were recovered by the surface-overlap method (Fig. 3); samples incubated in TSB permitted higher recovery than the samples incubated in water. Repair of injury
was very rapid for the first 30 min. Cell multiplication was not evident before 90 min. Similar observations have been made with stuffed flounder, deviled crab, crab cake, and ice cream. However, with some foods, such as deviled crab, incubation of samples in TSB, rather than in water, did not show any additional advantage. In this sample the cells repaired rapidly (within 30 min) and started multiplication after about 2 h (data not presented).

Samples from a frozen deviled crab were blended and incubated in TSB and water at 25 C and plated on TSA and VRBA by the surface-overlay method. TSA used as the overlay (on TSA plates) contained triphenyl-tetrazolium-chloride (0.005%) for easier recognition of bacterial colonies. It can be seen (Fig. 4) that coliforms constituted about 5% of the total bacterial population. Associated bacteria did not start multiplication before 2 h; however, early in this interval injured coliforms were repaired. These data suggested that repair of the injured coliforms was not affected by the growth of the associated flora. Similar results were obtained with ice cream (data not presented).

**Relative recovery of coliforms from frozen foods.** Coliforms in several frozen foods were enumerated on VRBA and DLA by the pour and surface-overlay methods and in BGB and LSB by the MPN method. Enumeration was done at

![Figure 3](image1.png)

**Fig. 3.** Effect of incubation (25 C) of a frozen breaded oyster sample on the recovery of coliforms. After blending in water, the sample was added to the TSB and water and incubated at 25 C. At intervals samples were plated on VRBA by pour and surface-overlay methods. For other explanations see Fig. 1 and Materials and Methods.

![Figure 4](image2.png)

**Fig. 4.** Effect of incubation (25 C) on the recovery of coliforms and associated bacteria from a frozen deviled crab sample. After blending in water, samples were inoculated in the TSB and water and inoculated at 25 C. At intervals samples were plated by surface-overlay method on VRBA (for coliforms) and TSA (for total counts). For other explanations see Materials and Methods.
0 h from foods suspended in water and after 1 h of incubation at 25 °C from foods suspended in TSB and water. In many samples MPN values were >1,100/g of food; therefore, average values for a particular type of food are not presented, but results for a single sample of each type of food are given (Table 2). However, a similar trend was observed with the other samples. In general, plating methods appeared to give values which were more consistent than the values obtained by the MPN method in both BGB and LSB; poor detection of coliforms from frozen foods by the MPN method has been observed by others (4, 7); many have preferred enumeration of coliforms in frozen foods by plating or solid media (4, 6, 7). In all samples, more coliforms were recovered by plating the samples after the 1-h repair in TSB; repair in water was not as extensive. The lowest injury was found in chicken chow mein. Plating on VRBA by the surface-overlay method permitted higher recovery of coliforms from samples incubated in water; however, for samples incubated in TSB, recovery was not as greatly influenced by the method of plating or by the media.

Average recovery of coliforms on VRBA and DLA from five kinds of foods indicated that plating the samples after 1 h of incubation at 25 °C was superior over that of plating them immediately after thawing. This increase occurred irrespective of media and methods of plating; recovery on VRBA was generally better than on DLA. Recovery from different foods and individual samples varied, but an increase in count of ×21 was observed as a result of the repair (Table 3).

Presumptive confirmation in BGB of the coliform colonies obtained on VRBA from frozen breaded oyster, devil crab, crab cakes, and stuffed flounder were studied (Table 4). The surface overlay provided about 25% more colonies than the pour method, and presumptive confirmation of colonies by both methods were essentially the same.

**DISCUSSION**

There are a number of studies which indicate that many semisemipreserved foods may contain injured microorganisms (5, 6; M. Warcek,

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**Table 2. Enumeration of coliforms from naturally contaminated frozen foods by plating and MPN procedures**

| Nature of sample and time of testing | Counts* (× 10⁵)/g by: | VRBA | DLA | VRBA | DLA | BGB | LSB |
|-------------------------------------|------------------------|------|-----|------|-----|-----|-----|
| Crab cake                           |                        | 80   | 0   | 100  | 100 | >110 | >110 |
| 0 h control                         |                        | 650  | 480 | 660  | 640 | >110 | >110 |
| 1 h TSB                             |                        | 700  | 570 | 710  | 680 | 29   | >110 |
| Deviled crab                        |                        | 10   | 10  | 30   | 30  | 24   | 46  |
| 0 h control                         |                        | 120  | 120 | 150  | 110 | 46   | 46  |
| 1 h TSB                             |                        | 190  | 210 | 180  | 180 | >110 | 46  |
| Breaded oyster                      |                        | 31   | 21  | 95   | 78  | 12   | 110 |
| 0 h control                         |                        | 84   | 42  | 126  | 111 | 110  | 46  |
| 1 h TSB                             |                        | 182  | 158 | 180  | 186 | 24   | 110 |
| Stuffed flounder                    |                        | 29   | 18  | 28   | 25  | 12   | 5   |
| 0 h control                         |                        | 180  | 180 | 420  | 300 | 12   | 46  |
| 1 h TSB                             |                        | 300  | 320 | 420  | 300 | 46   | 24  |
| Chicken chow mein                   |                        | 28   | 16  | 26   | 18  | 110  | 24  |
| 0 h control                         |                        | 42   | 10  | 26   | 10  | 110  | 110 |
| 1 h TSB                             |                        | 32   | 20  | 32   | 18  | 46   | 46  |

* Plate counts in crab cake sample and in certain other samples were actually made in 1₀ dilution; for uniformity in the presentation all the data were presented in 1₀ dilution.
* The food samples suspended in sterile water were used as control to compare its efficiency in recovery to foods suspended in Trypticase soy broth (TSB).
* Samples tested at 0 h had been incubated at 25 °C. For other explanations see Materials and Methods.

**Table 3. Increased coliform counts in frozen foods resulting from the 1-h repair period in water and TSB**

| Nature of sample and time of testing | Range of increase* in coliform counts by: |
|-------------------------------------|------------------------------------------|
| No. tested                          | VRBA | DLA | VRBA | DLA |
| Crab cakes                          | 4    |     | 1.7-7.9 | 1.5-6.0 | 1.4-6.6 | 1.5-6.4 |
| 1 h control                         |     |      | 1.6-8.8 | 1.0-7.1 | 1.6-7.1 | 1.8-6.8 |
| 1 h TSB                             |     |      | 1.1-12.0 | 1.2-12.0 | 1.0-9.2 | 1.8-8.8 |
| 1 h TSB                             |     |      | 1.5-19.0 | 1.7-21.0 | 1.5-9.5 | 1.7-8.2 |
| Deviled crab                        | 7    |     | 2.7-12.0 | 1.8-7.0 | 1.3-2.7 | 1.4-2.0 |
| 1 h control                         |     |      | 3.4-20.0 | 4.5-18.0 | 2.7-12.0 | 1.3-2.7 |
| 1 h TSB                             |     |      | 1.4-4.0 | 0.9-3.0 | 1.0-2.5 | 0.8-3.0 |
| 1 h TSB                             |     |      | 1.8-4.5 | 1.5-4.0 | 1.4-3.0 | 1.1-4.0 |
| Breaded oyster                      | 5    |     | 1.1-1.6 | 1.0-4.3 | 0.9-1.0 | 1.2-1.7 |
| 1 h control                         |     |      | 1.2-1.9 | 1.1-3.3 | 0.9-1.2 | 1.0-3.2 |

* Fold increase = count after incubating individual sample in water (control) and in TSB for 1 h at 25 °C compared to count of sample after thawing and homogenizing in water. For other explanations see Table 2 and Materials and Methods.
Table 4. Presumptive confirmation of coliforms from frozen foods enumerated by VRBA using two plating methods

| Plating method | No. of colonies on VRBA | No. of colonies inoculated in BGB | No. of colonies producing gas in BGB |
|----------------|-------------------------|----------------------------------|------------------------------------|
| Pour           | 1,089                   | 461                              | 423                                |
| Surface-overlay| 1,358                   | 476                              | 424                                |

*The surface-overlay method gave 25% higher recovery than the pour plating method; the latter method gave 3% higher confirmation. For other explanations see Materials and Methods.

M.S. thesis, N.C. State Univ., Raleigh, 1973). If an accurate evaluation of the bacterial population of such foods is to be obtained, it is essential that current methodology be revised so that such microorganisms can be detected and enumerated. Fortunately, the repair of injury proceeds at a sufficiently rapid rate so that this can be accomplished before cell multiplication takes place. Based on the present study, it would appear that a 60-min repair period at 25°C would be adequate without causing complications by cell multiplication of uninjured microorganisms.

To assure the repair of injured microorganisms in different kinds of foods, the repair must be done in a culture medium such as TSB, although it was observed that some foods allow repair per se.

The repair of injured cells appears to be essential for their detection regardless of the type of enumeration procedure employed. While the surface-overlay plating method is less stringent on damaged cells, the improved detection and enumeration of cells by this method would seem to warrant its use over the pour plate method. Furthermore, the repair of injured cells prior to MPN determination is also advisable. Fortunately, the associated flora in food samples so far tested do not have an adverse effect on the repair of coliform bacteria.

Obviously, the adoption of a repair step in the microbiological examination of foods may have a bearing on the acceptability of current standards for coliform bacteria in many foods. Only as the repair step is used routinely will information be available to evaluate the impact of repair on current standards.

The need for repair of other microorganisms is being studied in our laboratories at the present time. Information available to date indicates that most microorganisms related to food sanitation can exist in an injured state in semi-preserved foods. Studies are being conducted to establish repair procedures for such microorganisms.

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