A STUDY OF COLIFORM ORGANISMS IN MILK

I. Incidence and selectivity of media
II. Percentage distribution of *Escherichia*, "Intermediates" and *Aerobacter*
III. Probable source

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TABLE OF CONTENTS

INTRODUCTION

A general study of the coliform group in raw and pasteurized milk has been made in order to elucidate the complex coliform problem in milk. Particular attention was given to pasteurized milk. Various liquid and solid media, especially the latter, have been evaluated with a view of obtaining the highest coliform count in the shortest possible time. The present standard procedures require two to three days before the results are available and by this time the milk has been distributed.

An attempt has also been made to ascertain the percentage distribution of different sections or subgroups (1) of the coliform group of bacteria, i.e. Escherichia coli, Aerobacter aerogenes and the "Intermediates", as identified by the IMViC tests. The cultures used were isolated from pour-plates at random, a procedure rarely practiced by other workers.

A new way of proving contamination after pasteurization has been tried, but only a few samples were examined and the results are tentative rather than final.

HISTORICAL BACKGROUND

The terms 'B. coli', 'Colon', 'Colon-aerogenes', 'E. coli', 'Coli-aerogenes', 'Escherichia-Aerobacter', and 'Coliform' are all synonymously used for the "coliform group" (2) of bacteria by different authors at different times. The Standard Methods for the Examination of Dairy Products (3) defines the coliform group as "aerobic and facultative anaerobic, gram-negative, non-sporeforming bacteria which ferment lactose with gas formation".

Since the discovery of Escherichia coli and Aerobacter aerogenes by Escherich in 1885, this group has been extensively studied in water and milk in Public Health Laboratories. Coliform organisms are present in practically all raw milk and may come from barnyard manure, soil, dust, rarely from the udder (4) (5) and very frequently from feed and utensils (6). From the public health point of view, the coliform test in milk does not have the same significance as in water (7).

The Milk Ordinance and Code (8), therefore, does not provide for the use of the coliform test. However, the American Association of Medical Milk Commissioners provides for the examination of certified milk and demands less than ten coliform organisms per ml. The coliform test as an index for judging conditions under which the milk was produced is regarded of little significance by many

workers (9) (10). The Standard Methods for the Examination of Dairy Products (11) regards the presence of both fecal and nonfecal coliform organisms in raw and pasteurized milk as direct evidence of insanitary dairy practices. The author fully agrees with this interpretation of the significance of coliform organisms.

The presence of coliform organisms in pasteurized milk is viewed rather differently from that in raw milk. It has been interpreted to indicate heat resistant strains, recontamination, inadequate pasteurization, higher initial coliform counts in the raw milk, and growth of the organisms in the bottle.

The survival of very small numbers of coliform organisms, especially E. coli, in pasteurized milk, has always been a highly controversial problem. Survey of the literature leaves one in doubt about the pertinence of some of the conclusions which have been reported.

One school of bacteriologists (12) (13) (14) (15) (16) observed the survival of certain strains of coliform organisms at 143°F for 30 minutes, and reported that the presence of coliform organisms in pasteurized milk is neither to be interpreted as an index of inadequate pasteurization nor of subsequent contamination (17). The other school of workers (18) (19) (7) (20) stressed that the coliform test in pasteurized milk be used as a reliable supplementary laboratory method for the control of proper pasteurization and plant hygiene. Some of the investigators

belonging to the first school did not pay any attention to the number of cells and their physiological state when seeding milk for laboratory pasteurization, whereas this is important in a heat resistance study.. Absence of coliform organisms does not necessarily indicate proper pasteurization or even pasteurization at all, but the presence of typical coliform bacteria in small volumes of pasteurized milk indicates some fault in pasteurization or plant samitation. Thus, the coliform test as an index of pasteurization is applicable only to those milks which contain coliform organisms before pasteurization (7) (21).

The initial concentration of coliform organisms in raw milk does have a bearing on the survival of some cells after pasteurization.. Craig (22) clearly demonstrated this point when he tested eight heat resistant strains each of Escherichia, "Intermediate" and Aerobacter and found that all were able to survive 143.5° F for 30 minutes in milk when present in sufficient numbers. No strain tested survived when the concentration before pasteurization was less than 700 coliform bacteria per ml.

Contamination after pasteurization rather than the survival of heat resistant strains is adequately proved by many investigators (23) (24) (25).. Barkworth (20) has suggested the Incubation Coliform Test (ICT) for samples from vats or different points in the line to control plant hygiene and locate the pockets of contamination..

Besides improper pasteurization, heat resistant strains, high initial coliform counts in milk before pasteurization, and recontamination, growth of the coliform bacteria in the bottle is an important factor which may increase the coliform count before the milk is actually consumed.. Morris (26) stated that coliform cultures grow more rapidly in pasteurized than in raw milk due to the bactericidal substances present in the latter.. Robinton (27) found more rapid coliform growth in cream at 46.2° F than in skim milk. Dahlberg (28) kept pasteurized milk, having a coliform count 0.02 per cent of the standard plate count, at 45 to 50° F for 4 days and found that the coliform count increased to 88 per cent of the standard plate count. This work stresses fully the significance of growth in the bottle..

Ever since the discovery of coliform organisms a persistent search for new presumptive media for their detection and enumeration has been in progress.. Many media have been elaborated and discarded, being either too inhibitory to the coliform group or giving too many false positives. Many investigators (29) (30) (7) (31) (32) (33) (34) (35) (36) (37) (38) (39) (40) (41) have studied, comparatively and individually, the different media and have preferred one or the other.. Wilson recommended McConkey's broth and agar which are the official media for the presumptive coliform test in milk in England. In the United States, the Standard Methods for the Examination of Dairy

Products (11) recommends brilliant green (lactose peptone) 2% bile broth and formate ricinoleate broth as liquid and desoxycholate agar and violet red agar as solid presumptive media for the coliform test in milk. In the present work the author has further studied all these media to re-valuate them for pasteurized and raw milk..

The classification of coliform bacteria has been very confusing because it is a large group of closely related, intergrading and somewhat unstable bacteria. Since McConkey (42) (43) biochemically divided the coliform organisms into four groups many workers (44) (45) (46) (47) (48) (49) (50) (51) have recommended different schemes of grouping and classification based on different biochemical reactions. Clark and Lubs (52) in Roger's laboratory developed the methyl red test and Levine (53) first emphasized the "inverse correlation" of the methyl red and Voges-Proskauer tests, thus dividing coliform organisms into Aerobacter (V-P +, MR -) and Escherichia (V-P -, MR +) sections.. For some years it was held well-nigh perfect until Stuart and coworkers (54) found that almost 10 per cent of their cultures did not show "inverse correlation".

Koser's (55) citrate test resulted in the development and recognition of another group of coliform organisms, the "Intermediates", which in Bergey's Manual of Determinative Bacteriology (56) is termed Escherichia freundii. Mitchell and Levine (57) and Vaughn and Levine (58), by using nucleic acid and its degradation products as the source of nitrogen

for coliform organisms, have reported additional evidence of the "Intermediates" as a separate section and have added a new species, Escherichia intermedium, besides E. freundii, to this section.

Parr (59) (60) (1) analyzed the data presented in papers published from 1924 to 1937 and suggested that the IMViC quartet of tests, being most frequently used , should in the future be employed for coliform classification. Stuart and coworkers (54) incorporated cellobiose with the IMViC tests.

The author agrees with Parr's classification (1) and has followed it in the present work to determine the percentage distribution of E. coli, A. aerogenes and "Intermediate" sections of the coliform group of bacteria in milk. The section, E. coli, is comprised of three IMViC types, ++--, -+-- and +---, the section, "Intermediate", of ten types, -+-+, +-+-, -+++-, +-++, ++-+, ----, ++++, -+++, +++- and +--+, and the section, A. aerogenes, of three types, --++, --+- and ---+..

EXPERIMENTAL PROCEDURES

Collection of Samples

Raw milk

Samples were drawn aseptically with a 10 ml sterile pipette from cans at the M.S.C. dairy barn one hour after milking.. The temperature of samples at the time of collection ranged from 50 to 55° F.. Samples were brought to the laboratory in ice-cooled containers and tested immedi-

ately. These samples represented a herd of 60 cows.

The other samples of raw milk from each farmer were collected at the M.S.C. creamery in the forenoon.. These samples were aseptically drawn from the weighing pan holding milk of one farmer at a time.. The samples collected were from 48 individual producers.

Bottled samples

One of the very first few bottles from a vat was collected from the M.S.C. creamery within 3 hours of pasteurization. These bottles were kept below 40° F in mechanically refrigerated rooms..

Bottles were also obtained from individual vats from four other creameries; namely, Lansing Farm Products, Heatherwood Farms, Arctic Dairy, and Lansing Dairy.. These bottles were collected in November and December within 4 to 6 hours after pasteurization.. No ice-cooled containers were used as the atmospheric temperature was below 40° F and the samples were tested immediately..

Vat samples

Four ounce bottles, the neck and stopper of each connected with a long string, were wrapped in paper and sterilized in the oven so that when samples were drawn from the vat no contamination could occur due to the bottles. These bottles were used in duplicate for samples from each vat.. Immediately after pasteurization (pasteurization temperature and time ranging from 143 to 146° F for 30 to 35 minutes) the bottles, suspended by the string,

were dipped into the pasteurized milk and withdrawn. They were stoppered, the outside sanitized by 200 ppm chlorine solution, and cooled to 98° F at which temperature one bottle of each sample was incubated.

Media and Reagents

The following dehydrated culture media from Difco Laboratories (61) were used and the directions of the manufacturers strictly followed.

1. Bacto-Brilliant Green Lactose Bile Broth
2. Bacto-Formate Ricinoleate Broth (71)
3. Bacto-Violet Red Agar
4. Bacto-Lactose Broth
5. Bacto-Tryptone Glucose Extract Agar (to which had been added 1% sterile skim milk)
6. Levine Eosin Methylene Blue Agar
7. Bacto-M.R.-V.P. Medium
8. Bacto-Koser Citrate Medium
9. Bacto-Tryptose Agar
10. Bacto-Tryptone

Dehydrated desoxycholate agar (62), prepared by the Baltimore Biological Laboratories, was dissolved by heating to the boiling point, then dispensed in 10 to 15 ml quantities in sterile test tubes, sterilized in flowing steam for 30 minutes (11), and stored. Before use, the tubes were just melted by heating 3 to 4 minutes in the Arnold steamer.

Lauryl sulphate tryptose broth (63) was prepared as

described in the Standard Methods for the Examination of Water and Sewage (64).

The phosphatase (field) test was performed according to the instructions supplied with the Phosphatase Field Test Kit. The tablets for the substrate and the BQC solution (2,6-dibromoquinone chlorimide) were obtained from the Applied Research Institute, New York.

The reagents for the Gram stain were prepared according to Hucker's modification described in the Manual of Methods for Pure Culture Study of Bacteria, Leaflet IV (65).

For the indol test reagent five grams of cp. paradimethyl aminobenzaldehyde was dissolved in 75 ml of amyl alcohol to which 25 ml of concentrated HCl was added. The reagent should have a yellow color.

For the methyl red indicator solution 0.1 gram of methyl red was dissolved in 300 ml of 95 per cent ethyl alcohol and diluted to 500 ml with distilled water.

For the Voges-Proskauer test reagents a 5 per cent alphanaphthol solution was made in absolute ethyl alcohol and a 40 per cent KOH solution in distilled water.

Methods

The procedures recommended in the Standard Methods for the Examination of Dairy Products (3) were followed in diluting samples and inoculating tubes and plates. Two solid media, desoxycholate agar and violet red agar, and three liquid media, brilliant green lactose bile broth, formate-

ricinoleate broth, and lauryl sulphate tryptose broth were employed for the coliform count and tryptone glucose extract milk agar for the standard plate count.. Controls were used for each medium and technique.

Raw and bottled samples

Raw milk dilutions, ranging from 1:10 to 1:10,000, in geometric series, were planted in broths (5 tubes for each dilution) and from 1 to 1:100 in solid media in the summer season, while dilutions from 1 to 1:1,000 in broth and 1 to 1:10 in solid media were used in fall and winter.. For the standard plate count dilutions 1:100, 1:1,000 and 1:10,000 were used.. Pasteurized milk was used in portions of 1 ml and 0.1 ml for broths, 1 ml and 2.5 ml for solid media, and dilutions of 1:100 and 1:1,000 for the standard plate count.

To prevent the formation of atypical coliform colonies, violet red agar and desoxycholate agar, after inoculation and solidification, were covered with 3 to 4 ml of the respective sterile agar.. Standard plates and broth tubes were incubated at 35 to 37° C for 48 hours while desoxycholate and violet red plates were incubated at 35 to 37° C for 20 to 24 hours..

Standard plate colonies were counted with the Quebec counter.. Typical dark red colonies, at least 0.5 mm in diameter, were counted on the desoxycholate and violet red plates.. Hoskin's table given in the Standard Methods for the Examination of Dairy Products (3) was used to

compute the "MPN" (Most Probable Number) in the broth tubes. Those broth tubes having less than 10 per cent gas were confirmed on eosin methylene blue agar and then the "MPN" was determined.

Vat samples

One of the duplicate bottles was used for the phosphatase field test (66). Each of 5 brilliant green lactose bile broth tubes was planted with 1 ml of milk for the initial coliform count and tryptone glucose extract milk agar plates with 1:100 and 1:1,000 were poured for the initial standard plate count.

The other bottle, containing about 100 ml of milk, was incubated at 35° C for 6 to 8 hours and then 4 plates of desoxycholate agar were seeded each with 2.5 ml of milk and 3 plates with 1 ml, 0.1 ml and 0.01 ml of milk. These plates were incubated at 37° C for 18 to 24 hours, the positive plates being used for purified culture isolation and typing as described below.

Isolation of Coliform Cultures

Purification

From presumptive positive desoxycholate and violet red plates having less than 10 typical deep red colonies, each colony was picked and transferred to individual brilliant green lactose bile broth tubes whereas from plates having a larger number of such colonies, 4 to 6 representative colonies were picked at random and transferred to individual brilliant green lactose bile broth

tubes.. The inoculated tubes were incubated at 37° C for 24 hours and a trace of each liquid culture was streaked with a bent needle on solid eosin methylene blue plates, which were incubated at 37° C for 24 hours.. A discrete colony from each plate was picked and transferred to an individual lactose broth tube which was incubated at 37° C and immediately after the appearance of gas was restreaked on eosin methylene blue agar plates as above. From each of these plates a discrete colony was picked and streaked on a tryptose agar slant and incubated for 24 hours at 37° C. This was the procedure followed throughout for the isolation and purification of cultures. These cultures were next subjected to gram staining for the completed coliform test.

Gram stain

Hucker's modification of the Gram stain was followed and the smears were stained as described in Leaflet IV of the Manual of Methods for Pure Culture Study (65). The cultures which were gram-negative, non-sporeforming rods were next typed.

Identification of Cultures

The IMViC reactions were utilized for the identification of coliform organisms (purified as described above) and 24 hour slope cultures were used. A trace of the culture was transferred with a needle into a tryptone broth tube, the needle was shaken a few times, and the same needle was next used to seed a M.R.-V.P. broth tube.

Then the needle was sterilized, cooled, and a trace of the same culture was used to seed two tubes, one of citrate and the other of M.R.-V.P. broth, care being taken to inoculate the citrate medium with very light inoculum so as not to carry any nutritive matter into the broth. The methyl red and Voges-Proskauer tubes were incubated at 30° C for 5 days and 24 to 48 hours respectively (67) (64). The tryptone broth and citrate tubes were incubated at 35 to 37° C for 24 hours and 72 hours respectively.

IMViC reactions

1. Indol differential test. To 5 ml of 24-hour tryptone broth coliform culture was added 0.2 to 0.3 ml of amyl alcohol indol reagent and shaken. A dark red color, developing on the surface within 10 minutes, constituted a positive test, the original color of the indol reagent a negative test, and the intermediate a questionable one.

2. Methyl red differential test. To 5 ml of a 5 day old M.R.-V.P. broth culture 5 drops of methyl red indicator solution were added. A distinct red color was recorded as positive and a distinct yellow color as negative.

3. Voges-Proskauer differential test. About 0.6 ml of 5 per cent alphanaphthol in absolute alcohol and 0.2 ml of 40 per cent KOH solution were added to 1 ml of 24 to 48 hour M.R.-V.P. broth culture and shaken until the white

precipitate just dissolved. The development of a crimson to ruby color within 2 to 4 hours was reported as positive and no color formation as negative.

4. Sodium citrate differential test. Koser's sodium citrate broth tubes were observed for growth after 72 to 96 hours incubation at 37° C. Visible growth was reported as positive and no growth as negative.

The results of these differential tests for each culture were recorded in the sequence of Indol, Methyl red, Voges-Proskauer, and Citrate test, together known as the IMViC tests. All coliform cultures thus were divided into three subgroups or sections: E. coli, "Intermediate", and A. aerogenes.

RESULTS

Raw Milk

Coliform and standard plate counts were determined for each of the 48 producers' milk supplied to the M.S.C. creamery during August, 1946. Fifty-six samples (16 from M.S.C. creamery producers and 40 from the M.S.C. dairy barn) were studied thoroughly in January, February, March, August, September and October, 1947. As the number of samples was not large, the results presented are only tentative.

Comparison of the coliform and standard plate counts

Brilliant green lactose bile broth, formate ricinoleate broth, desoxycholate agar and violet red agar were used for the coliform count whereas tryptone glucose extract

milk agar was utilized for the standard plate count. Lauryl sulphate tryptose broth (63) proved unsatisfactory (68) when inoculated with 1 ml milk and hence was rejected in further work. In higher dilutions it gave fairly comparable results.

The samples from the M.S.C. producers had very high coliform and standard plate counts. The M.S.C. dairy barn samples during the same season gave a much lower incidence of coliform and standard plate counts.

A general correlation was found between the coliform and the standard plate counts as shown in Graph I. To avoid plotting 88 samples on the graph, the arithmetic averages of the coliform and standard plate counts of about every 4 samples, the counts of which were in a certain range (i.e., coliform count from 100 to 1,000 and standard plate count 10,000 to 100,000), were plotted. This curve clearly shows that a higher coliform count is concurrent with a higher standard plate count and vice versa. The lower incidence of coliform and standard plate counts in M.S.C. dairy barn samples was due to clean production and lower keeping temperature of the milk.

The seasonal variations in coliform and standard plate counts in milk from the same producers, presented in table 1, show how sharply the coliform count falls in winter below that of the summer.

GRAPH I

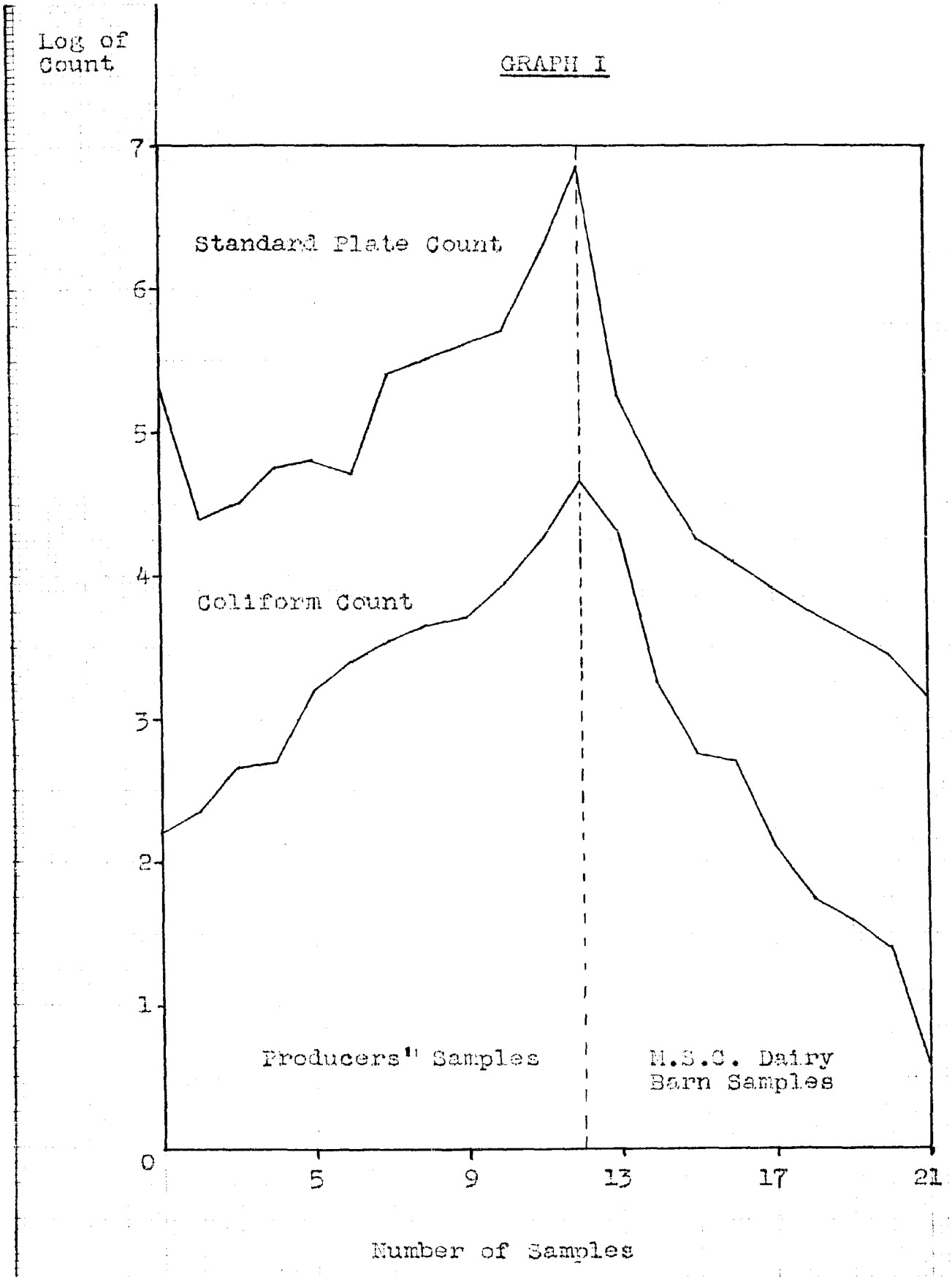

Log of Count
7
6
5
4
3
2
1
0
Standard Plate Count
Coliform Count
Producers' Samples
M.S.C. Dairy Barn Samples
5
9
13
17
21
Number of Samples

TABLE 1

Seasonal Variation of Coliform and Standard Plate Counts

Producer No.	Coliform Count per ml		S. P. Count per ml	
	Summer	Winter	Summer	Winter
1	18,000	3,500	810,000	100,000
2	2,400	900	30,000	65,000
5	2,800	12	135,000	63,000
8	79	0.5	176,000	8,000
9	4,900	4	236,000	17,000
13	1,600	4.5	172,000	1,100
18	150	2	90,000	20,000
30	600	8	20,000	4,800
59	6,350	130	658,000	81,000
61	79	5	250,000	8,000

Comparison of brilliant green lactose bile broth with desoxycholate agar

In comparing the various media for coliform counts one medium was arbitrarily selected as standard, the count by that medium was assigned a value of 100 per cent, and the counts on other media were compared for calculating efficiency. Regarding brilliant green lactose bile broth as a standard, desoxycholate agar was compared and its per cent efficiency calculated. Desoxycholate agar gave higher coliform counts in 26 samples and brilliant green lactose

bile broth in 22 samples while in 5 samples the counts were equal. Arithmetic average of the total coliform count of all samples of brilliant green lactose bile broth was higher than such an average of desoxycholate agar. If brilliant green lactose bile broth was 100 per cent efficient, desoxycholate agar proved 95.5 per cent efficient. The two media were very closely comparable as shown in table 2.

TABLE 2

Comparison of Standard Presumptive Liquid Media with Solid Media in Milk

Media	Number of Samples			Average Coliform Count per ml	Per cent Efficiency
	Total	Higher	Equal		
Brilliant green lactose bile broth	53	22	5	73.45	100
Desoxycholate agar		26		70.13	95.5
Formate ricinoleate broth	30	6	0	212.8	100
Violet red agar		24		333.6	160

Comparison of formate ricinoleate broth with violet red agar

Thirty samples of milk from M.S.C. creamery producers were tested and the coliform counts obtained by formate ricinoleate broth and violet red agar were compared exactly as above. When formate ricinoleate was regarded 100 per cent efficient, violet red agar was found 160 per cent efficient as shown in table 2.

From violet red plates representing 16 samples, 154 typical deep red colonies were subjected to the completed coliform test and it was revealed that only 85.2 per cent proved coliform positive while under similar experimental conditions desoxycholate agar gave 96 per cent coliform positive colonies. Hence, the higher coliform count obtained with violet red agar may be due to a higher number of false positives.

Percentage distribution of IMViC types and sections

Fifty-five samples were subjected to IMViC reactions. About 10 colonies, either from a desoxycholate or violet red plate, representative of a single sample were picked at random. Completed coliform tests were performed on 558 such colony cultures which had first been purified. Out of 16 possible IMViC types, 13 were identified. The average percentage distribution of these types and their per cent occurrence are given in table 3. For percentage distribution of sections as E. coli, "Intermediate", and A. aerogenes. Parr's (1) grouping of types is followed. It has been found that the percentage distribution of these

TABLE 3

Average Percentage Distribution of Various Types and Sections of Coliform Group Organisms Determined in 55 Milk Samples by IMViC Reactions

Cultures Typed	Coliform Section	IMViC Types	Per cent Occurence	Type %	Section %	% Non-inversely Correlated
558	*E. coli*	++--	54	20.27	23.52	
		-+--	13	3.25		
	"Intermediate"	-+-+	30	12.24	21.68	
		-++-	10	3.38		3.38
		++++	6	2.13		2.13
		++-+	4	1.90		
		-+++	8	0.70		0.70
		----	6	0.60		0.60
		+--+	5	0.55		0.55
		+-++	2	0.18		
	A. aerogenes	--++	85	47.46	54.80	4.39
		---+	25	4.39		
		--+-	13	2.95		
Total				100.00	100.00	11.75

three sections varies with the locality (69) and conditions of production at the farm and the handling and hauling of the milk. The average percentage distribution in 16 samples from M.S.C. creamery farmers was E. coli, 47 per cent; A. aerogenes, 12 per cent; and "Intermediate", 41 per cent while 39 samples from the M.S.C. dairy barn included E. coli, 16 per cent; A. aerogenes, 73 per cent; and "Intermediate", 11 per cent. These figures illustrate the previous statement.

Of the 13 types obtained, 6 showed no "inverse correlation" (53) of methyl red and Voges-Proskauer tests. These types represent about 12 per cent of the total type percentage.

Aberrant coliform organisms

The term "aberrant" has been used for the "slow lactose fermenters" as suggested by Stuart and coworkers (70) and is comprised of 4 types:

1. Microaerogenic coliform bacteria
2. Papilla-forming coliform bacteria
3. Pseudoaerogenic coliform bacteria
4. Anaerogenic coliform bacteria

An examination of 40 samples showed 18 samples positive for aberrant coliform bacteria. Their average percentage, determined from 40 samples, was 13 and composed of 47 out of 388 cultures. Only microaerogenic and anaerogenic types were isolated. The former, 4.5 per cent (15 cultures) and the latter, 8.5 per cent

(32 cultures). None of these aberrant forms belonged to the E. coli section on the basis of Parr's IMViC reactions.

Among the anaerogenic coliform bacteria the "Intermediate" type having the IMViC formula -++- was the most prevalent. Three chromogenic (yellow) cultures, with the IMViC formula -++-, were isolated from one sample. Yale (32) also found yellow cultures in his aberrant strains and classified them as Flavobacterium. Thirty anaerogenic cultures produced acid but no gas in lactose broth while two cultures produced neither acid nor gas. All these cultures were gram-negative, non-sporogenous rods, giving typical deep red colonies on desoxycholate or violet red agar. Coliform-like surface colonies, some with metallic sheen, appeared on eosin methylene blue plates.

The microaerogenic cultures produced a bubble of gas in lactose broth in 3 to 9 days and were white to pink on eosin methylene blue plates after 24 hours. Dark centers gradually developed concurrently with the development of gas in lactose broth tubes. These cultures belonged mostly to A. aerogenes, a few to "Intermediate", but none to E. coli.

Probable source of contamination

No definite source of contamination was ascertained. Aseptically drawn milk from 11 cows gave negative coliform results when 10 ml quantities were tested and all cows were free from any udder infection. Rinse water from cans, milking machines, pails, and swabs from the surface cooler

and milk tank were tested for coliform bacteria and proved negative except for one milking machine, having a 1.6 coliform count per ml of 500 ml rinse water. Milk samples were collected the same day from cans soon after milking and cooling, and were then tested immediately so as not to give any chance for growth of bacteria. Forty to 130 coliform bacteria per ml were found which on typing proved to be A. aerogenes or "Intermediate" but not E. coli, indicating non-fecal contamination. None of the 40 samples gave more than 50 per cent E. coli and the average distribution was only 16 per cent.

On the other hand, of 16 samples from M.S.C. creamery producers, 7 samples showed 67 to 100 per cent E. coli in January and February as shown in table 4.

TABLE 4

Predominant Distribution of E. coli in Certain Producers' Samples

Producer Number	Coliform Count per ml	Standard Plate Count	% E. coli (++--)	Cultures Typed
2	80	8,200	100	8
9	5	17,000	83	6
61	4	8,000	100	4
18	2	11,000	67	3
8	0.5	8,500	100	4
30	6	4,800	92	12
59	130	81,000	100	10

However, these coliform and standard plate counts were not high but the overwhelming prevalence of E. coli indicated fecal contamination.

Pasteurized Milk

One hundred and forty-seven bottled samples from 5 creameries were studied exhaustively during January, February, October, November and December, 1947. No ice containers were used due to the lower atmospheric temperature. Two standard liquid media, brilliant green lactose bile broth and formate ricinoleate broth, and two solid media, desoxycholate agar and violet red agar, recommended by the Standard Methods for the Examination of Dairy Products (3) were evaluated. Twenty to twenty-two ml (about 5 ml in each medium) of milk from each sample were tested for coliform organisms. Of 147 samples, 96 (about 65 per cent) proved positive by one or more of the media. This higher percentage of positives is partly due to the recovery of coliform organisms by different media and partly due to the larger quantities of milk tested. In some cases a small amount of growth of coliform bacteria in the bottle may have occurred.

The coliform count in positive samples, being very low as compared to that of raw milk, was determined per 100 ml.

Incidence of coliform organisms

The samples tested from Lansing creameries were sometimes 6 to 8 hours old and slight growth might have taken

place during this interval (28).. Bottles collected from the M.S.C. creamery consisted of one of the first few from each vat and it has been demonstrated that such bottles have a higher incidence of coliform organisms. Therefore, much importance should not be attached to the incidence of coliform organisms in these positive samples. Four samples out of 96 gave all positive broth tubes in the highest dilution and hence their counts could not be compared with those obtained from solid media.

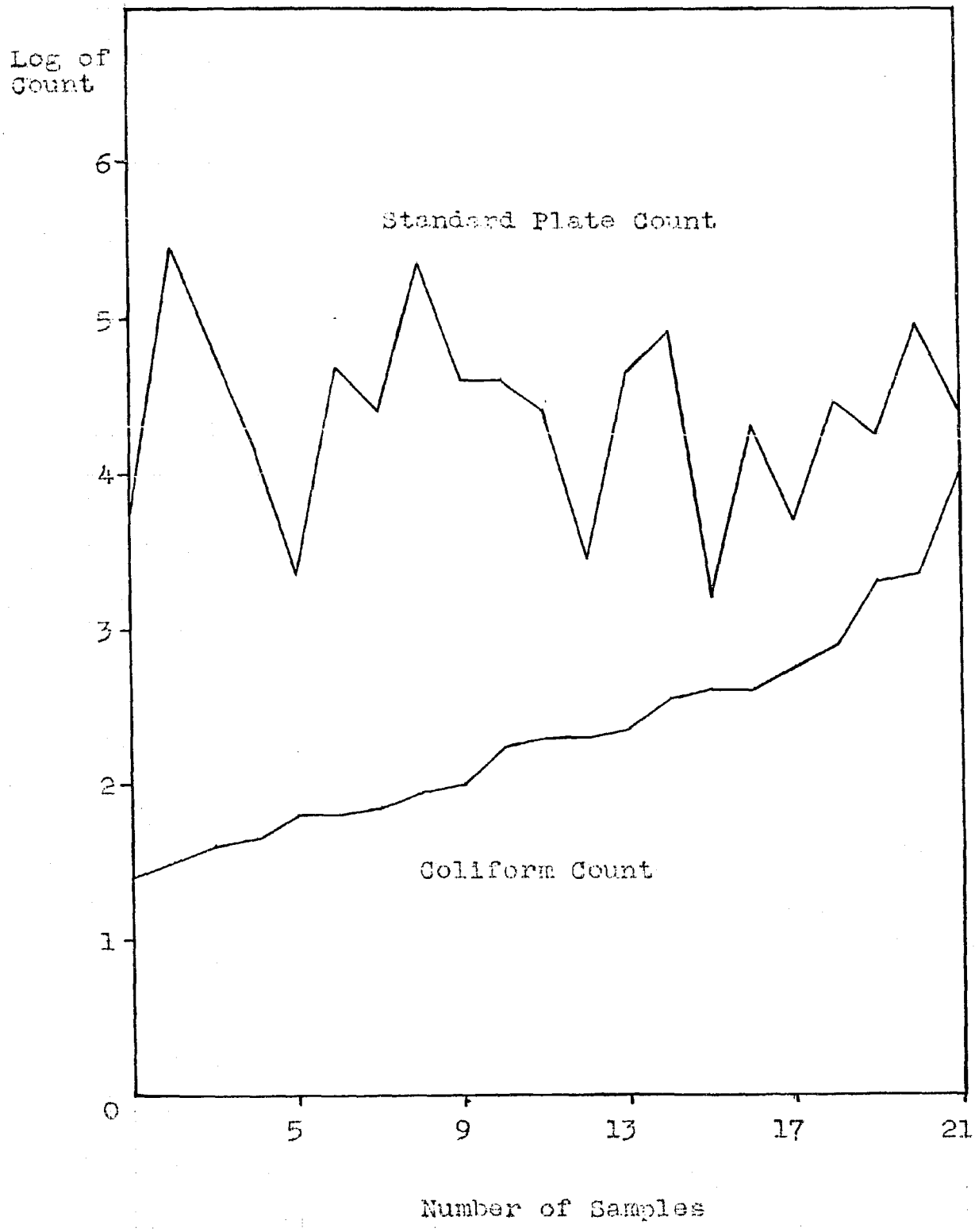
The coliform count ranged from 20 to 5,000 per 100 ml of which about 75 per cent of the positive samples gave a count below 500.

Comparison of coliform and standard plate counts

No definite correlation could be elicited from the coliform and standard plate counts. Coliform negative samples, in 20 ml portions, sometimes gave standard plate counts higher than 100,000 per ml, while many times, when the standard plate count was below 50,000 per ml, thousands of coliform organisms were present.. This is depicted in Graph II in which, to avoid plotting 96 samples on the graph, the arithmetic averages of the coliform and standard plate counts of about every 5 samples, the counts of which were in a certain range (e.g., coliform count from 100 to 1,000 and standard plate count 10,000 to 100,000), were plotted.

It is therefore concluded that the standard plate count of pasteurized milk is of little significance as an

GRAPH II


Log of Count
6
5
4
3
2
1
0
Standard Plate Count
Coliform Count
5
9
13
17
21
Number of Samples

index of improper pasteurization or recontamination. It has been found in the M.S.C. creamery that sometimes a higher incidence of thermoduric and thermophilic bacteria in raw milk results in a fairly high count in that milk after pasteurization although the milk proved negative to the coliform test.. This point contributes to the conclusion that there is no definite correlation between the coliform and standard plate counts. Such pasteurized milk, on the basis of the standard plate count, may wrongly be interpreted as recontaminated or improperly pasteurized..

Comparison of brilliant green lactose bile broth with two solid media

The coliform counts of 90 positive samples were compared with brilliant green lactose bile broth and desoxycholate agar. Of these, 52 samples gave higher counts in desoxycholate agar and 35 samples gave higher counts in brilliant green lactose bile broth while 3 samples had equal counts. The average brilliant green lactose bile broth coliform count and the desoxycholate agar coliform count were determined. Representing brilliant green lactose bile broth as 100 per cent efficient, the per cent efficiency of desoxycholate agar (180) was obtained.

Seventy-four positive samples were compared in brilliant green lactose bile broth and violet red agar. Twenty-five samples gave a higher coliform count in brilliant green lactose bile broth and 46 samples in

violet red agar while 3 samples gave equal counts. Assuming brilliant green lactose bile broth to be 100 per cent efficient, violet red agar proved 169.3 per cent efficient (see table 5, page 28).

Comparison of brilliant green lactose bile broth and formate ricinoleate broth

Fifty-one coliform positive samples were compared in the two broths, namely brilliant green lactose bile broth and formate ricinoleate broth. Twenty samples gave a higher coliform count in brilliant green lactose bile broth and 2 samples in formate ricinoleate broth while 10 samples had equal counts. These results were very closely comparable but the per cent efficiency of formate ricinoleate broth was 120.7 per cent (determined as above) when that of brilliant green lactose bile broth was 100. It was noticed that formate ricinoleate broth produced a more copious amount of gas than brilliant green lactose bile broth in the same length of time..

As some members of the Salmonella group give false positives (33) in formate ricinoleate broth, all coliform presumptive positive tubes of this medium, numbering 159, were confirmed by transferring 4 loopfuls from each tube into a brilliant green lactose bile broth tube and incubating the latter at 37° C for 48 hours. These brilliant green lactose bile broth tubes were all positive although some of them formed only a bubble of gas. From each of the tubes having a bubble of gas a trace of culture was

streaked on eosin methylene blue agar plates and produced typical coliform colonies showing thereby not a single false positive in formate ricinoleate broth.

TABLE 5

Comparison of Standard Liquid and Solid Media Used for the Presumptive Coliform Test in Milk

Media	No. of Samples			Average Coliform Count per 100 ml	Per cent Efficiency
	Total	Higher	Equal		
B.G.L.B. broth	51	20	10	172.1	100
Formate ricinoleate broth		21		207.6	120.7
B.G.L.B. broth	74	25	3	162.3	100
Violet red agar		46		274.8	169.3
B.G.L.B. broth	90	35	3	172.8	100
Desoxycholate agar		52		312.2	180
Violet red agar	74	24	18	274.8	100
Desoxycholate agar		32		326.7	119

Comparison of violet red agar and desoxycholate agar

During the study of these two media it was experienced that duplicate plates, each seeded with 1 ml of milk, when compared to duplicate plates, each seeded with 2.5 ml of milk, gave a lower coliform count per ml; for this reason 2.5 ml of milk per plate was used for solid media thereafter.

Of 74 samples compared, desoxycholate agar gave higher counts in 32 samples and violet red agar in 24 samples while 18 samples gave equal counts. When violet red agar was assumed to be 100 per cent efficient, desoxycholate agar proved 119 per cent efficient (see table 5). These results show that the two media are approximately equally superior to brilliant green lactose bile broth or formate ricinoleate broth, desoxycholate agar being slightly superior to violet red agar. In addition, desoxycholate agar has the advantage of producing more and larger typical dark red colonies.

Colonies picked by random selection from violet red agar plates representing 67 samples were transferred to brilliant green lactose bile broth and subjected to the Completed Coliform Test. It was found that of the 186 violet red agar colonies picked, 97 per cent were coliform cultures. Under similar conditions, 383 desoxycholate agar colonies, representative of 83 samples, after the Completed Test, gave 96.7 per cent coliform cultures. These results showed how few false positives these two

solid media produced.

One noticeable point was that colonies having less than 0.5 mm diameter (which the author calls pin point colonies) occurred very rarely in positive plates of both media. These pin point colonies will be described later.

A comparison of the detection of different IMViC types in 48 coliform positive samples was made with desoxycholate agar and violet red agar. About 3 colonies each from violet red agar and desoxycholate agar plates, of a positive sample, were picked at random and purified cultures were obtained from them. One hundred and thirty-one cultures from desoxycholate agar plates and 131 from violet red agar plates (representing 48 positive samples) were typed by IMViC reactions and 11 IMViC types were identified. A comparison of the numbers of cultures belonging to one type and recovered from each medium showed that practically all the 11 types were equally detectable on both media, excepting 2 "Intermediate" types, namely, +-++ and ++++, which were not at all detected by desoxycholate agar. The number of times one type occurred in 48 samples in one medium was also found comparable with that obtained on the other medium (see table 6). Thus it was concluded that desoxycholate agar and violet red agar, in general, are about equally good for growing coliform bacteria, when present in small numbers in pasteurized milk.

TABLE 6

Comparative Detection of IMViC Types in 48 Coliform Positive Samples by Violet Red Agar and Desoxycholate Agar

IMViC Section	IMViC Types	No. of Samples (out of 48) Positive on		No. of Cultures Obtained from	
		Des. agar	V-Red agar	Des. agar	V-Red agar
E. coli	++--	16	12	26	20
	-+--	6	6	9	6
"Intermediate"	-+-+	25	25	41	38
	+-++	0	3	0	3
	++-+	1	2	1	3
	++++	0	1	0	1
	----	2	1	4	1
	-+++	2	1	3	3
A. aerogenes	--++	21	23	32	39
	---+	10	13	11	15
	--+-	2	2	4	2
Total----------		85	89	131	131

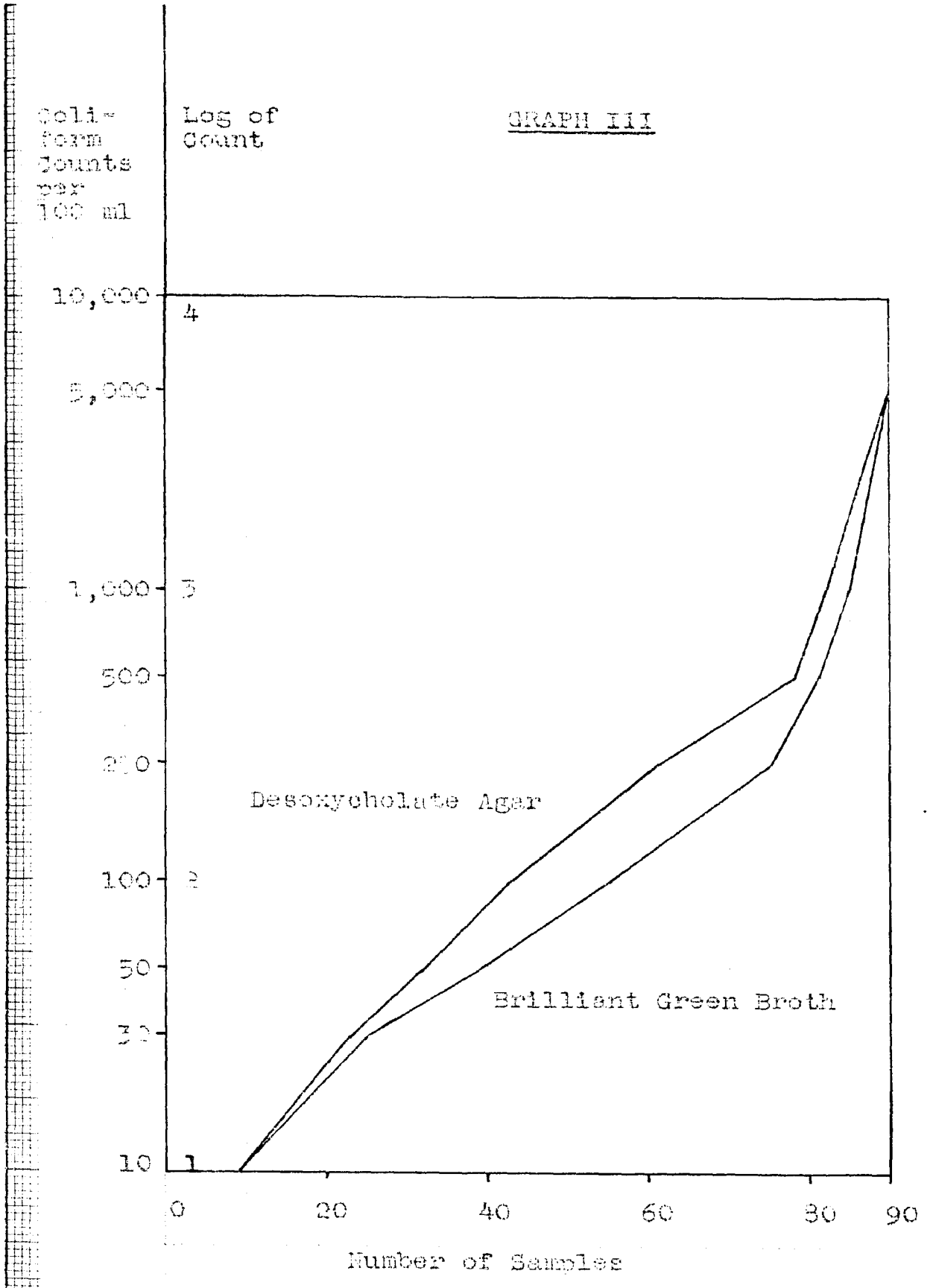
Superiority of desoxycholate agar over brilliant green lactose bile broth

All these comparisons of desoxycholate agar and violet red agar showed that there was little choice between the two media. However, desoxycholate agar, giving a higher per cent efficiency, and larger, typical, dark red colonies, was adopted as the best solid medium for the Presumptive Coliform Test.

A further comparison of desoxycholate agar with brilliant green lactose bile broth was thus made to determine the superiority of the former. Of 90 positive samples, the numbers of samples having coliform counts below 10, 30, 50, 100, 250, 500, 1000 and 5000 were determined in desoxycholate agar and brilliant green lactose bile broth. As desoxycholate agar gave a higher coliform count than brilliant green lactose bile broth in milk, it usually gave a smaller number of samples below a certain coliform count than did brilliant green lactose bile broth (see table 7, page 33).

Plotting the number of samples according to the group in which they occur, curves for brilliant green lactose bile broth and desoxycholate agar were obtained as shown in Graph III.

A study of these curves showed that the greater bulk of samples had coliform counts between 30 and 500 and it was in this range that desoxycholate agar proved far superior to brilliant green lactose bile broth. Below


Coli-form Counts per 100 ml
Log of Count
GRAPH III
10,000
5,000
1,000
500
100
50
10
4
3
2
1
Desoxycholate Agar
Brilliant Green Broth
0
20
40
60
80
90
Number of Samples

a coliform count of 30 and above a coliform count of 500, brilliant green lactose bile broth and desoxycholate agar tended to give parallel counts.

TABLE 7

The Numbers of Samples below Certain Coliform Counts as Obtained by Two Media

Coliform Positive Samples	Coliform Count Below	No. of Samples in	
		B.G.L.B. broth	Desoxy. agar
90	10	9	9
	30	25	23
	50	39	32
	100	55	43
	250	75	61
	500	81	78
	1,000	85	82
	5,000	90	90

Percentage distribution of IMViC types and sections

Of 92 positive samples, 445 coliform cultures were obtained. All these purified cultures had been derived from original, typical, dark red colonies selected at random from violet red agar and desoxycholate agar plates

and subjected to the Completed Coliform Test. The cultures were typed by IMViC reactions (59) which had been generally utilized by workers in the past two decades.

The per cent distribution of a type was determined by calculating the average of various percentages in which that type occurred in different samples. Eleven types and their respective percentages and the percentage of the coliform sections, viz. E. coli, "Intermediate" and A. aerogenes, to which those types belonged (1) are given in table 8.

The "Intermediate" having the IMViC formula -+-+ was the most prevalent type, 30 per cent of the cultures being of this type. In 1932, Werkman and Gillen (49) placed this organism in the genus Citrobacter and Bergey's Manual of Determinative Bacteriology (56) classifies it as Escherichia freundii. On eosin methylene blue agar plates this type gave colonies with a bright, metallic sheen and large, dark centers resembling very much the typical E. coli colonies. The highest percentage of the "Intermediate" section of coliform organisms in pasteurized milk was largely due to this coli-like type.

Four "Intermediate" types which showed no "inverse correlation" (53) of the Voges-Proskauer and methyl red tests, being either positive or negative to both, constituted 8.7 per cent of the total type percentages. They are expressed as non-inversely correlated "Intermediate" types in table 8.

TABLE 8

Percentage Distribution of Various Types and Sections of Coliform Bacteria in Pasteurized Milk

Positive Samples	Cultures Typed	Coliform Sections	IMViC Types	Type %	Section %	% Non-inversely correlated
92	445	<u>E. coli</u>	++--	20.2	27.6	
			-+--	7.4		
		"Intermediate"	-+-+	30.8	41.7	
			+-++	4.3		
			++-+	2.2		
			+++	1.5		1.5
			----	1.5		1.5
			-+++	1.4		1.4
		<u>A. aerogenes</u>	--++	25	30.7	4.3
			---+	4.3		
			--+-	1.4		
Total----------				100	100	8.7

The majority of the samples contained two or more IMViC types representing at least two sections. However, in 27 per cent of the samples only single types were identified. IMViC type -+-+ was present in 9.8 per cent of the samples, ++-- in 13 per cent of the samples and --++ in 4.3 per cent of the samples. Similarly, in 37 per cent of the samples only single coliform sections i.e. E. coli, "Intermediate", and A. aerogenes were found in 13 per cent, 18.5 per cent and 5.4 per cent of the samples respectively. In every respect the most prevalent type, as determined in 92 positive samples, was the "Intermediate" -+-+, next were types ++-- and --++ (see table 9, page 37).

The number of samples (out of 92) and the per cent of samples from which a certain type was isolated are shown on table 9.

Comparison of the percentage distribution of coliform sections determined by two methods

Method I

In this method, generally used by other workers, only the cultures representing definite coliform types present in a certain sample were used for the determination of percentage distribution of coliform sections. All the other cultures from the same sample, which on identification proved duplicates, were discarded. In all, 208 such cultures were derived after identification of 445 cultures from 92 positive samples. The percentage

distribution of various coliform sections was calculated by the following formula..

$$\% \text{ distribution of section} = \frac{\text{no. of cultures of section}}{\text{total cultures (208)}} \times 100$$

TABLE 9

Distribution of 11 IMViC Types in 92 Coliform Positive Samples

IMViC Types	Isolated from		% of Samples having 100 %	
	No. of Samples	% of Samples	One Type	One Section
E. coli				
++--	34	37	9.8	13
-+--	19	20.7	0	
"Intermediate"				
-+-+	55	60	13	18.5
+-++	8	8.7	0	
++-+	6	6.5	0	
++++	5	5.4	0	
----	4	4.3	0	
-+++	4	4.3	0	
A. aerogenes				
--++	48	52	4.3	5.4
---+	19	20.7	0	
--+-	6	6.5	0	
Total	208		27.1	36.9

Method II

In the second method employed in this work, 4 to 6 (an arbitrary number) typical coliform colonies were selected at random from 4 violet red agar and desoxycholate agar plates representing 1 sample. Purified coliform cultures, obtained from these colonies and confirmed by the Completed Test, were typed by IMViC tests and the percentage distribution of different coliform sections in that sample was determined. Ninety-two samples were examined as above and the average percentage for each section was determined.

The percentages determined by the two methods are compared in table 10. The second method gives a much more accurate picture of the percentage distribution of coliform sections and is recommended.

TABLE 10

Comparison of Average Percentage Distribution of Coliform Sections by Two Methods

Sections	Average % Distribution Determined by	
	Method I (208 cultures)	Method II (445 cultures)
E. coli	25.5	27.6
"Intermediate"	39.4	41.7
A. aerogenes	35.1	30.7
Total	100	100

Pin point colonies

Pink colonies, 20 to 24 hours old and 0.5 mm or less in diameter on violet red agar or desoxycholate agar, are designated as pin point colonies. These colonies did not characteristically precipitate bile salts as is so commonly done by larger colonies..

Of thousands of colonies on 368 violet red agar and desoxycholate agar plates representing 92 samples, only 39 were pin point colonies. Of these 39 colonies isolated from 13 samples, 28 belonged to the microaerogenic type of aberrant coliform bacteria (70) as they formed a bubble of gas within 3 to 9 days in lactose broth, while 11 colonies did not produce any gas in 14 days.

No further study was made because of the insignificantly low incidence of these colonies on desoxycholate or violet red agar plates seeded with pasteurized milk.

The source of coliform organisms in pasteurized milk will be discussed under Vat Samples.

Vat Samples

An examination of 26 duplicate samples, drawn directly from vats in the M.S.C. creamery, was made in October, 1947. Routine bottled samples of pasteurized milk, numbering 23, were also collected in that month from the same creamery and tested. Five of these bottled samples corresponded to 5 vat samples and will be discussed later.

The phosphatase field test was read on all the 26 samples, of which 21 produced upto 2 phosphatase units,

3 produced 2 to 5 units, and 2 produced 50 to 100 units respectively. The last 2 samples were not properly pasteurized as indicated by the phosphatase test and the initial coliform count. Two samples which gave 2 to 5 phosphatase units were positive in brilliant green lactose bile broth before incubation of the milk. Samples number 4, 6, 11, 13 and 20 (see table 11) were properly pasteurized, as shown by 2 phosphatase units, and gave no positives in brilliant green lactose bile broth before incubation. They proved coliform positive after incubation showing that at least one coliform cell per 100 ml did survive proper pasteurization.

TABLE 11

Phosphatase Test and Bacterial Counts Before and After Incubation of Vat Samples

Sample Number	Phosphatase Units	Coliform Count per 100 ml		Standard Plate Count per ml	
		Initial	Final	Initial	Final
4	2	0	340	2,100	180,000
6	2	0	300	22,300	5,000,000
7	2-5	51	10,000	29,000	2,300,000
8	2-5	22	9,800	85,000	15,200,000
11	2	0	1,090	32,000	4,200,000
13	2-5	0	1,800	4,600	600,000
18	100	180	unsat.	441,000	unsat.
20	2	0	1,600	99,000*	242,000
21	50	180	unsat.	148,000	unsat.

*A great majority of colonies were pin point colonies.

All the 26 samples were incubated for 6 to 8 hours. Initial and final standard plate counts were made before and after incubation and a comparison of the counts showed that the bacteria reproduced 6 to 8 times. Initial and final coliform counts also confirmed this.

Types isolated and their percentage distribution

Samples 18 and 21 were not typed as no isolated colonies could be obtained from desoxycholate agar plates. Eight colonies from a positive desoxycholate agar plate of each of the remaining 7 samples were selected at random and purified cultures were typed by IMViC reactions. None of the colonies proved to be a false positive. Five samples had 100 per cent E. coli, with IMViC formula ++--, and 2 samples had 100 per cent A. aerogenes, with IMViC formula --++. None of these samples contained more than one type as shown in Table 12 (page 42).

Probable source of coliform bacteria in pasteurized milk

Of 26 incubated vat samples 17 did not have a single coliform cell in 100 ml while 1 out of 23 bottled samples, from the same creamery, examined during the same month proved negative to the coliform tests in 20 ml.

Five vat samples did not have any coliform bacteria per 100 ml but when 5 bottles, corresponding to these vat samples, were tested, 50, 325, 250, 200 and 575 coliform organisms per 100 ml were found and 2 to 4 IMViC types were present in each sample.

In 92 pasteurized samples only 27 per cent of the

samples had 100 per cent of one type while 73 per cent of the samples had two or more IMViC types. None of the 7 incubated vat samples gave more than one type in each sample showing thereby that if any cells survive after pasteurization they belong to one type, predominantly of IMViC formula ++--. None of the 7 incubated vat samples had any "Intermediate" types but in pasteurized bottled milk these were isolated from 60 per cent of the samples.

TABLE 12

Distribution of *E. coli* and *A. aerogenes* Sections in 9 Incubated Coliform Positive Vat Samples

Total Samples Run	No. of Positive Samples	Cultures Typed	Percentage Distribution		
			E. coli (++--)	"Intermediate"	*A. aerogenes* (--++)
26	4	8	100	-	-
	6	8	-	-	100
	7	8	100	-	-
	8	8	100	-	-
	11	8	100	-	-
	13	8	-	-	100
	18	unsatisfactory.			
	20	8	100	-	-
	21	unsatisfactory.			

It was therefore concluded that the presence of coliform organisms in pasteurized milk resulted from recontamination.

The greater the number of coliform types present in a bottled sample the more varied are the sources of contamination.

DISCUSSION

The environment of the cow and the conditions under which milk is produced and handled are such that the presence of coliform organisms in fresh raw milk is almost universal. Not one sample of 104 proved negative to the coliform test when about 10 ml of milk was examined. However, Slack and Haddeford (72) reported that 14 per cent of their samples of raw milk were negative to the coliform test when 1 ml was tested and Bartram and Black (73) found that 36.4 per cent of the samples which had standard plate counts of 10,000 or less were free of coliform bacteria in 10 ml tested. In the present study none of the 29 samples having a standard plate count of 10,000 or less gave a negative coliform test, even though these samples were properly refrigerated and tested within 3 hours after milking. Either very sanitary production of the milk or less sensitive media used seem to be the cause of so many coliform negative samples in the hands of those workers.

The author's observations are similar to Finkelstein's (74) who found that milk initially contaminated on the farm had 100 coliform bacteria per ml under careful production and 588 per ml when no care was used. Sherman and Wing (9) suggested that a standard of 100 coliform

bacteria per ml in high grade milk and 10 in certified milk was not stringent.

In properly pasteurized milk a positive coliform test as a result of the survival of heat resistant coliform organisms is highly improbable. Of the 24 vat samples tested only 2 samples were positive, having 51 and 22 coliform bacteria per 100 ml. Recontamination or improper pasteurization were definitely the cause of the presence of coliform organisms in bottled milk.

Chilson and coworkers (24) stated that the coliform test "should supplement and not supplant the standard plate count" in detecting recontamination; but, the present studies revealed that of the 92 coliform positive samples 63 had standard plate counts below 30,000 per ml with a logarithmic average of 9,000 per ml while of the 41 coliform negative samples 20 had standard plate counts above 30,000 per ml with a logarithmic average of 63,000 per ml. Those 63 samples, which on the basis of the coliform index were regarded as recontaminated, might not be reported so by the standard plate count while the 20 coliform negative samples might rightly or wrongly be reported recontaminated as a result of the standard plate count. The higher standard plate count might possibly be the result of slight growth after pasteurization or the presence of thermoduric and thermophilic bacteria in raw milk. Therefore, the coliform index is more reliable (18) (30) (19) (23) (20) in detecting recontamination than the standard

plate count which might be used to supplement and not supplant the coliform test.

The media developed for the determination of coliform organisms in water have, in the past, been used for the coliform test in milk. However, milk is rich in fat and proteinacious matter and behaves differently from water. This was demonstrated by Barkworth (75) who inoculated coliform organisms into milk and water at the same rate but found that milk gave a lower percentage of positives than water. Raw milk possesses a germicidal property which may supplement the inhibitory power of a medium when seeded with milk.

McAuliffe and Farrell(38) recommended that the dye concentration of brilliant green lactose bile broth be increased 2.5 times of the standard concentration as it is partly adsorbed by the solids in 1 ml of milk. This, they claimed, would eliminate false positives.

Stark and Curtis (71) (33) introduced formate ricinoleate broth and claimed its superiority over brilliant green lactose bile broth as it gave very few false positives in milk. Farrell(37) reported that formate ricinoleate broth gave 100 per cent higher "MPN" than brilliant green lactose bile broth in raw milk. In the present study it proved slightly superior to brilliant green lactose bile broth in pasteurized milk and did not give a single false positive, but when compared with violet red agar in raw milk it was found quite inferior.

The need for a good solid medium (7) (31) has always been felt. Yale (32) compared 10 solid media developed for the enumeration of coliform bacteria and found desoxycholate agar and violet red agar to be the most promising when 1 ml of milk was used and that duplicate agar plates gave as reliable counts as 15 broth tubes. The superiority of desoxycholate agar has also been emphasized by other workers (39) (41) (40) (76).

However, Bartram and Black (36) compared 2 strains each of Escherichia, "Intermediate" and Aerobacter in different solid media and graded desoxycholate agar as sixth in the descending effectiveness of solid media. Neutral red agar and violet red agar were placed first and second. They stated that the inferiority of desoxycholate agar was due to its inhibitory action against the "Intermediate" strains used. In this study desoxycholate agar proved just as good as brilliant green lactose bile broth for raw milk but for pasteurized milk it showed a marked superiority over the broths with a slight margin of efficiency over violet red agar. No inhibitory effect of desoxycholate agar as compared with violet red agar was discerned in the detection of the "Intermediate" and other types of coliform organisms in pasteurized milk.

In pasteurized milk desoxycholate agar has the following advantages:

1. It gives a higher coliform count.
2. Up to 2.5 ml of milk can be added to each plate.

(Buchbinder has used 4 ml per plate of 15 ml agar)

3. It gives larger and more distinct typical, dark red colonies than does violet red agar.
4. Typical, dark red colonies prove to be coliform organisms in 97 per cent of the cases.
5. The occurrence of atypical colonies (less than 0.5 mm in diameter) is rare and when they do occur the majority of them belong to the aberrant coliform group. The presence of this group indicates recontamination.
6. It is most efficient when coliform organisms are present in small numbers and so is especially applicable to pasteurized milk.
7. The results are available after 18 hours incubation.
8. Duplicate desoxycholate agar plates, each seeded with 2.5 ml of milk, gave higher counts than were obtained with 15 brilliant green lactose bile broth tubes.
9. It is as productive as violet red agar in detecting all coliform types when present in small numbers in milk.

On the basis of the above advantages it is strongly recommended that duplicate desoxycholate agar plates, each seeded with 2.5 ml of milk, be used for routine examination of pasteurized milk in Public Health Laboratories. Tiedeman (39) and Buchbinder (40) have recommended one plate seeded with 4 to 5 ml of milk but it is always safer to seed duplicate plates than a single plate.

Perry's E. C. medium (77) to detect coliform bacteria

at 37° C and *E. coli* at 45.5° C and Leifson's (78) newly developed hyodesoxycholate agar and broth have been reported to give good results in the examination of milk. A comparison of desoxycholate agar with these media for testing pasteurized milk would be worth while.

In the determination of the percentage distribution of coliform types three provisions should be met.

First, samples of milk, in which no growth has taken place, should be employed so that the relative proportion of various coliform types remains similar and truly representative of the initial coliform contamination. Malcolm (79) seeded raw milk with bovine feces, containing predominantly *E. coli*, and after 36 hours incubation at 62.5° F found that the majority of coliform bacteria belonged to the *A. aerogenes* section. This shows that higher storage temperatures may change the proportion of coliform types from that initially present; therefore, the source of contamination may be interpreted inaccurately.

Second, pour-plate cultures should always be used for typing because in broth cultures one type may overgrow the other types.

Third, random selection of colonies from a pour-plate, rather than isolating the different types from a sample, should be used.

Many authors (30) (35) (37) (69) (76) (80) (81) have determined the percentage distribution of coliform sections but as they did not follow the above three

provisions their percentages cannot be compared with the percentages found in this study. In addition, the distribution of coliform bacteria in milk differs according to locality, climate and conditions of production.

The percentage distribution of coliform sections has rarely been studied in pasteurized milk. Vaughn and Levine (58) summarized the incidence of "Intermediate" bacteria in feces, urine, soil, milk, eggs, etc., and in none of them was the percentage distribution more than 29.5. In the present study 41 per cent of the coliform bacteria in pasteurized milk belonged to the "Intermediate" section. In 13 per cent of the samples the "Intermediate" type -+--+ was the only coliform type present. The cause of this very high incidence of the "Intermediate" section could not be ascertained. Minkewitsch and coworkers (82) reported the change of E. coli, seeded in soil, into citrate utilizing "Intermediates". It would be interesting to determine if under dairy plant sanitizing procedures some E. coli change into "Intermediates".

Of the 16 possible IMViC types, 11 from pasteurized and 13 from raw milk were isolated. Three types with IMViC formulae +++--, +-+- and +---- were not isolated in this study. Sanborn (83) reported the isolation of all the 16 types in a study of slime producing coliform organisms. Ruchhoft and coworkers (84) asserted that only 4 fecal coliform types, ++--, -+--, -+-+ and --++, exist and the others are either mixed cultures or extraneous

to feces. Wilson and coworkers (7) stated that cultures which show no "inverse correlation" of Voges-Proskauer and methyl red reactions are mixtures. Later workers (54) (60) have consistantly found non-inversely correlated cultures, some of which, after numerous serial plantings in lactose broth for purification, have shifted to inversely correlated cultures while the majority of them have proved non-inversely correlated. Stuart and coworkers (54) reported about 10 per cent of non-inversely correlated cultures in 1358 raw samples that they examined and in the present work 8.7 per cent of these types in pasteurized and 11.75 per cent in raw milk have been found. Parr classifies them in the "Intermediate" section but Mitchell and Levine (57), on the basis of the dissimilation of nucleic acid degradation products, have grouped them with the A. aerogenes section. The author agrees with Parr's classification. Stuart and coworkers (54) described the inversely correlated types ++++ and -+++ as "Irregulars".

This study shows that the presence of aberrant coliform bacteria in pasteurized milk is rather infrequent but in raw milk they constituted 13 per cent of the total coliform count and the IMViC type -++- was the most prevalent. It is interesting to note that this anaerogenic type was never isolated from pasteurized milk.

The Standard Methods for the Examination of Dairy Products is not designed to detect these bacteria and

their insignificant presence in pasteurized milk makes them of no importance to dairy bacteriologists. Besides, their sanitary significance has yet to be determined.

Ostman (85) found that 47 of the 50 Friedländer's bacilli cultures, belonging to serological types A, B, C and X, gave biochemical reactions, such as acid and gas production in glucose and lactose and IMViC reactions, characteristic of typical and aberrant coliform organisms. He concluded that "valid criteria have not yet been established for the differentiation of organisms of the Friedländer and coli-aerogenes group". In the absence of biochemical tests to differentiate Friedländer's bacilli from the Escherichia-Aerobacter group our understanding of the pathogenicity of coliform bacteria is not complete.

Standard Methods for the Examination of Dairy Products (11) regards the presence of all coliform organisms, of both fecal and non-fecal origin, as direct evidence of unsanitary dairy practices. It is immaterial whether the presence of E. coli is the result of direct or indirect (as through utensils) fecal contamination of milk. The occurrence of the E. coli section in pasteurized milk is decidedly not directly fecal but due to growth of these bacteria in nooks and corners of equipment that escape usual cleaning practices. The A. aerogenes section is non-fecal but the "Intermediate" section is found in small numbers in human and animal feces. Parr (60)

reported 15.3 per cent of this section in human feces.

Frequent occurrence of two or more types in pasteurized milk indicates varied sources of recontamination as from (86) vats, valves, pumps, pipes, cooler, homogenizer, bottler, bottle and the personnel and reflects improper cleaning practices. Bottled samples containing two or more types generally had a higher incidence of coliform bacteria. Presence of only one type shows a possible bacterial pocket of contamination somewhere in the equipment. The rare occurrence of only one coliform type in a raw sample shows a diversity of sources of contamination.

SUMMARY AND CONCLUSION

Raw Milk

1. A high coliform count was concurrent with a high standard plate count and vice versa.
2. Samples from the same producers when examined in summer gave a higher coliform and standard plate count than in winter.
3. Desoxycholate agar proved equal to brilliant green lactose bile broth with 53 samples while violet red agar gave a 1.6 times higher average coliform count than formate ricinoleate broth with 30 samples.
4. From 55 samples, 558 cultures were selected at random and utilized for the determination of the average per cent distribution of coliform types and sections. The E. coli section constituted 23.5 per cent, "Intermediate" 21.9 per cent, and A. aerogenes 54.8 per

cent.

5. About 12 per cent of the coliform organisms present in milk belonged to non-inversely correlated IMViC types. Of Parr's 16 possible IMViC types 13 were isolated from 55 samples and the IMViC type --++ constituted about 48 per cent of the total percentage of coliform types.

6. Aberrant coliform bacteria composed 13 per cent of the total coliform count, 4.5 per cent being microaerogenic and 8.5 per cent anaerogenic with IMViC type -++- the most prevalent. None of these aberrant coliform organisms belonged to the E. coli section.

7. No definite source of contamination could be ascertained. However, in 6 of the 56 samples the E. coli type ++-- composed 92 to 100 per cent of the coliform count and indicated direct fecal contamination.

Pasteurized Milk

8. No definite correlation could be established between the standard plate and coliform counts in 96 coliform positive samples, showing that the standard plate count as an index of recontamination is not dependable but the coliform index is reliable and the former may supplement but not supplant the latter

9. In a study of media when the per cent efficiency of brilliant green lactose bile broth, arbitrarily chosen as 100, was compared to other media it was found that the per cent efficiency of formate ricinoleate broth

was 121, violet red agar 169, and desoxycholate agar 180.

10. As desoxycholate agar gave 1.8 times higher average coliform count than brilliant green lactose bile broth, it is tentatively recommended that duplicate desoxycholate agar plates, seeded with 2.5 ml of milk each, be used for the routine examination of bottled milk in Public Health Laboratories.

11. Average percentage distribution of coliform types and sections was determined in 92 coliform positive samples. The coli-like "Intermediate" type -+-+ was the most prevalent (31 per cent) of all the 11 IMViC types isolated.

12. The non-inversely correlated IMViC types constituted 8.7 per cent of the total percentage of coliform types.

13. Single types were present in 27 per cent of coliform positive samples while two or more types were present in 73 per cent.

14. A few pin point colonies (less than 0.5 mm in diameter) were observed on desoxycholate agar and violet red agar plates and a majority of them belonged to microaerogenic aberrant coliform bacteria.

15. All vat samples which proved coliform positive only after incubation, contained only one IMViC type per sample but 73 per cent of the bottled samples showed two or more types which indicated recontamination.

16. It was concluded that the greater the number of coliform types present in a sample the more varied are the sources of contamination.

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