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Effects of Freezing and Storage on Microorganisms in Frozen Foods: A Review

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ABSTRACT

The fate of bacteria contained in food during freezing, storage, and thawing is usually one that is detrimental. However, many microorganisms considered to have been killed by such treatments actually are only injured. Their viability can be determined by allowing the injury to repair in a non-selective medium before testing for their presence on selective media. Injured cells of pathogens have been found to be as pathogenic as uninjured ones; injured spoilage microorganisms can cause spoilage if permitted to repair and grow. Injured microorganisms and their potential importance in food safety and shelf-life constitute and important problem for the frozed food industry as well as the food sanitarian.

Freezing has become a very important means for food preservation. Frozen foods have two properties that control microbiological activity. One is the limiting a_w; the other is that the temperature of the product is too low to allow microbial growth. Freezing also can maintain a more desirable texture and flavor in foods that can not be accomplished by other preservation procedures. Yet, freezing and frozen storage of foods result in something of a dilemma for the food processor because the attributes of freezing, which permit preservation of the cellular structure of foods, have similar effects on microorganisms that are contained in the foods. Therefore, while some of the micoorganisms may be killed during freezing, many do survive and exist in different states of viability during frozen storage. As a consequence, the microbiologist must now deal with different states of microbial viability in assessing the microbiolobical quality of frozen foods.

FATE OF BACTERIA IN FROZEN FOODS

The successful freezing of food must be done with only minor consideration being given to reducing the bacterial load; yet, depending upon various factors, freezing can be lethal to many bacteria present in foods. As a result, frozen foods contain dead and surviving bacterial cells; many survivors may be in an injured condition. This differentiation is based on the ability of the bacterial cells to form colonies on different types of solid media (*17*). Among gram-negative bacteria, survivors are differentiated from dead cells by their ability to form colonies on a nonselective medium, such as trypticase soy agar (TSA). Among the survivors, injured cells are able to form colonies on TSA, but not on commonly used selective (for "structurally injured cells") or minimal (for "metabolically injured cells") media. The injured cells vary in the extent of their injury; where injury extends beyond the ability of a cell to multiply and form a colony, it is regarded as dead. Differentiation between "structural" and "metabolic" injury probably can be ascribed by the extent of cell damage; all injured cells have damage to cell structure; when injury is more extensive metabolic systems are damaged and such cells are considered metabolically injured.

The numbers of dead, injured, and uninjured bacterial cells in a frozen food are dependent upon many factors. While these factors have been studied primarily with pure cultures (*3, 6, 13, 17*), similar effects can be expected in bacteria present in foods.

Type of bacteria

Bacterial spores are exceedingly resistant to freezing. The vegetative cells of micrococci, staphylococci, and streptococci are very resistant to freezing and frozen storage. However, there is considerable variation in resistance among strains. Gram-negative bacteria generally are more sensitive to freezing than are the gram-positive types. Cells in the stationary phase are more resistant than those in the log phase (*13, 17*).

Composition of food.

Composition of the food in which bacteria are contained can increase or reduce the resistance of bacterial cells to freezing damage. Increased resistance usually is provided by viscous foods and by such food components as proteins, simple and complex carbohydrates, and by triglycerides; reduced resistance is associated with the presence of certain ions, inorganic salts, acids, surface active components, and certain enzymes (e.g., lysozyme, proteases) (*10, 11, 16, 17, 19, 22, 24*).

Treatments of food before, during and after freezing

Freezing can be expected to be more lethal if the food

containing the bacteria is subjected to some other sublethal treatment before freezing. Such treatments could be low heat, irradiation, refrigeration, salting, or acidification.

Within limits, fast freezing is generally less lethal to bacterial cells than slow freezing. The smaller intracellular ice crystals resulting from rapid freezing are less damaging to the cell. Also, rapid thawing is less damaging than slow thawing. Repeated freezing and thawing are highly lethal.

Bacterial cells die rapidly during the initial period of frozen storage and at a reduced rate thereafter. Death rate during frozen storage may increase at higher temperatures of storage, due to fluctuations in temperature and the presence of oxygen (*3, 6, 13, 17*). In frozen foods, pathogenic and indicator bacteria can survive in variable quantities for extended periods (*5, 9*).

IMPORTANCE OF DEAD, INJURED, AND UNINJURED BACTERIA IN FROZEN FOODS

The state in which bacterial cells exist in a frozen food can cause a number of problems with respect to evaluation of its microbiological quality. While freezing and frozen storage can reduce bacterial numbers considerably, this can not be depended upon qualitatively or quantitatively. Therefore, freezing can not replace sanitary production and handling of frozen foods. Consequently, examination of frozen foods for indicator or pathogenic bacteria is important in monitoring frozen food quality. Indicator bacteria, such as the coliform group and *Escherichia coli*, are detected in foods by selective enumeration methods (*1, 4*). Injured cells, which can constitute 90% or more of those surviving, are sensitive to the selective media customarily used to enumerate coliforms and therefore will not be detected (*16, 17, 24*). Consequently, coliform counts on frozen foods can not be depended upon to indicate the quality of sanitary practices used in processing and handling (*2*). It has been shown that the uninjured salmonellae could be equally pathogenic (*21*). Yet injured pathogens are also sensitive to the selective conditions used in their isolation and enumeration (*10, 11, 14*). Therefore, frozen foods contaminated with pathogenic bacteria can be an unsuspected health hazard to consumers.

Effect of selective solid media

Freeze-injured coliforms are extremely sensitive to violet red bile agar (VRBA) and other solid media used for the selective enumeration (*7, 12, 16, 22, 24*). Under some conditions 99% or more of the survivors may be injured and remain undetected. Productivity of VRBA medium varies with the method used in plating; pour plating is more inhibitory than the surface and surface-overlay plating methods (*15, 16, 22, 23, 24*). Increased sensitivity has been shown for freeze-injured *Salmonella* to xylose lysine deoxycholate agar, *Shigella* to Hektoen Enteric agar, *Vibrio parahaemolyticus* to thiosulfate citrate bile salts sucrose agar, and *Staphylococcus aureus* to Vogel-Johnson agar (*10, 11, 14, 17*).

Effect of selective liquid media

Cells of coliforms injured by freezing die rapidly in selective liquid media (*16, 17*). Exposure of such cells for 1 to 2 min in lauryl sulfate tryptose or brilliant green bile broth (LST-BGB) causes them to lose their ability to form colonies on TSA. Similar observations have been made with freeze-injured *Salmonella* in tetrathionate and selenite cysteine broth, with *V. parahaemolyticus* in glucose salt Teepol broth (GSTB), and with *S. aureus* in TSB containing 10% NaCl (Ray, unpublished data).

METHODOLOGY FOR REPAIR OF INJURY AND SELECTIVE ENUMERATION OF COLIFORMS IN FROZEN FOODS

Repair of injured cells cannot occur in selective environments used for their detection. In a non-selective environment injured cells repair their damage and then can proceed with growth and multiplication. Efforts therefore were directed to the development of methods to effect repair of any injured cells before their selective enumeration.

Repair in liquid media

In this method injured cells are allowed to repair in Trypticase Soy Broth (TSB). It has been studied extensively with pure cultures, with sterile foods inoculated with coliforms before freezing, and with commercially processed foods containing coliforms (*16, 17, 22, 24*). Results have indicated that the cells repaired freeze-injury rapidly in TSB; most cells repaired within 1 h at 25 C and were no longer susceptible to VRBA. Resistance of repaired coliforms to liquid selective media has been reported (*16*). Frozen foods, without thawing, can be blended with TSB, incubated 1 h at 25 C to effect repair and then can be enumerated for coliforms with VRBA by plating or with LST by the MPN (most probable number) method.

Repair in liquid media has the advantage of being applicable to foods with small and large coliform populations. For foods having low coliform limits (e.g. $\leq$10/g), a 10-ml portion containing 1 g of a sample could be plated on three plates with VRBA; a smaller amount can be used for foods having high limits. However, this method has several disadvantages. All strains of coliforms do not repair equally well in TSB within 1 h. There is also the possibility that during the repair period uninjured cells may start multiplication; this has been observed with fresh isolates, but not with laboratory cultures or with coliforms in commercial foods. This possibility has made the liquid-repair method of questionable value, especially for regulatory purposes. The other possible disadvantage of the liquid media repair is that certain food components, such as lysozyme, protease, acids, and NaCl, might be detrimental to repair which could limit the recovery of injured coliforms after

incubation in TSB.

Similar repair of freeze-injured *Salmonella, Shigella, V. parahaemolyticus,* and *S. aureus* in TSB has been studied (*10, 11, 14,* Ray, unpublished data).

Repair on solid media

The applicability of this method for enumeration of coliforms in frozen and other semi-preserved foods is being studied currently in our laboratory (*18, 23*). The method consists of either surface or pour plating of blended samples using TSA or PCA (Plate Count Agar); this is followed by a 1 to 2 h of incubation at 25 to 35 C to effect repair. The plates are then overlaid with VRBA in an effort to permit only selective growth of coliform cells during subsequent incubation at 35 C for 24 h. Red to pink colored colonies are enumerated as coliforms. Recent studies have indicated that up to 1 ml of the blended samples could be plated with 5 ml of TSA repair medium. At present, several modifications relating to incubation time and temperature are being studied with the objective to increase coliform colony formation and better recognition of the colonies. In general, for frozen non-dairy products a 50-g sample is blended with 450 ml of 0.1% peptone without prior thawing; coliforms are enumerated by plating (1 ml/plate) by the solid repair procedure (TSA followed by VRBA, to determine the total coliform survivors), by VRBA alone (to determine the uninjured coliforms), and by the standard three tube-three dilution MPN method (*4*) using LST broth followed by confirmation in BGB broth. For frozen dairy products a 50-g sample was thawed by rotating in a waterbath and then plated (1 ml/plate) by the solid repair method and by the standard procedure (*1*). Enumeration of coliforms in 20 commercial ice cream samples by the solid repair procedure resulted in a 4-fold increase in counts. It was of particular interest that 73% of these samples met the customary limit of 10 or less coliforms/g by the standard procedure; by the solid-repair method only 25% were found to meet this standard (*18*). This method has also been found to increase detection of coliforms from different frozen foods, such as seafoods, T.V. dinners, meat products, and vegetable products. Increased counts by use of this procedure also were obtained from other semipreserved foods such as salads, soft ripened cheese, sausage, frankfurters, bacon, sandwiches, refrigerated meat products, and spices (Ray, unpublished data). In most samples coliform counts by the solid-repair method were considerably higher than by the MPN method.

The above results indicate that the solid-repair method could probably be effectively used for enumeration of coliforms from frozen foods as well as from other semi-preserved foods and samples where coliforms may be present in an injured state. This method not only recovered injured coliforms, but also reduced variation in the population enumerated in subsamples, and permitted a reduction of 24 h in the time required for the MPN method. Studies are now in progress to optimize enumeration of coliforms from frozen and other semi-preserved foods by the solid-repair method. Some variation of this method has been reported recently by others (*8, 20*).

CONCLUSIONS

It is becoming increasingly clear that the number of viable microorganisms in frozen foods may markedly exceed that which has been determined by conventional methodology. This presents new problems with respect to assessment of frozen food safety by microbiological analyses. Certainly frozen foods have contained undetected injured microorganisms during the years that conventional analytical methods have been used for monitoring these foods. Whether or not this situation has caused frozen foods to have constituted major dangers to consumers could rightfully be questioned. At the same time, there is no reason for not detecting any pathogen, or enumerating all index bacteria, if they are present in frozen foods and the technique for their detection is available. Furthermore, if other injured cells might affect the shelf-life of the foods during storage or upon thawing, certainly the industry would want to take corrective actions. Conceivably, microbiologal standards for frozen foods, particularly index bacteria, may need some relaxation for a period after regulatory agencies have adopted newer procedures for enumerating injured as well as uninjured bacteria. Similar consideration should be given in the evaluation of microbiological quality and in setting up microbiological standards for different types of semi-preserved products because conditions in such foods may produce sublethal injury to the microbial population present.

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