The effect of cold stress on the recovery of coliform organisms from milk
by Gundlagutta Mahadeva Reddy

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Microbiology
Montana State University

Abstract:
The effect of "cold stress" on the recovery of coliform organisms was studied. Raw milk samples .containing natural coliform populations and sterile milk inoculated with pure cultures of Escherichia coli,, Aerobacter aerogenes and atypical coliform organisms were stored at various temperatures between -23 and 9 C. The results indicated: 1. A substantial reduction in numbers of coliform organisms in milk occurred when the samples were frozen at -23 C and stored at -15 C, Almost total destruction occurred when samples were pre-incubated, 2. A substantial decrease in coliform populations occurred at temperatures between -3 and 6 C. Destruction was greater when samples were pre-incubated, 3. The temperature of maximum stability of coliform populations during 4 days storage appears to be around 7 C.

4. Substantial increases in coliform numbers were observed at 8 and 9 C during 4 days, 5. Uncooled raw milk samples showed a decrease in coliform numbers after one day while pre-cooled samples did not.

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ABSTRACT

The effect of "cold stress" on the recovery of coliform organisms was studied. Raw milk samples containing natural coliform populations and sterile milk inoculated with pure cultures of Escherichia coli, Aerobacter aerogenes and atypical coliform organisms were stored at various temperatures between -23 and 9 C. The results indicated:

1. A substantial reduction in numbers of coliform organisms in milk occurred when the samples were frozen at -23 C and stored at -15 C. Almost total destruction occurred when samples were pre-incubated.

2. A substantial decrease in coliform populations occurred at temperatures between -3 and 6 C. Destruction was greater when samples were pre-incubated.

3. The temperature of maximum stability of coliform populations during 4 days storage appears to be around 7 C.

4. Substantial increases in coliform numbers were observed at 8 and 9 C during 4 days.

5. Uncooled raw milk samples showed a decrease in coliform numbers after one day while pre-cooled samples did not.

INTRODUCTION

Refrigeration is playing an ever increasing part in the preservation and handling of food supplies. For example, milk is almost always subjected to rapid cooling and is often stored at near freezing temperatures for extended periods of time.

Centralized processing and widespread distribution of foods has led public health officials, as well as industry, to rely, among other things, on compliance with microbial standards as indicators of sanitary conditions surrounding the production of many foods. Dairy products are good examples.

The coliform population of foods, especially milk, is a common sanitation indicator. The U. S. Public Health Service milk code and ordinance (43) sets a maximum of 10 coliform organisms per ml. for pasteurized milk. No standards for raw milk are included but the Montana Livestock Sanitary Board, which is responsible for milk sanitation in Montana, and many commercial dairy concerns use the coliform count as an indicator of raw milk quality. Standards vary, but coliform counts of more than 100 per ml. are generally considered to be evidence that better sanitary procedures should be employed.

Standard Methods for the Examination of Dairy Products (2), the recognized procedure upon which the U. S. Public Health Service milk code and ordinance sets its standards, states that milk samples should be cooled immediately after taking to 4 C or lower and stored at these temperatures until analyzed. It does not specify whether the samples shall be frozen. The U. S. Public Health Service publication "Examina-

tion of Foods for Enteropathogenic and Indicator Bacteria" (22) states:

> "Once a specimen has been placed in a sterilized container, it should, of course, be treated so that the bacterial content does not change." and further, "ideally, the food should be examined within an hour after sampling, but, where longer periods are unavoidable, prompt refrigeration is essential."

To avoid freezing, the specimen can be kept cold by packing in crushed ice.

In Montana, it is often necessary to hold samples taken for bacterial examination, for a period of time before shipping long distances. Sometimes it is further necessary to store the samples prior to analysis.

Sanitarians, while generally pleased with coliform counts as indicators of sanitary conditions, invariably report instances where the counts and visual inspection do not seem to be in agreement.

It was the objective of this study to: 1) evaluate the changes in the recoverable coliform organisms due to cooling and storage of milk samples at different refrigerator temperatures; 2) to determine a range in storage temperatures that will result in minimum change in the recoverable coliform organisms; and 3) to determine, if possible, whether various cooling and storage temperatures might be responsible for the occasional inconsistencies reported by sanitarians between coliform counts and visual inspections.

LITERATURE REVIEW

The coliform group. The coliform group of bacteria includes all aerobic and facultatively anaerobic gram-negative non-sporeforming rods capable of fermenting lactose with the production of acid and gas at 32 C. within 48 hr. (2).

To minimize potential hazards to public health and assure processing and handling of milk under sanitary conditions, bacterial indices are used. The coliform organisms are commonly employed in the dairy industry, as sanitary indicator organisms, because of their wide distribution, their destruction by pasteurization (7), and the availability of comparatively rapid and simple testing procedures.

Escherichia coli and Aerobacter aerogenes--the two main organisms in the coliform group, grow well in milk and ferment lactose rapidly. In absence of a fermentable carbohydrate, Escherichia coli may be mildly proteolytic and produce indol, whereas, Aerobacter aerogenes is unable to do this. Escherichia coli is found in manure, dirty utensils and soil and it always occurs in the intestinal tract of man and vertebrate animals. Aerobacter aerogenes is frequently found on grains and feeds, and also in the intestines of man and many warm blooded animals.

Most raw milk supplies harbor these organisms, which, owing to their unrestricted occurrence, are not necessarily of fecal origin. Since they invariably are destroyed by proper pasteurization, their presence in heat treated dairy products signifies either inadequate pasteurization or post-pasteurization contamination (7).

The coliform group is also responsible for producing gas and a number of flavor defects in milk and milk products. Aerobacter aerogenes may cause ropiness in milk (7).

Media for coliform bacteria. Several liquid and solid media are available for the detection and enumeration of coliform organisms. Lactose broth, lauryl sulfate tryptose broth and brilliant green lactose broth are commonly used as liquid media. The first is the least inhibitory and the last is the most inhibitory (18, 32, 46). Among the solid media, violet red bile agar (VRB agar) and desoxycholate lactose agar are generally used. Kereluk and Gunderson (19) reported that VRB agar and desoxycholate lactose agar gave comparable results. Silverman et al. (37) and Nickerson (31) found that desoxycholate lactose agar gave excellent results. Yale (47) reported desoxycholate lactose agar gave the best results among ten media studied, including VRB.

Deep red colonies 0.5 mm. or larger on VRB and desoxycholate lactose agar are considered coliform organisms (2). In addition to the coliform group, Proteus, Alcaligenes and Achromobacter species produce small, atypical red colonies (11).

The confirmation and differentiation of the coliform group is based on a series of physiological tests known as IMViC reactions. These letters represent indole production, methyl red reaction, Voges-Proskauer reaction, and citrate utilization respectively. Lewis and Angelotti (22) gave the following classification:

Table I. Types or varieties of coliform organisms

Organism	Indole	Methyl Red	Voges-Proskauer	Citrate
Escherichia coli:				
Variety I	+	+	-	-
Variety II	-	+	-	-
Escherichia freundii:				
Variety I	-	+	-	±
Variety II	+	+	-	+
Aerobacter aerogenes:				
Variety I	-	-	+	±
Variety II	±	-	+	+

Cold and heat stressing. Considerable work has been done on the effect of "heat stressing" on the recovery of microorganisms (15), whereas, little information can be found on the influence of "cold stressing" on the recovery of microorganisms except where the cold stress involves freezing of the product (5).

Kereluk and Gunderson (20) inoculated E. coli into chicken gravy and stored it at -21 C. The number of organisms recovered was reduced from 5,600,000 per ml. to 66 per ml. in 481 days. Larkin et al. (21) inoculated E. coli into green beans and found a significant decrease in the numbers recovered when stored at -18 C. for several weeks. Fitzerald (6) found a marked decrease in numbers of E. coli in frozen

pork stored at -4 C. Hilliard and Davis (13) concluded that, with freezing temperatures, there is much less destruction of bacteria in milk or cream than in pure tap water. They attributed this to the physical protection of the bacteria by the colloidal and solid matter in suspension.

Weiser and Osterud (44) reported there are two kinds of bacterial death that occur due to freezing. These are: 1) freezing effect, which is the result of freezing itself, and 2) storage effect, which is a slow but continual attrition of cells when held in the frozen state.

Sedgwick and Winslow (34) compared the death rates of typhoid organisms suspended in tap water at 0 C. with similar suspensions held at 1 C. and found the reduction of viable organisms to be essentially the same in each case. They concluded that the process of destruction at low temperatures was continuous above and below the freezing point, depending upon the factors of time and temperature.

Borgstrom (3) reported that at temperatures where ice is formed in the suspending liquid, a certain proportion of microorganisms in the suspension is always killed, irrespective of the rate at which the suspension is frozen. There is some controversy, however, as to whether ice formation takes place in bacterial cells, and if it does, what its role is in microbial death during freezing (3). Luyet (24) found no ice particles in frozen staphylococci. Majur (26) found no intracellular ice formation in Saccharomyces cerevisiae when frozen slowly, whereas, on rapid freezing, there was evidence of ice formation within the cell.

Keith (17) suggested that microbial death during freezing was due to an enormous increase in the concentration of dissolved solutes in the unfrozen portion of the medium and the consequent rapid loss of water from the cells by osmosis. Mechanical crushing by the ice formed is held to be the cause of death by others (15, 26).

Lion et al. (23) and Dimmick et al. (4) considered free radical formation to be responsible for storage death. It is suggested that this may explain the lower death rate in cultures preserved by freeze drying. Measuring paramagnetic resonance, Lion et al. (23) found the approximate number of magnetic centers produced by oxygen in dried E. coli cells to be about 2×10^{16} spins per gram, or approximately 5,000 free radicals per dried cell. In Serratia marcescens, 200 free radicals were formed per cell (4). A correlation was indicated between the death process and the concentration of free radicals.

When bacteria are held in the frozen state, they continue to lose viability at a slow rate, which is dependent on the storage temperature. In general, the lower the holding temperature, the lower the rate of killing (15). There is some evidence that temperature ranges between -4 C. and -10 C. are the most lethal (5).

Ingraham (15) stated that the percentage of survival of a culture of bacteria when frozen and thawed is dependent, in a complex manner, on: 1) the susceptibility of the particular strain to death by freezing and the physiological state of the cells; 2) the rate of cooling; 3) the medium in which the cells are suspended; 4) the maximum temperature to

which the cells were exposed; 5) the period of time the cells are held in the frozen state; 6) the rate of subsequent warming; and 7) the medium on which the cells are plated to observe colony formation.

Straka and Stokes (39) termed "injured" cells incapable of growth on a simple medium but were capable of growth on a complex medium. Cells that grew on either medium were considered unharmed and those that could not develop on either, dead.

There are conflicting claims regarding the relative susceptibilities of microorganisms to death by freezing. Haines (9) found that Pseudomonas aeruginosa, Achromobacter, E. coli and Staphylococcus varied in susceptibility to death by freezing; the first being the most susceptible and the last, the least. Straka and Stokes (39) found very little difference among the susceptibilities of P. fluorescens, P. ovalis, P. geniculata and E. coli. It is generally agreed (3) that yeasts and molds are less susceptible than bacteria, and that psychrophiles and mesophiles are equally susceptible. Gram-positive species are more resistant to cold temperatures than gram-negative species (10, 25) and spores are more resistant than vegetative species (3).

The effect of the rate of freezing on death seems to be a minor one (15). Also, there is some controversy about the specific effect of fluctuating temperature on microorganisms in the freezing temperature range (3). If the fluctuating temperature passes through the thawing point of the product, it might be a decisive factor, but in most systems freezing and thawing are not clearly defined and a definite freezing

point is hard to locate (3). Whether the microbial cells are frozen is doubtful (3).

It has been reported that non-sporeforming bacteria are the most sensitive to temperature drops (42) during the logarithmic phase while they show little susceptibility during the lag and the intermediary phases (12). The longer the culture is held at 0 C. the greater is its susceptibility to low temperatures and the greater the increase in its sensitivity (12). This would imply that cooling of foods prior to freezing would induce a greater killing effect.

Adaptation to a new temperature range. The lowest temperature for bacterial growth seems to be in the vicinity of -10 C. (14). The optimum temperature for growth is not changed by cultivating at temperatures above or below the optimum. For example, Jennison (16) found no significant changes in the generation times of E. coli, A. aerogenes, Serratia marscescens, and Chromobacter violaceum. The generation time at a given temperature was the same whether the cultures used for inoculation were first subcultured for several weeks at room temperature (22 C.) or at 22, 27, 32, 37 or 42 C. Numerous other investigators have been unable to change the growth temperature ranges (15) by: 1) serial transfer at the extremes of the growth temperature ranges; 2) brief exposures to temperatures above or below the range; or 3) exposure to high osmotic pressures. However, Allen (1) found a decrease in the minimum temperature of aerobic sporeformers on continued laboratory culture. Ingraham (15) concluded that, in general, the temperature range for growth of an

organism is not readily changeable.

Effect on metabolism. A number of individual metabolic activities of microorganisms are influenced by low temperatures and in many instances the effects are different from those on growth (5). Weiser (45) stated that most metabolic activities are greatly slowed at low temperatures. Thiel (41) showed an increase in the ratio of acetic acid formed to the sugar utilized as the incubation temperature is lowered. Spores of many bacilli germinate at temperatures as low as 5 C. (30).

Less is known about the effects of low temperature on microorganisms than about the effects of heat, perhaps because of the shorter commercial history for freezing (3).

In a review of low temperature microbiology, Farrell and Rose (5) point out that comparatively little data has been reported on the effects of cooling mesophilic microorganisms to temperatures below the minimum for growth but above the freezing point of the suspending liquid.

Borgstrom (3) surmised whether some microorganisms, like some higher plants, have critical temperature levels which cause death far above freezing.

Marth and Frazier (28) reported that the predominant bacteria in raw milks held at farm bulk cooling tank temperatures were gram-negative rods of the following genera: Achromobacter, Aerobacter, Alkaligenes, Flavobacterium and Pseudomonas. When inoculated into raw milk and held at 3 C. for four days, most of the Pseudomonas cultures grew rapidly and steadily for the first two days but more slowly during

the last two days. The Achromobacter and Alcaligenes cultures grew rapidly for the first three days and more slowly during the fourth, while the Aerobacter culture grew the first day, then stopped for two days and resumed growth again. Flavobacterium cultures failed to grow appreciably.

Cold shock. First reported by Sherman and Albus (35), cold shock is the phenomenon by which certain bacteria can be killed by sudden exposures to low temperatures, although no freezing occurs. According to Meynell (29), cells must be in the logarithmic phase of growth to be susceptible to killing by cold shock and the degree of killing is dependent on the rate of chilling, as well as the composition of the medium in which they are chilled. Strange and Dark (40) found that loss of viability was accompanied by release from the bacteria of endogenous solutes including nucleotides, amino acids and adenosine triphosphate. The magnitude of this effect depended on the density of the suspension, the nature of the diluent, the rate of cooling and the temperature employed. Loss of viability was prevented by including either magnesium ions or filtrate from a suspension of shocked organisms in the suspending liquid. Thus, the effect is mainly exerted on the cell membrane (40).

Cold shock has been reported for E. coli (12, 29, 35, 36, 45), A. aerogenes (40) and P. aeruginosa (8) among the mesophilic, gram-negative organisms. Ninety-five percent of the young cells of E. coli were killed by rapid cooling from 45 to 10 C. (36, 45). Farrell and

Rose (5) reported that a psychrophilic strain of Pseudomonas exhibited cold shock when a suspension of organisms grown at 30 C. was cooled to 0 C.

Gram-positive organisms have also been shown to display some aspects of cold shock. Marshall et al. (27) reported 52% decrease in colony forming units of Staphylococcus aureus inoculated into sterile NFDM (10%) and stored at 4 C. for 5 days. Ring (33) showed that the permeability of Streptomyces hydrogenans increases rapidly on cooling a suspension from 30 to 0 C. This effect is reversed on returning to 30 C. Ring (33), Meynell (29) and Strange and Dark (40) attributed the lethal shock to an irreversible depletion of cellular constituents, due to an intensively increased permeability.

EXPERIMENTAL PROCEDURE

Determination of coliform populations. Raw milk samples containing natural coliform populations or sterile milk inoculated with pure cultures of coliform organisms were used to determine the effect of cold stress on coliforms in milk. Coliform populations were determined by the plate count method according to the procedures described in the Standard Methods for the Examination of Dairy Products (2).

One ml. and 0.1 ml. quantities of milk (diluted in 99 ml. phosphate buffered water blanks where needed) were pipetted into five plates each and poured with desoxycholate lactose agar or violet red bile agar. After incubation for 24 hr. at 32 C. the plates were counted using a Quebec colony counter. All the colonies in case of pure cultures and only typical coliform colonies (2) in case of raw milk samples were counted. An arithmetic average of counts from either five or ten plates was calculated.

Subdivision, storage, and pre-incubation. After preparing plates, milk was pipetted using 11 ml. sterile pipettes into a series of 6 oz. sterile plastic bags (Nasco, Fort Atkinson, Wisconsin). These were placed at various storage temperatures. Some samples were incubated at 32 C. in plastic bags for various periods before transfer to storage stations to increase the count and to adjust the stage of growth.

The following day, after counting the initial plates, one bag for each culture and treatment was withdrawn from the storage stations. The bags were shaken in a reciprocating type mechanical shaker for one

minute (150 oscillations) to mix the contents thoroughly and to determine the coliform populations. This procedure was repeated for four or five days, using a separate unopened sample bag for each determination.

Collection of raw milk samples. Uncooled raw milk samples were collected from the milk parlor pipe line of Montana State University dairy farm in sterile containers. Each sample was immediately transported to the laboratory and treated as described above. Some samples of cooled stored raw milk were obtained from the bulk tank at the pilot plant of the University. These samples usually consisted of a mixture of six milkings rapidly cooled and stored at about 2 C.

Isolation of pure coliform cultures from raw milk. To determine the nature of coliforms occurring in raw milk and to obtain pure cultures for use as test organisms, 50 coliform cultures were isolated as follows: Raw milk was plated using desoxycholate lactose agar and incubated for 24 hr. at 32 C. All the typical colonies from six plates (2 plates each from 3 different days) were picked and transferred to lactose broth. After incubation for 24 hr. the cultures were gram stained and only those which contained gram negative rods were streaked on trypticase soy agar (TSA agar) plates. After incubation, well isolated colonies were picked and streaked on TSA agar slants, which were then incubated and stored at about 4 C. Fresh cultures were prepared from these stock cultures for all subsequent testing.

Gas production in brilliant green lactose bile broth (BGB broth),

appearance on eosin methylene blue agar (EMB agar), indole production, methyl red reaction, Voges-Proskauer reaction and citrate utilization tests were performed on each culture according to the procedures outlined in the Manual of Microbiological Methods (38). The coliform cultures were then classified, using the key in Examination of Foods for Enteropathogenic and Indicator Bacteria (22). Those organisms which did not fall under E. coli or A. aerogenes will be referred to as "atypical".

Identity of test organisms. One culture of E. coli and one of A. aerogenes was obtained from the collection of the Department of Botany and Microbiology, Montana State University. These were designated as E. coli (MB) and A. aerogenes (MB). Eighteen of the 50 cultures isolated from raw milk were used and these were designated as: E. coli M6, M7, M26, M27, M34, M35, M42; A. aerogenes M13, M20, M21, M29, M30, M32; and atypical M15, M16, M23, M24, M39.

Inoculation of pure cultures. TSA broth cultures about 24 hr. old were diluted, using phosphate buffered water blanks (2), to obtain a final concentration, preferably not lower than 10 and not more than 100 organisms per ml., in sterile milk.

Temperature control. A series of refrigerators and cold rooms were used for obtaining various temperatures. These were thermostatically controlled. The sample bags were placed in water baths at all the above-freezing temperatures in order to minimize temperature changes in the samples. All of the thermometers used for determining the temperatures of the refrigerated areas were tested for accuracy. Temperatures

at all the stations were recorded twice daily during the experimental work. For convenience, the temperatures are shown as whole numbers (1 C., 2 C., etc.) but a range of about 1 C. on either side of the reported temperatures should be allowed for, considering the accuracy of the thermometers and the difficulty of maintaining temperatures.

RESULTS AND DISCUSSION

Preliminary investigations. In order to find the effect of cold stress on coliform bacteria some preliminary experiments were conducted on both natural populations in raw milk and pure cultures inoculated into sterile milk. These included: 1) evaluation of desoxycholate lactose agar and violet red bile agar for enumeration of cold stressed coliform organisms in milk, 2) determination of the relative populations of *E. coli*, *A. aerogenes* and atypical coliforms in raw milk so that pure culture studies could be conducted using comparable organisms, and 3) the effect of cooling and storing at low temperatures on the recovery of coliform in milk.

Slightly higher counts were obtained on desoxycholate lactose agar than on VRB agar. The arithmetic average of counts from 60 plates each, on the two media, were 960 and 880 respectively. These data are in agreement with other reports (31, 37, 47). Desoxycholate lactose agar also seemed to give better defined colonies and, therefore, was used in all further work.

To determine the nature of coliforms occurring in raw milk, 50 cultures were isolated by methods previously outlined. Their physiological reactions are shown in Table II.

Table II. Physiological reactions of 50 coliform cultures isolated from raw milk

Gas in BGB	Growth on EMB agar	Metallic sheen on EMB agar	Indol production	Methyl red reaction (positive)	Voges-Proskauer reaction (positive)	citrate utilization
50	50	29	10	39	11	20

The cultures were typed as follows, based on IMViC reactions and the key in Table I (see page 5).

Table III. Types of coliform organisms among 50 cultures isolated from raw milk

Type	Number of cultures
E. coli variety I	11
E. coli variety II	18
E. freundii variety I	10
A. aerogenes variety II	11

E. freundii is referred to as atypical in the tables and the text that follow.

E. coli, *A. aerogenes* and atypical coliform organisms are well represented in the raw milk, as can be expected, from the nature of contamination in commercial production of raw milk.

To determine the effect of low temperature storage on coliform populations, sterile milk samples inoculated with a pure culture of

E. coli were stored at -3, 0, 3, 7 and 9 C. The same samples pre-incubated for 4 hr. at 32 C. prior to storage were also stored at these temperatures. The results obtained are shown in Tables IV and V respectively.

At 7 C. there was only a small change in numbers during 4 days storage in both unincubated (25% decrease) and pre-incubated samples (30% increase). There was a 19-fold increase occurred in unincubated samples (Table IV) at 9 C. and appreciable decreases occurred at 3 C. or lower over a 5 day storage period. Similar changes occurred in pre-incubated samples (Table V).

Contrary to expectation, storage at -3 C. did not seem to be more lethal than at 0 or 3 C. in unincubated samples (Table IV). Numbers of *E. coli* decreased by 34% at -3 C. during 5 days storage, whereas the decreases were 55% at 0 C. and 53% at 3 C.

In incubated samples (Table V) decreases at 0 and 3 C. were less than at -3 C. on the 1st and 2nd days of storage but were more on the 4th and 5th days of storage.

In order to further investigate this point, experiments were carried out using pure cultures of *E. coli* and *A. aerogenes* at -3 and 1 C. The results are shown in Table VI. The same samples incubated for 3 hr. at 32 C. prior to storage at -3 and 1 C. gave the results shown in Table VII.

In unincubated samples (Table VI) the mean decrease during 4 days storage at -3 C. was 31%, whereas this was 60% at 1 C. In the

Table IV. Changes in numbers of E. coli (MB)* inoculated into sterile milk and stored at -3, 0, 3, 7 and 9 C.

Storage (days)	Organisms/ml. of milk				
	Temperature (C.)				
	-3	0	3	7	9
0	89	89	89	89	89
1	82	80	81	85	120
2	74	68	62	86	200
3	70	65	50	72	420
4	65	54	42	68	860
5	59	40	42	67	1,700

*Indicates culture obtained from the Department of Botany and Microbiology, Montana State University

Table V. Changes in numbers of E. coli (MB)* inoculated into sterile milk, pre-incubated for 4 hours at 32 C. and stored at -3, 0, 3, 7 and 9 C.

Storage	Organisms/ml. of milk				
	Temperature (C.)				
	-3	0	3	7	9
0	4,600	4,600	4,600	4,600	4,600
1	3,300	4,200	4,500	4,500	7,900
2	1,400	1,800	1,900	4,500	8,500
3	1,200	600	1,000	4,700	11,000
4	950	40	300	5,700	26,000
5	730	6	34	6,000	32,000

*Indicates culture obtained from the Department of Botany and Microbiology, Montana State University

pre-incubated samples (Table VII) the mean decrease was greater at -3 C. (95%) than at 1 C. (88%). Individual samples, however, varied from the general trends of greater decreases at 1 C. in Table VI and at -3 C. in Table VII--especially after one or two days storage.

Results in Tables IV, V and VI seem to indicate -3 C. as less lethal than 1 C. over a 4 or 5 day storage period. However, results in Table VII seem to show freezing at -3 C. to be somewhat more detrimental.

E. coli seems to be slightly less susceptible to death than A. aerogenes (Table VI). E. coli population was reduced 21% at -3 C., whereas A. aerogenes was reduced 40%. At 1 C. the reduction was 58% for E. coli and 60% for A. aerogenes.

At -3 C. milk samples are not frozen to hard solid but are soft and can be easily mashed by finger pressure. Borgstrom (3) pointed out that there is no fixed freezing point in biological materials. The protective action of milk colloids and solids (13), concentrated by partial removal of water as ice (17), may be the cause of higher recovery when frozen at -3 C., the destructive effect of freezing not having been manifested fully. The results may be due to both the rate of cooling and freezing (3) and the ratio of frozen to unfrozen milk. Another factor may be the uneven migration of bacteria into the unfrozen concentrate.

In all the incubated samples (Table VII) the counts were drastically reduced, reaching less than 1% of the original population at the end of the 4 day storage period. This effect is also apparent in Table V at 3 C. and below. These decreases are in agreement with the

Table VI. Changes in numbers of E. coli and A. aerogenes inoculated into sterile milk and stored at -3 C. and 1 C.

Storage (days)	Organisms/ml. of milk							
	-3 C.				1 C.			
	E. coli (MB)**		A. aerogenes (MB)		E. coli (MB)		A. aerogenes (MB)	
	1	2	1	2	1	2	1	2
0	20	44	26	17	20	44	26	17
1	20	42	25	15	20	39	22	9
2	19	39	22	13	17	33	16	9
3	18	39	17	9	15	23	12	7
4	18	39	12	5	13	14	10	7

* MB indicates culture obtained from the Department of Botany and Microbiology, Montana State University

** Results from 2 different runs

Table VII. Changes in numbers of E. coli and A. aerogenes inoculated into sterile milk, incubated for 3 hours at 32 C. and stored -3 C. and 1 C.

Storage (days)	Organisms/ml. of milk							
	-3 C.				1 C.			
	E. coli (MB)*		A. aerogenes (MB)		E. coli (MB)		A. aerogenes (MB)	
	1**	2	1	2	1	2	1	2
0	620	1,900	2,700	700	620	1,900	2,700	700
1	600	50	1,600	30	580	1,400	2,400	30
2	390	40	1,000	10	580	890	1,700	20
3	230	30	260	10	510	470	490	20
4	260	4	16	1	480	26	220	1

* MB indicates culture obtained from the Department of Botany and Microbiology, Montana State University

** Results from 2 different runs

reported greater sensitivity to temperature drops during the logarithmic phase (42).

Table VIII shows results obtained by freezing samples at -23 C. then stored at -15 C. Table IX gives results obtained from samples pre-incubated for 3 hr. at 32 C., frozen at -23 C. then stored at -15 C. Rapid freezing at -23 C. and storage at -15 C. appear to have an adverse effect on coliform population, both when pre-incubated and not pre-incubated (Tables VIII and IX). Almost total destruction occurred in the pre-incubated samples (Table IX). Early logarithmic phase of growth, in which these incubated cultures were, appears to have caused this destruction (42, 29, 35). The effects of cold shock, of freezing itself and storage at the low temperatures may together be responsible for this phenomenal decrease. The relative roles of these factors are, however, not known (3).

E. coli cultures appear to be slightly less susceptible to destruction as compared to A. aerogenes and atypical cultures. The means of the decreases in unincubated samples (Table VIII) were 40%, 66% and 69% respectively over a four day storage period.

Determination of the temperature of minimum change. From the results of preliminary experiments presented earlier, it was apparent that the temperature at which minimum change in coliform populations in milk occurs is above 3 C. and below 9 C. In order to increase the number of observations and arrive at a closer range, similar experiments,

Table VIII. Changes in numbers of coliform organisms inoculated into sterile milk, frozen at -23 C. and stored at -15 C.

Storage (days)	Organisms/ml. of milk						
	E. coli		A. aerogenes			Atypical	
	(MB)*	(M6)*	(MB)	(M20)	(M21)	(M23)	(M24)
0	38	24	26	30	31	34	20
1	27	26	11	15	20	14	9
2	20	16	15	15	12	11	11
3	20	14	10	12	15	11	9
4	22	15	9	11	11	9	8

*MB, M6 etc. designate the cultures used.

Table IX. Changes in numbers of coliform organisms inoculated into sterile milk, incubated for 3 hr. at 32 C., frozen at -23 C., and stored at -15 C.

Storage (days)	Organisms/ml. of milk				
	E. coli		A. aerogenes		Atypical
	(MB)*	(M6)	(MB)	(M21)	(M23)
0	660	580	1,000	600	300
1	4	2	1	1	1
2	2	1	1	1	1
3	2	<1	<1	<1	<1
4	1	<1	<1	<1	<1

*MB, M6, etc. designate the cultures used.

using temperatures between 3 and 9 C., were conducted.

Figure 1 shows the behaviour of populations of pure cultures of coliform organisms stored at 3, 6, 7, 8 and 9 C., plotted to the common base of 100 to give a better pictorial representation of the changes and to facilitate comparison. Each of the plotted values represents the average of several individual values (see appendix, Tables X through XIV). Plots of actual numbers without conversion to a common base are also included in the appendix. Tables X through XIV in the appendix show the individual behaviour of the different cultures.

The temperature of maximum stability of these coliform populations (pure cultures) appears to be around 7 C. (Figure 1). There was a destructive effect at 3 and 6 C. The destruction continued with increase in storage time. At 3 C. this amounted to only 80% recovery after 1 day and 40% after 4 days, while at 6 C. this was 85% and 57% respectively. At 7 C., where the temperature of maximum stability lies, 93% of the organisms could be recovered after 1 day. There was a decrease to 85% the 2nd day, followed by an increase to 89% after 3 days and 100% after 4 days. However, at 8 C. an increase of 18% occurred after 1 day and continued until the 4th day when it reached 57%. At 9 C. the increases are much higher (35 times the original number after 4 days).

Therefore, it appears that there is a range near 7 C. where coliform populations from pure cultures inocuated into sterile milk are most stable.

E. coli, A. aerogenes and atypical organisms showed slight