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HYGIENIC INDICATOR ORGANISMS : A COMPARISON OF SURVIVAL AND ENUMERATION OF THE GROUP D STREPTOCOCCI AND *Escherichia coli* FOLLOWING FREEZING AND FROZEN STORAGE

FOOD HYGIENE DIVISION
DEPARTMENT OF NUTRITION
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Hygienic indicator organisms: A comparison of survival and enumeration of the Group D streptococci and *Escherichia coli* following freezing and frozen storage

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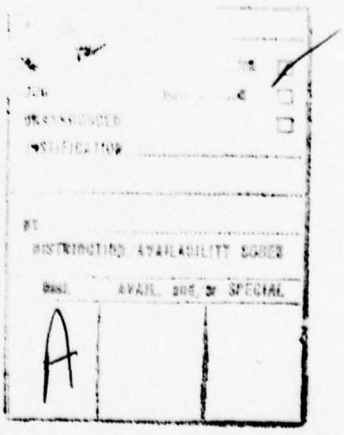

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The Group D streptococci and Escherichia coli were compared as to their ability to survive freezing and frozen storage as well as the capability for enumeration on selective bacteriologic media. The non-enterococcal portion of the Group D streptococci, including Streptococcus bovis and Streptococcus equinus, exhibited marked sensitivity to all parameters measured. Escherichia coli was able to survive freezing and frozen storage, however cellular injury to frozen cells prevented enumeration on selective media. The enterococcal portion of the Group D streptococci demonstrated resistance to effects of freezing and frozen

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ABSTRACT

The Group D streptococci and *Escherichia coli* were compared as to their ability to survive freezing and frozen storage as well as to the capability for enumeration on selective bacteriologic media. The non-enterococcal portion of the Group D streptococci, including *Streptococcus bovis* and *Streptococcus equinus*, exhibited marked sensitivity to all parameters measured. *Escherichia coli* was able to survive freezing and frozen storage, however cellular injury to frozen cells prevented enumeration on selective media. The enterococcal portion of the Group D streptococci demonstrated resistance to effects of freezing and frozen storage. Enumeration of the enterococci with the use of selective media was not affected by either freezing or frozen storage. Results of this study have implications in the hygienic analysis of frozen food products.

PREFACE

The authors wish to thank SP6 Richard L. Okoluk for his technical assistance throughout this study. Our appreciation is extended to Mrs. Karen Trefz for her typing of the manuscript. Colonel James L. Fowler's present address is P.O. Box O, Live Oak, FL 32060.

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A safe and wholesome food supply is one of mankind's basic needs. References to the first attempts at regulation or practices designed to ensure food safety can be found in the Old Testament. Throughout the centuries, lack of a safe food supply severely handicapped military forces. Microbiologists have contributed much to the store of scientific knowledge about standards for water, food, and dairy products in attempts to help provide safe food supplies for military and civilian communities. In the late 1800s, microbiological techniques were adapted for use in the hygienic examination of water. When microbiologists found that isolation and identification of enteric pathogens were difficult and time-consuming, they focused their interest on the use of "indicator" organisms. Schardinger, cited by Buttiaux (1,2), suggested the use of Escherichia coli for this purpose. The proposal was adopted because it was reasoned that, if other organisms belonging to the family Enterobacteriaceae were absent, pathogens would be absent as well.

E. coli was chosen as the indicator organism for several reasons (2-5): 1) E. coli was part of the normal intestinal flora of man and other animals; 2) E. coli, Shigella sp. and Salmonella sp. displayed similar resistance to unfavorable external conditions; and 3) with procedures in use at that time, E. coli was easily isolated and differentiated from other Enterobacteriaceae.

Currently, the family Enterobacteriaceae is composed of the genera Escherichia, Edwardsiella, Shigella, Salmonella, Arizona, Citrobacter, Klebsiella, Enterobacter, Serratia, Proteus, Providencia, and Yersinia. All of these organisms are glucose fermenting, nitrate reducing, gram-negative rods of similar microscopic appearance. At times, differentiation between these genera and their member species is an arduous task requiring numerous biochemical and serological procedures for definitive speciation.

In 1894, the idea of using E. coli as a hygienic indicator organism was expanded to include several other members of the Enterobacteriaceae having similar physiological characteristics. These Enterobacteriaceae, given the collective term coliform, were described as gram-negative, non-sporeforming rods capable of fermenting lactose with the production of acid and gas within a 48 hour period. In 1920, Schardinger's original idea was adopted for use in examination of pasteurized milk and ice cream. Later, the use of coliform organisms as indicators of hygienic quality was extended to general usage in the examination of food products. Soon after the coliform modification

1. Buttiaux, R., J Appl Bacteriol. 22:153, 1959
2. Buttiaux, R., and Mossel, D.A.A., J Appl Bacteriol. 24:353, 1961
3. Mossel, D.A.A., J Sci Fd Agri. 10:662, 1959
4. Mossel, D.A.A., J Appl Bacteriol. 22:184, 1959
5. Mossel, D.A.A., J Assoc Off Agri Chem. 59:91, 1967

was put into general usage, it was demonstrated that some coliforms could be found outside the intestinal tract. This finding necessitated the development of tests designed to differentiate between coliforms of fecal and non-fecal origin.

The use of indicator organisms remains the technique of choice in food microbiology for several reasons: 1) The isolation of enteric pathogens, such as Salmonella and Shigella, from food products remains a difficult task; 2) In addition, these organisms may be present in foods as a minority in comparison with other bacterial species; 3) Proving the absence of pathogens in a food sample is significant only for that sample, while continued failure to find indicator organisms demonstrates that food prepared in such a manner will not be dangerously contaminated (3-7); 4) Food can be the vehicle of transmission for other pathogens such as viruses, the ova of intestinal worms and protozoan parasites, the detection of which is complicated and time-consuming.

Since biblical times, achievements in food technology have been numerous. One of the great developments grew out of recognition that cellular processes are extremely slow at low temperatures. As a result, the use of freezing temperatures for food preservation was begun and the frozen food industry was created.

Large scale use of precooked frozen foods by the military began in 1951 with the introduction of frozen meals to the United States Air Force in-flight feeding program (8). Introduction of this type of feeding program coincided with the publishing, by the U.S. Army Quartermaster Corps, of a military specification for these items. The specification (MIL-M-13966) imposed the following bacterial standard for the finished product: standard plate count not to exceed 100,000 per gram and coliform count not to exceed 10 per gram. The allowable coliform count has been increased to not more than 100 per gram as per specification MIL-M-0013966 D 1968. When the coliform count is below 100 per gram, further testing for E. coli is performed in E.C. broth incubated for 24 hours at 45.5 C ± 0.2 C. Any positive tube from this test constitutes grounds for rejection of the product.

During recent years, however, it has been demonstrated that many coliform organisms, especially E. coli suffer sub-lethal cellular injury when exposed to a variety of physiological stresses that include heating, freezing, irradiation, and exposure to germicides (9). This injury to the organism may be manifested in one of several ways, including increased susceptibility to antimicrobial agents, inability to grow on selective media, leakage of intracellular material, and altered metabolic activities (10).

6. Mossel, D.A.A., J Food Technol. 3:401, 1969
7. Mossel, D.A.A. et al, J Appl Bacteriol. 20:265, 1957
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It has been demonstrated that the growth of E. coli cells, after injury by freezing, is severely impaired on both violet red bile and desoxycholate lactose agars (11,12). In violet red bile agar, the medium used for coliform plate counts of food products, both the bile salts and crystal violet components of the medium have been found inhibitory to the growth of freeze-injured E. coli cells.

Coliform organisms suffering injury due to freezing have the ability to repair when in the presence of small peptides and amino acids (12-16). However, since various coliforms repair at different rates, a repair step before coliform enumeration does not appear to be feasible when attempting to enumerate a mixed coliform population as may be found in a frozen food sample.

Recently, there has been intense interest in setting up bacterial standards for various food items. The increase in production and use of frozen products has generated pressure for development of bacterial standards for these products as well. If freeze-injured coliform bacteria are unable to produce colonies on selective media, then any coliform count obtained using a selective medium may not be a true representation of the population of that product. This decrease in count is surely significant when coliform counts are used as a basis for acceptance or rejection of frozen food products.

Although the enterococci have been considered unreliable as reflections of direct fecal contamination, they can be used as reliable indices of levels of sanitation and holding conditions which have been used during preparation of a product (17). Additionally, gram-positive bacteria are known to exhibit resistance to cold injury. This project was undertaken to explore the possibility of using the Group D streptococci as hygienic indicators in frozen food products.

MATERIALS AND METHODS

For each organism studied, a 10 ml aliquot from an overnight culture grown in trypticase soy broth (TSB) was inoculated into each of four flasks containing 250 ml TSB. Flasks were incubated for 24 h at 37 C in a shaking waterbath set for 80 oscillations per minute. Following this incubation period, cultures were harvested by centrifugation at 3000 rpm for 45 min at 4 C (International Equipment Corporation, Model PR-6000, Head No. 284). Harvested cells were washed three times in 0.2 M phosphate buffer, pH 6.8, before they were suspended in 100 ml

11. Ray, B., and Speck, M.L., Appl Microbiol. 25:494, 1973
12. Ray, B., and Speck, M.L., Appl Microbiol. 24:258, 1972
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16. Ray, B., and Speck, M.L., Appl Microbiol. 25:499, 1973
17. Diebel, R.H., and Hartman, P.A., Compendium of Methods for the Microbiological Examination of Foods, 1976

of the same buffer. Next, cells were diluted 1:10 in the medium in which they were to be frozen. Cells were diluted to a final density of approximately 3 x 10^9 cells/ml in water (pH 6.94), 0.2 M phosphate buffer pH 7.0 (prepared using solutions of 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4), 5% glycerol, 10% sucrose or a 15% slurry of mashed potatoes in water. Cell suspensions, in five ml aliquots, were distributed into sterile 13 x 100 mm screw cap test tubes. A tube of each medium was withheld for determination of the initial viable cell count and all other tubes were frozen at -20 C for 2 h. At this time, samples of cells diluted in each medium were placed in storage at -4 C, -20 C, or -80 C.

Following preselected storage time periods of 1 day, 7 days, 30 days, 60 days, and 180 days, one tube of cells from each medium stored at each temperature was thawed and a determination of the remaining viable cell populations was made. Thawing of frozen cell suspensions was accomplished by swirling the tube in a 45 C waterbath for 25 sec and then mixing at top speed on a vortex for 25 sec. This procedure was repeated twice.

Duplicate pour plates, trypticase soy agar (TSA) and either KF streptococcal agar (Group D streptococci) or violet red bile agar (E. coli), were prepared with either undiluted or 10^{-1} through 10^{-9} dilutions of thawed cell suspensions in phosphate buffered water. Plates were incubated at 37 C for 48 h before colony counts were made by using a Quebec Colony Counter with a hand tally.

Statistical analysis of the data was conducted with the use of a 2-way analysis of variance with distribution-free multiple comparisons based on Friedman's rank sums (Friedman's test) (18). The eight organisms tested were ranked according to percent recovery for each set of conditions (temperature, suspension medium, and storage time) with the highest percent recovery receiving a rank of one. The rank sums for each organism under all conditions were computed, and an arbitrary level of significance of $\leq$ 5% was chosen to determine significant differences in percent recovery between organisms. To assess whether differences were due to temperature or type of suspension medium, similar comparisons were made for each. For temperature comparisons, the calculated level of significance was $\leq$ 5%; for comparisons of suspension media, the calculated level of significance was $\leq$ 3.7%.

RESULTS

For each organism studied, the percentage of the population which was able to survive frozen storage and produce colonies on all-purpose as well as selective media can be seen in Tables I and II. In general,

18. Hollander, M., and Wolfe, D.A., Non-parametric Statistical Methods. 1973

all organisms except S. bovis and S. equinus were recovered on the all-purpose medium, TSA, following frozen storage. This observation suggests that only S. bovis and S. equinus were sensitive to lethal injury following frozen storage. Lethal damage to S. bovis and S. equinus were observed to a greater extent following storage at -4 C as compared to -20 C or -80 C. On selective media, E. coli was poorly recovered in addition to S. bovis and S. equinus. This observation suggests that E. coli is more susceptible to sublethal freezing injury than the enterococcal portion of the Group D streptococci. Again, a greater amount of sublethal injury to E. coli occurred following storage at -4 C than following storage at -20 C or -80 C.

When all of the values for percentage of recovery on TSA for all storage treatments (temperature, suspension media, and storage time) were ranked according to a Friedman's analysis (Table III), the recovery of S. bovis was ranked significantly lower than the recovery of all other organisms. In addition, S. liquefaciens showed significantly lower recoveries on TSA than did S. faecalis, S. faecium or S. durans. The recovery of S. durans on TSA was significantly higher than that of S. equinus or E. coli. Since all storage temperature treatments are grouped together, effects of a particular temperature, such as -4 C, on a particular organism, such as S. equinus, may be somewhat overshadowed.

When recovery on selective media for all treatments was ranked by a Friedman's analysis (Table IV), E. coli was ranked at a significantly lower rate than all other organisms except S. bovis. Recovery of S. bovis was significantly lower than that for the other streptococci studied except S. equinus. The recovery of S. equinus was significantly lower than that for S. faecalis, S. faecium, S. faecium var. casseliflavus and S. durans. In addition, recovery of S. liquefaciens was significantly lower than that for S. faecalis, S. faecium, and S. faecium var. casseliflavus. In general, the number of days of frozen storage contributed no differences in the relationships between organisms on either all purpose or selective agar media.

The results of a Friedman's test based on percent recovery on non-selective (i.e., TSA) and selective media with the data grouped according to storage temperature are presented in Tables V and VI. On non-selective media it can be seen that the percent recovery of S. bovis is consistently and significantly lower than the other organisms tested. On selective media, E. coli and S. bovis both show poor recovery percentages no matter which temperature of frozen storage is considered. S. equinus was shown to recover poorly also, especially when storage was at -4 C. S. equinus may be protected by storage at -80 C. Otherwise, storage at the three different temperatures had essentially equivalent effects on the various organisms.

Tables VII and VIII show the results of a Friedman's test when the data are grouped according to the type of suspension medium. Some differences were noted in the percent of the organisms recovered depending

on the suspension medium. For example, the relative recovery of S. liquefaciens on TSA was markedly improved following storage in buffer as compared to other types of suspension. In general, however, percent of the various organisms recovered varied only a little by differences in suspension media.

The rank order of recoverability of the various organisms based on Friedman's rank sums is presented in Table IX. The rank order changed very little as a function of either storage temperature or the type of suspension medium. Several appreciable changes were noted such as the protection of S. equinus at -80 C and the improved recovery of S. liquefaciens on TSA after storage in buffer. While the rank order of recoverability did not change appreciably with the type of suspension, the rate and extent of cell injury or death was greater at -4 C than -80 C (Tables I and II). Similarly, the extent of cell injury or death appears to be greater in certain types of suspension media, e.g., water and buffer as compared to glycerol, sucrose, and mashed potatoes for S. bovis, even though the rank order of recoverability was essentially unchanged.

DISCUSSION

Correct interpretation of data generated in food microbiology requires recognition of the fact that many of the treatments to which foods are subjected may result in sublethal injury to bacterial cells which may be contained therein. Although the effects of freezing on bacterial cells have been of interest since the early 1900s, food microbiologists have, until recently, chosen to ignore literature reports on this subject when developing methodology for the hygienic evaluation of food products.

Straka and Stokes (19) elucidated three conditions of bacterial cells following exposure to low temperatures. Injured cells were defined as those which grew on TSA and did not grow on a minimal medium after exposure to cold, while dead cells were those failing to grow on TSA. Unharmed cells were defined as cells which were able to grow on minimal media after low temperature storage. These definitions were expanded in a review of freeze-injury to bacterial cells by Ray and Speck (10). The freezing and thawing of bacterial cells result in a population containing injured and non-injured cells. Uninjured cells are those members of a population which are undamaged or considered normal. These cells are able to multiply and form colonies equally well on complete and minimal or selective and non-selective agar media. Following freezing and thawing, injury to bacterial cells may be classified as either non-lethal or lethal in nature. Lethal injury results in cells which are non-viable or incapable of forming colonies on a non-selective complete agar medium and can be expressed as:

$$\text{Percent dead} = \left(1 - \frac{\text{colony count after freezing}}{\text{colony count before freezing}}\right) \times 100$$

19. Straka, R.P., and Stokes, J.L., J Bacteriol. 78:1181, 1959

Non-lethal injury has been broken down into two categories: structural injury and metabolic injury. Metabolic injury is seen in those surviving cells which can form colonies on a non-selective complete agar medium but not on a minimal medium. Structural injury is exhibited by those surviving cells which are capable of forming colonies on a non-selective complete agar medium while being unable to form colonies on a selective complete agar medium. It can be expressed mathematically as:

$$\text{Percent structurally injured} = \left(1 - \frac{\text{colony count on selective medium}}{\text{colony count on non-selective medium}}\right) \times 100$$

The emphasis of this investigation centered around the occurrence of structural rather than metabolic injury following freezing and frozen storage since the standard methodology for enumeration of various groups of organisms contained within a food product revolves around the use of selective media and the pour plate technique.

Several investigators (10,20) have alluded to the greater resistance to freezing and frozen storage of the streptococci over coliform organisms. Yet no quantitative evaluation of this survival ability has been made for organisms other than E. coli. Our data indicate that the enterococcal portion of the Group D streptococci and E. coli survive freezing equally well; however, when comparisons are made of the ability to enumerate these organisms on selective media, the enterococci are far superior to E. coli. Therefore, E. coli must be more susceptible to structural injury due to frozen storage than the enterococcal portion of the Group D streptococci.

Acceptance or rejection of military subsistence is partially based on the coliform level of the product as determined by a plate count performed with the use of violet red bile agar. Analyses as described above are performed on a variety of frozen products including ice cream, TV dinners and meat and seafood products, as well as semipreserved foods which might include salads, cheese, sausage, sandwiches, refrigerated meat products, and spices. In all of these foods one may find coliform bacteria suffering cellular injury. Injury such as this must be taken into consideration when evaluation of these products is undertaken. It may be important that recovery of E. coli on selective media was particularly poor when stored frozen in mashed potatoes (Table II). Glycerol, sucrose, and buffer may have provided some cryoprotection to E. coli, but the mashed potato suspension would be expected to portray closely the effects to be found with food products in general. In a study of commercial ice cream samples, Ray and Speck (21) demonstrated that 73% of those ice creams tested met the customary

20. Woodburn, M.J., and Strong, D.H., Appl Microbiol. 8:109, 1960
21. Ray, R., and Speck, M.L., Abstr Ann Meeting Am Soc Microbiol. 1975

coliform limit of 10/g or less. When the analyses of these products were performed with a procedure including the cellular repair step, only 25% of the samples met the standard for acceptance.

In an examination of freeze injury to E. coli, Ray and Speck (11) demonstrated the inability to enumerate this organism by either the pour plate or the MPN techniques. Our results in this study are in agreement with these investigators. The ineffectiveness of current coliform analysis procedures with frozen products was further substantiated by Guthertz et al. (22) in an examination of frozen, comminuted turkey meat. It should be clear from these studies that coliform organisms, particularly E. coli, cannot be used as indicators of the hygienic quality in frozen products without development of analytical techniques allowing for recovery of injured cells.

Several research groups have conducted studies dealing with repair of injured organisms (9,12,17-19) following freezing and frozen storage of products and some general procedures for repair of cells in pure cultures have been proposed. However, since frozen food products generally contain mixed bacterial culture systems and organisms have been shown to repair at different rates, a single repair step before analysis of a frozen product does not appear to solve the problem. Repair procedures should not be ignored totally, but instead should be experimented with and incorporated into Salmonella analyses and analyses for other organisms of specific public health significance. Routine analyses for the determination of the hygienic quality of frozen products should use a group of organisms other than coliforms as indices.

Several conditions are known to influence viability in frozen bacteria. Among these are included temperature of frozen storage, length of frozen storage, concentration and age of cells when frozen, the use of cryoprotective agents, variation in bacterial strains, as well as the freezing and thawing processes used. However, the results of this study would suggest that the type of organism may be the most important factor in determining viability following frozen storage. Obviously, S. bovis is more susceptible to the lethal effects of frozen storage than the other organisms regardless of storage temperature or type of suspension (Tables I and IX). Similarly, E. coli is more sensitive to sublethal freezing injury than the other organisms regardless of storage temperature of suspension medium (Tables II and IX).

Though most investigations have been conducted with gram-negative bacterial species, it is generally accepted that the amount of storage death increases with the duration of storage and that initially the rate of death is rapid but levels off. Additionally, the lower the storage temperature and its fluctuation the slower is the rate of storage death. Storage death at temperatures below -60 C is generally low. Results of this investigation do not completely correlate with these generalities. When cells frozen and stored in water at -4, -20,

22. Guthertz, L.S. et al, J Food Sci. 42:1344, 1977

and -80 C were plated, little storage death was observed for S. faecalis, S. liquefaciens, S. faecium, S. durans, or E. coli irrespective of the storage temperature. This was not the case, however, with the non-enterococci S. bovis or S. equinus. As seen in Table I, these organisms show a continual decline in the cell population with storage with S. bovis declining in numbers faster than S. equinus. We did observe here that the lower the storage temperature, the less death occurred in the population, particularly with S. equinus. The sublethal injuries observed with E. coli (Table II) also increased with the time of storage and were more pronounced at -4 C than -80 C. A limited ability of glycerol, sucrose, and buffer to act as cryoprotective agents for sensitive organisms was also evident (Tables I and II). However, the cryoprotective effect was not observed with the mashed potato suspension, which most closely resembles a true food system.

RECOMMENDATIONS AND CONCLUSIONS

Based on the findings in this study, it can be concluded that E. coli, S. bovis, and S. equinus are poorly suited as hygienic indicators for frozen food products. S. faecalis, S. faecium, or S. durans due to their ability to survive frozen storage and be enumerated on selective media may be acceptable substitutes as hygienic indicator organisms. The following recommendations are made for further work in this regard:

1. The coliform analyses portion of military specifications for frozen food products should be reassessed and suspended if further studies show them to be inappropriate.

2. If E. coli is to remain the indicator organism of choice, analytical methodology for the selective repair of freeze injured organisms in mixed culture systems should be developed.

3. Analytical methodology for rapid differentiation within the Group D streptococci should be developed to speed the definitive identification of organisms within the group. Research should be conducted to develop appropriate guidelines for levels of Group D streptococci in frozen products.

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TABLE I: Percent recovery of original population on trypticase soy agar

1 Day Frozen Storage

	Streptococcus faecalis ATCC 19433	*Streptococcus liquefaciens* FRCC 274	*Streptococcus faecium* ATCC 19434	*Streptococcus faecium* var. *casseliflavus* ATCC 25789	*Streptococcus durans* ATCC 19432	*Streptococcus bovis* ATCC 9809	*Streptococcus equinus* ATCC 9812	*Escherichia coli* ATCC 11303
Water								
-4 C	99	95	100	99	99	61	99	98
-20 C	98	96	100	99	100	84	98	99
-80 C	99	96	100	99	101	87	100	99
Glycerol								
-4 C	99	99	100	99	100	98	99	99
-20 C	100	99	101	99	100	98	99	100
-80 C	99	98	101	99	102	99	100	99
Sucrose								
-4 C	100	93	97	99	101	92	101	98
-20 C	101	93	97	99	103	97	101	98
-80 C	101	93	98	100	103	96	102	99
Buffer								
-4 C	99	99	99	98	100	67	97	98
-20 C	98	100	99	99	101	81	95	98
-80 C	99	100	99	99	103	88	100	99
Mashed Potatoes								
-4 C	99	97	97	98	98	87	99	97
-20 C	99	97	97	99	100	98	99	98
-80 C	100	98	96	98	100	95	99	99

TABLE I: Percent recovery of original population on trypticase soy agar (Cont)

7 Days Frozen Storage

	Streptococcus faecalis ATCC 19433	*Streptococcus liquefaciens* FHCC 274	*Streptococcus faecium* ATCC 19434	*Streptococcus faecium* var. *casseliflavus* ATCC 25789	*Streptococcus durans* ATCC 19432	*Streptococcus bovis* ATCC 9809	*Streptococcus equinus* ATCC 9812	*Escherichia coli* ATCC 11303
Water								
-4 C	99	92	100	99	97	52	95	98
-20 C	98	91	100	98	99	83	92	99
-80 C	100	95	100	99	100	88	100	100
Glycerol								
-4 C	99	98	100	99	100	97	99	100
-20 C	100	98	101	98	100	97	99	99
-80 C	99	98	100	99	100	99	99	100
Sucrose								
-4 C	101	91	96	99	100	88	95	98
-20 C	100	95	97	99	102	95	101	99
-80 C	101	97	97	98	203	96	101	98
Buffer								
-4 C	100	100	97	99	100	56	92	99
-20 C	98	98	97	97	97	73	88	97
-80 C	100	99	100	99	102	85	98	99
Mashed Potatoes								
-4 C	99	97	97	98	97	76	97	96
-20 C	99	96	96	98	99	93	98	97
-80 C	99	96	97	99	99	96	99	98

TABLE I: Percent recovery of original population on trypticase soy agar (Cont)

30 Days Frozen Storage

	Streptococcus faecalis ATCC 19433	Streptococcus liquefaciens FHCC 274	Streptococcus faecium ATCC 19434	Streptococcus faecium var. casselliflavus ATCC 25789	Streptococcus durans ATCC 19432	Streptococcus bovis ATCC 9809	Streptococcus equinus ATCC 9812	Escherichia coli ATCC 11303
Water								
-4 C	97	91	99	99	95	35	95	98
-20 C	95	93	98	98	97	71	89	97
-80 C	99	95	100	99	100	88	100	99
Glycerol								
-4 C	95	98	101	97	98	89	93	100
-20 C	99	98	100	98	101	92	98	98
-80 C	99	99	101	99	100	99	99	99
Sucrose								
-4 C	99	94	96	97	97	84	80	98
-20 C	100	93	97	98	101	93	100	98
-80 C	101	92	97	98	102	95	101	98
Buffer								
-4 C	96	99	99	99	100	36	79	97
-20 C	94	97	96	96	97	53	83	94
-80 C	100	99	100	99	100	86	99	98
Mashed Potatoes								
-4 C	98	97	97	97	96	65	95	97
-20 C	99	96	97	98	100	89	98	97
-80 C	99	98	97	98	98	95	100	99

TABLE I: Percent recovery of original population on trypticase soy agar (Cont)

60 Days Frozen Storage

	Streptococcus faecalis ATCC 19433	*Streptococcus liquefaciens* FHCC 274	*Streptococcus faecium* ATCC 19434	*Streptococcus faecium* var. *casseliflavus* ATCC 25789	*Streptococcus durans* ATCC 19432	*Streptococcus bovis* ATCC 9809	*Streptococcus equinus* ATCC 9812	*Escherichia coli* ATCC 11303
Water								
-4 C	96	90	98	97	96	29	67	98
-20 C	96	01	97	97	98	73	85	96
-80 C	98	94	100	99	101	89	99	100
Glycerol								
-4 C	98	98	100	98	96	68	73	100
-20 C	98	99	101	97	101	94	98	99
-80 C	99	100	100	99	102	101	99	100
Sucrose								
-4 C	98	93	95	96	98	76	70	98
-20 C	99	92	96	99	101	95	98	99
-80 C	100	92	96	99	102	99	101	99
Buffer								
-4 C	95	98	98	97	98	39	65	97
-20 C	91	96	96	94	94	63	76	92
-80 C	99	99	99	98	101	88	99	99
Mashed Potatoes								
-4 C	98	98	96	97	94	59	90	97
-20 C	98	96	97	96	98	93	96	93
-80 C	99	97	96	98	100	98	99	98

TABLE I: Percent recovery of original population on trypticase soy agar (Cont)

180 Days Frozen Storage

	Streptococcus faecalis ATCC 19433	Streptococcus liquefaciens FHCC 274	Streptococcus faecium ATCC 19434	Streptococcus faecium var. casseliflavus ATCC 25789	Streptococcus durans ATCC 19432	Streptococcus bovis ATCC 9809	Streptococcus equinus ATCC 9812	Escherichia coli ATCC 11303
Water								
-4 C	98	88	99	97	93	19	18	97
-20 C	93	89	97	98	94	66	99	91
-80 C	99	94	100	99	101	87	99	100
Glycerol								
-4 C	90	97	99	96	51	0	4	98
-20 C	98	97	100	98	101	74	97	98
-80 C	100	97	100	99	101	99	100	100
Sucrose								
-4 C	98	91	96	95	98	50	29	98
-20 C	98	91	96	99	102	93	99	99
-80 C	100	91	97	99	105	96	100	99
Buffer								
-4 C	95	93	98	98	93	40	26	96
-20 C	91	91	96	93	90	45	76	86
-80 C	99	100	99	100	102	86	99	99
Mashed Potatoes								
-4 C	97	95	96	96	93	42	72	94
-20 C	97	94	96	97	98	87	95	94
-80 C	98	95	96	98	90	96	100	99

TABLE II: Percent recovery of original population on selective media[a,b]

1 Day Frozen Storage

	Streptococcus faecalis ATCC 19433	*Streptococcus liquefaciens* FHCC 274	*Streptococcus faecium* ATCC 19434	*Streptococcus faecium var. casseliflavus* ATCC 25789	*Streptococcus durans* ATCC 19432	*Streptococcus bovis* ATCC 9809	*Streptococcus equinus* ATCC 9812	*Escherichia coli* ATCC 11303
Water								
-4 C	101	99	99	99	96	56	93	35
-20 C	100	98	100	99	97	79	90	78
-80 C	101	99	100	99	98	82	100	77
Glycerol								
-4 C	100	93	100	99	98	99	99	86
-20 C	100	93	100	98	100	99	100	85
-80 C	100	91	99	99	100	99	99	86
Sucrose								
-4 C	101	98	93	100	99	90	93	70
-20 C	101	98	94	99	100	98	101	85
-80 C	101	97	95	100	101	91	102	86
Buffer								
-4 C	101	97	99	98	100	60	87	93
-20 C	100	98	100	98	100	78	86	91
-80 C	101	97	100	99	102	84	99	97
Mashed Potatoes								
-4 C	98	94	100	98	96	86	94	0
-20 C	99	94	100	98	97	96	97	64
-80 C	99	94	100	99	97	97	97	81

[a] Organisms 1 through 7 - selective medium = KF streptococcal agar

[b] Organism 8 - selective medium = violet red bile agar

TABLE II: Percent recovery of original population on selective media (Cont)

7 Days Frozen Storage

	Streptococcus faecalis ATCC 19433	*Streptococcus liquefaciens* FHCC 274	*Streptococcus faecium* ATCC 19434	*Streptococcus faecium* var. *casseliflavus* ATCC 25789	*Streptococcus durans* ATCC 19432	*Streptococcus bovis* ATCC 9809	*Streptococcus equinus* ATCC 9812	*Escherichia coli* ATCC 11303
Water								
-4 C	100	96	100	98	93	43	80	12
-20 C	99	97	99	98	95	75	87	52
-80 C	101	99	100	99	98	78	97	75
Glycerol								
-4 C	99	92	100	99	98	87	96	98
-20 C	100	91	101	97	99	86	97	71
-80 C	97	92	100	99	99	89	99	94
Sucrose								
-4 C	101	96	93	100	99	76	72	75
-20 C	100	98	93	99	100	87	97	85
-80 C	100	97	93	99	100	87	100	84
Buffer								
-4 C	100	97	97	98	98	43	64	79
-20 C	98	96	97	94	96	66	71	81
-80 C	100	97	101	99	102	80	83	100
Mashed Potatoes								
-4 C	98	94	99	98	95	73	93	16
-20 C	98	93	100	98	97	89	96	53
-80 C	98	93	100	98	97	93	98	66

TABLE II: Percent recovery of original population on selective media (Cont)

30 Days Frozen Storage

	Streptococcus faecalis ATCC 19433	Streptococcus liquefaciens FHCC 274	Streptococcus faecium ATCC 19434	Streptococcus faecium var. casseliflavus ATCC 25789	Streptococcus durans ATCC 19432	Streptococcus bovis ATCC 9809	Streptococcus equinus ATCC 9812	Escherichia coli ATCC 11303
Water								
-4 C	98	95	99	94	92	23	61	0
-20 C	98	96	98	98	94	61	85	42
-80 C	101	100	100	100	99	78	98	58
Glycerol								
-4 C	95	92	100	98	97	88	93	91
-20 C	100	93	99	98	100	91	100	90
-80 C	99	93	100	98	99	89	98	96
Sucrose								
-4 C	99	98	93	99	98	74	54	75
-20 C	100	98	93	99	100	97	67	84
-80 C	101	98	93	100	101	99	103	97
Buffer								
-4 C	99	96	100	96	98	27	48	72
-20 C	96	94	97	94	96	60	76	72
-80 C	100	99	100	99	101	80	94	94
Mashed Potatoes								
-4 C	98	94	100	97	94	68	92	13
-20 C	98	92	100	98	97	95	96	37
-80 C	98	93	100	97	95	100	98	61

TABLE II: Percent recovery of original population on selective media (Cont)

60 Days Frozen Storage

	Streptococcus faecalis ATCC 19433	Streptococcus liquefaciens FHCC 274	Streptococcus faecium ATCC 19434	Streptococcus faecium var. casseliflavus ATCC 25789	Streptococcus durans ATCC 19432	Streptococcus bovis ATCC 9809	Streptococcus equinus ATCC 9812	Escherichia coli ATCC 11303
Water								
-4 C	97	93	98	98	92	17	45	0
-20 C	97	95	97	98	94	68	82	27
-80 C	100	98	99	99	99	81	98	76
Glycerol								
-4 C	97	92	100	98	93	68	48	85
-20 C	100	91	100	99	100	84	95	72
-80 C	100	93	100	99	100	96	98	95
Sucrose								
-4 C	97	98	92	97	97	58	50	68
-20 C	99	98	93	100	99	89	91	73
-80 C	100	97	92	100	100	93	101	83
Buffer								
-4 C	95	95	97	94	95	15	36	55
-20 C	94	94	96	94	91	53	69	71
-80 C	101	98	100	99	100	81	97	98
Mashed Potatoes								
-4 C	97	95	99	97	93	59	85	4
-20 C	98	92	100	97	95	93	94	28
-80 C	99	95	100	98	97	97	98	56

TABLE II: Percent recovery of original population on selective media (Cont)

180 Days Frozen Storage

	Streptococcus faecalis ATCC 19433	*Streptococcus liquefaciens* FHCC 274	*Streptococcus faecium* ATCC 19434	*Streptococcus faecium* var. *casseliflavus* ATCC 25789	*Streptococcus durans* ATCC 19432	*Streptococcus bovis* ATCC 9809	*Streptococcus equinus* ATCC 9812	*Escherichia coli* ATCC 11303
Water								
-4 C	94	92	97	90	87	0	0	0
-20 C	95	93	96	97	91	54	93	22
-80 C	99	98	99	100	98	77	98	75
Glycerol								
-4 C	93	89	97	97	43	0	0	85
-20 C	96	89	99	98	98	53	79	79
-80 C	99	90	99	99	100	83	99	89
Sucrose								
-4 C	96	96	92	94	94	36	0	60
-20 C	98	97	93	100	99	77	85	84
-80 C	100	96	92	101	102	80	100	93
Buffer								
-4 C	100	88	97	98	79	17	10	31
-20 C	95	89	95	92	84	34	61	64
-80 C	99	96	99	99	101	76	82	102
Mashed Potatoes								
-4 C	96	92	98	97	91	33	57	0
-20 C	95	91	99	97	95	82	89	12
-80 C	97	94	99	98	97	97	96	63

TABLE III: Friedman rank sums based on percent recovery of organisms on trypticase soy agar

Organism	Friedman Rank Sum[1,2]
Streptococcus faecalis	413[a,b]
Streptococcus liquefaciens	251[c]
Streptococcus faecium	401.5[a,b]
Streptococcus faecium var. casseliflavus	362.5[b,c]
Streptococcus durans	490.5[a]
Streptococcus bovis	114.5[d]
Streptococcus equinus	307.5[b,c]
Escherichia coli	357.5[b,c]

[1] Sums with the same superscript are not significantly different ($P < 0.05$).

[2] The critical values for differences between Friedman rank sums used in determining significant differences were 125 for $P < 0.05$ and 137 for $P < 0.01$.

TABLE IV: Friedman rank sums based on percent recovery of organisms on selective media[1]

Organism	Friedman Rank Sums[2,3]
Streptococcus faecalis	504.5[a]
Streptococcus liquefaciens	307[b,c]
Streptococcus faecium	478.5[a]
Streptococcus faecium var. *casseliflavus*	438.5[a]
Streptococcus durans	409[a,b]
Streptococcus bovis	159[d,e]
Streptococcus equinus	267.5[c,d]
Escherichia coli	141[e]

[1] Selective media used were violet red bile agar for *E. coli* and KF streptococcal agar for all streptococci

[2] Sums with the same superscript are not significantly different ($P < 0.05$).

[3] The critical values for differences between Friedman rank sums used in determining significant differences were 125 for $P < 0.05$ and 137 for $P < 0.01$.

TABLE V: Friedman rank sums based on percent recovery of organisms on trypticase soy agar after frozen storage at -4 C, -20 C and -80 C

Organism	Friedman rank sum[1,2]		
	-4 C	-20 C	-80 C
Streptococcus faecalis	147.5[a]	137[a,b]	128.5[a,b]
Streptococcus liquefaciens	102.5[a,b]	82.5[c]	67[e,d]
Streptococcus faecium	150[a]	136[a,b]	115.5[b,c]
Streptococcus faecium var. casseliflavus	134.5[a]	127.5[a,b,c]	100.5[b,c,d]
Streptococcus durans	134[a]	176.5[a]	180[a]
Streptococcus bovis	30[c]	32.5[d]	52[d]
Streptococcus equinus	67.5[b,c]	104.5[b,c]	137.5[a,b]
Escherichia coli	132[a]	107.5[b,c]	118[b,c]

[1] Sums with the same superscript within each storage temperature grouping are not significantly different ($P < 0.05$).

[2] The critical values for differences between Friedman rank sums used in determining significant differences were 52.5 for $P < 0.05$ and 61 for $P < 0.01$.

TABLE VI: Friedman rank sums based on percent recovery of organisms on selective media after frozen storage at -4 C, -20 C and -80 C[1]

Organism	Friedman rank sums[2,3]		
	-4 C	-20 C	-80 C
Streptococcus faecalis	173[a]	169.5[a]	162[a]
Streptococcus liquefaciens	122[a]	101.5[b,c,d]	80.5[c,d]
Streptococcus faecium	168[a]	163.5[a]	147[a]
Streptococcus faecium var. casseliflavus	155[a]	148[a,b]	135.5[a,b]
Streptococcus durans	122.5[a]	138.5[a,b]	148[a]
Streptococcus bovis	48.5[b]	51.5[d,e]	57[d]
Streptococcus equinus	58.5[b]	90[c,d,e]	93[b,c,d]
Escherichia coli	52.5[b]	37.5[e]	51[d]

[1] Selective media used were violet red bile agar for E. coli and KF streptococcal agar for all streptococci

[2] Sums with the same superscript within each storage temperature grouping are not significantly different ($P < 0.05$).

[3] The critical values for differences between Friedman rank sums used in determining significant differences were 52.5 for $P < 0.05$ and 61 for $P < 0.01$.

TABLE VII: Friedman rank sums based on percent recovery of organisms on trypticase soy agar after frozen storage in various suspension media

Organism	Friedman rank sums[1,2]				
	Water	Glycerol	Sucrose	Buffer	Mashed Potatoes
Streptococcus faecalis	$73^{a,b,c}$	$65^{b,c,d}$	96.5^{a}	$72.5^{a,b,c}$	106^{a}
Streptococcus liquefaciens	$34^{c,d}$	$52^{c,d}$	25.5^{c}	$90.5^{a,b}$	$51^{b,c}$
Streptococcus faecium	106^{a}	108^{a}	$48^{b,c}$	$87^{a,b}$	$53.5^{b,c}$
Streptococcus faecium var. casseliflavus	$80.5^{a,b}$	$53^{c,d}$	$74^{a,b}$	$74^{a,b,c}$	$81^{a,b}$
Streptococcus durans	$90.5^{a,b}$	$100^{a,b}$	113^{a}	102^{a}	$85^{a,b}$
Streptococcus bovis	16^{d}	30^{d}	29.5^{c}	16^{d}	24^{c}
Streptococcus equinus	$61.5^{b,c}$	$51.5^{c,d}$	$77^{a,b}$	$40^{c,d}$	$77.5^{a,b}$
Escherichia coli	$78.5^{a,b}$	$81.5^{a,b,c}$	$76.5^{a,b}$	$59^{b,c}$	$62^{b,c}$

[1] Sums with the same superscript within each suspension grouping are not significantly different ($P < 0.037$).

[2] The critical values for differences between Friedman rank sums used in determining significant differences were 42 for $P < 0.037$, 43 for $P < 0.030$, and 47 for $P < 0.011$.

TABLE VIII: Friedman rank sums based on percent recovery of various organisms on selective media after frozen storage in various suspension media[1]

	Friedman rank sums[2,3]				
Organism	Water	Glycerol	Sucrose	Buffer	Mashed Potatoes
Streptococcus faecalis	108.5^{a}	$94^{a,b}$	103.5^{a}	103^{a}	$95.5^{a,b}$
Streptococcus liquefaciens	$76^{a,b,c}$	$42^{c,d}$	$73.5^{a,b}$	$66^{a,b}$	$46.5^{c,d}$
Streptococcus faecium	$104^{a,b}$	106.5^{a}	$46.5^{b,c}$	101.5^{a}	119.5^{a}
Streptococcus faecium var. casseliflavus	$94.5^{a,b}$	$81.5^{a,b,c}$	94^{a}	$75^{a,b}$	$92.5^{a,b}$
Streptococcus durans	$63^{b,c,d}$	$89.5^{a,b}$	97.5^{a}	94.5^{a}	$67.5^{b,c}$
Streptococcus bovis	$30^{d,e}$	33^{d}	$35.5^{b,c}$	16^{c}	$42.5^{c,d}$
Streptococcus equinus	$50.5^{c,d,e}$	$60.5^{b,c,d}$	$62.5^{a,b,c}$	$34^{b,c}$	$60^{b,c}$
Escherichia coli	16^{e}	33^{d}	27^{c}	$50^{b,c}$	15^{d}

[1] Selective media used were violet red bile agar for E. coli and KF streptococcal agar for all streptococci

[2] Sums with the same superscript within each suspension grouping are not significantly different ($P < 0.037$).

[3] The critical values for differences between Friedman rank sums used in determining significant differences were 42 for $P < 0.037$, 43 for $P < 0.030$, and 47 for $P < 0.011$.

TABLE IX: Rank order of recoverability of various organisms on trypticase soy agar or selective media after frozen storage[1]

Treatment	Rank order of recoverability[2]	
	Trypticase Soy Agar	Selective Media
All	6-2-7-8-4-3-2-5[3]	8-6-7-2-5-4-3-1
-4 C	6-7-2-8-5-4-1-3	6-8-7-2-5-4-3-1
-20 C	6-2-7-8-4-3-1-5	8-6-7-2-5-4-3-1
-80 C	6-2-4-3-8-1-7-5	8-6-2-7-4-3-5-1
Water	6-2-7-1-8-4-5-3	8-6-7-5-2-4-3-1
Glycerol	6-7-2-4-1-8-5-3	8-6-2-7-4-5-1-3
Sucrose	2-6-3-4-8-7-1-5	8-6-3-7-2-4-5-1
Buffer	6-7-8-1-4-3-2-5	6-7-8-2-4-5-3-1
Mashed Potatoes	6-2-3-8-7-4-5-1	8-6-2-7-5-4-1-3

[1] Selective media used were violet red bile agar for *E. coli* and KF streptococcal agar for all streptococci

[2] The organisms were ranked from the lowest to the highest percent recovery based on the Friedman rank sums presented in Tables III through VIII.

[3] Organisms were numbered in the following manner: 1 - *S. faecalis*, 2 - *S. liquefaciens*, 3 - *S. faecium*, 4 - *S. faecium* var. *casseliflavus*, 5 - *S. durans*, 6 - *S. bovis*, 7 - *S. equinus*, and 8 - *E. coli*.