UNIVERSITY OF EDINBURGH

STUDIES OF THE OCCURRENCE AND BEHAVIOUR OF BACILLUS CEREUS AND STREPTOCOCCUS THERMOPHILUS IN MILK

by

Kathleen Owen Donovan, B.Sc.Agr.(Sydney)

~~THE UNIVERSITY OF EDINBURGH~~
~~DEPARTMENT OF AGRICULTURE~~
~~10 GEORGE SQUARE~~
~~EDINBURGH 8.~~

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PART I

STUDIES OF BITTY CREAM AND THE OCCURRENCE AND BEHAVIOUR OF BACILLUS CEREUS IN MILK

INTRODUCTION

Milk presents a micro-environment of extraordinary complexity. There exists a dynamic inter-relationship between organism and environment, any change in the one causing some sort of response in the other. For example, the destruction of a heat-susceptible group by the process of pasteurization causes a relatively resistant group to assume a dominant role in the population.

The need to prolong the keeping time of milk in response to a growing consumer demand has dictated most of the major changes in dairy practice over the years. Naturally, the most common and active groups of spoilage organisms received first attention. However, because of the nature of milk, it was inevitable that new faults should appear as the old ones were brought under control. It is characteristic of these new faults that they are increasingly difficult to control. A good example is provided by bitty cream.

Bitty cream has only attracted attention in the last twenty years. During this time, the situation has progressed from a few small outbreaks in the Reading area in 1938 (Davis, 1940) to a perennial problem - at least in the summer months - in many of the major creameries of southern England and Wales (Stone & Rowlands, 1952), and there are reports of a gradual spread northwards (Proctor, 1953).

As its name implies, the chief manifestation of bitty cream is a breaking up of the cream layer into small 'bits'

which will not re-emulsify when the milk is shaken. It has been shown that the condition is caused by the action of the aerobic spore-former Bacillus cereus, or occasionally by the closely related species Bacillus mycoides (Stone & Rowlands, 1952). These organisms both produce a lecithinase and it is the action of this enzyme on the milk fat globule membrane that is at least partly responsible for the development of bitty cream (Stone, 1952a). The fat globule membrane, which imparts emulsion stability to the fat globule (Wiese & Palmer, 1932) is lipo-protein in nature (Palmer & Samuelsson, 1924), the phospholipid fraction consisting largely of lecithin (Palmer & Wiese, 1933). By breaking down the lecithin molecule, the lecithinase causes the fat globules to demulsify. The demulsification process does not, however, go to completion, for although the globules agglomerate they do not show the phenomenon of 'oiling-off' characteristic of complete demulsification, as in churning (King, 1955). Thus, the action of the lecithinase is only part of the sequence of events leading to the 'bitty' appearance. Stone (1952a) suggests that a second enzyme able to coagulate protein may also be involved.

Attempts to control bitty cream have been considerably hampered by our incomplete knowledge of the conditions leading to outbreaks of the fault. The aim of the work embodied in the first part of this thesis has therefore been to try to throw some light on this problem.

Section I deals with the development of a medium for

detecting small numbers of B. cereus in raw milks, previous work by other people having suggested that initial contamination was likely to be low.

Section II is devoted to a survey of a number of milks from different sources to determine the incidence of the organism in the Edinburgh area and its relationship to the occurrence of bitty cream, and to detect any handling methods particularly favourable to the development of large numbers of the organism.

Sections III and IV led on from the first two sections in a way which will be described and deal with some aspects of sporogenesis and spore germination of B. cereus in milk.

Note: B. mycoides is so closely related to B. cereus that Smith, Gordon & Clark (1952) regard it as a variety of B. cereus. That view has been favoured here, especially because both organisms can produce bitty cream. For this reason, no reference to B. mycoides as a separate species has been made in the account which follows. Although from time to time the typical rhizoid colonial form of the variety mycoides was encountered in cases of bitty cream, it was felt that no useful purpose would be served in making the distinction.

SECTION I

The development of a medium selective for *Bacillus cereus* in raw milk

REVIEW OF LITERATURE

In establishing the identity of the causative organism of bitty cream, Stone & Rowlands (1952) used a qualitative method. For the present purpose their method has two disadvantages. Firstly, the use of a preliminary heat treatment makes it impossible to determine the role of vegetative cells as contaminants in milk developing bitty cream. Secondly, the use of a period of enrichment incubation makes it impossible to assess the degree of original contamination of samples by B. cereus.

For detecting small numbers of spore-formers in milk, Burton, Akam, Thiel, Grinstead & Clegg (1953) used a dilution count technique together with the probability tables worked out by McCrady for water analysis (Ministry of Health, 1939). However, this method also involved a preliminary heat treatment.

The most satisfactory method seemed to be a direct plating technique for detecting as few as one or two cells in 1 ml. of raw milk. The problem consisted in developing a medium in which colonies of B. cereus could be readily distinguished, and in which interference from other colonies on the plate was at a minimum. In the early stages of the work two properties of B. cereus stood out as being likely to be of value. These were its ability to produce a lecithinase and its relatively simple nutritive requirements.

The lecithinase-producing ability of B. cereus

The value of this property for our purpose is that it is

at least partly responsible for the development of bitty cream and that it provides a means of distinguishing the organism from all other aerobic spore-formers other than the closely related species B. mycoides and B. anthracis (Colmer, 1948; McGaughey & Chu, 1948).

The method of detecting bacterial lecithinase production was originally developed for the α-toxin of Clostridium welchii which was found to be a lecithinase C. (Macfarlane & Knight, 1941) and happens to be identical with the lecithinase of B. cereus (Chu, 1949). A chance observation of Nägler (1939) that Cl. welchii α-toxin produced opalescence in human serum led to the discovery that the reaction was even more marked in egg yolk saline (Macfarlane, Oakley & Anderson, 1941) and this culminated in the egg yolk agar of McClung, Heidenreich & Toabe (1946) in which lecithinase-producing organisms could be distinguished by the formation of a zone of opacity around the colonies. A method involving this egg yolk reaction was particularly suitable for the present work because it depends on the same general principle as that operating in the case of bitty cream, namely liberation of a fatty substance as a result of the breakdown of the lecithin molecule in a lipo-protein (Macfarlane et al., 1941).

Reports have appeared from time to time of lecithinase activity in certain strains of other organisms. For example, it has been observed in the genus Vibrio (Felsenfeld, 1944), the genus Serratia (Monsour & Colmer, 1952) and the genus

Pseudomonas (Villecourt & Jacobelli, 1953; Paton, 1956). A very similar reaction in egg yolk due to the action of a lipase has also been reported in some staphylococci (Gillespie & Alder, 1952). However, it was thought that any of these organisms, should they occur in milk, would be readily distinguishable from B. cereus on the basis of colonial morphology.

The simple nutritive requirements of B. cereus.

Knight & Proom (1950) showed that B. cereus will grow in the presence of a mixture of amino-acids without any added growth factors. The lactic acid bacteria, on the other hand, which seemed likely to form a large proportion of the competing population in milk, are characteristically extremely fastidious in their nutritive requirements (Orla-Jensen, 1919).

As a first step in the development of the medium, therefore, an agar containing only egg yolk and vitamin-free casamino acids was tested.

EXPERIMENTAL AND RESULTS

Four strains of B. cereus isolated from soil were used as the test organisms for development of the medium. Spore suspensions were prepared by washing the growth off slopes of nutrient agar (see appendix, p.i) which had been incubated at 30° for 1 week, using sterile glass-distilled water. The resulting suspensions were centrifuged, washed four times in sterile glass-distilled water, held at 65° for 30 min. to destroy vegetative cells and stored in a refrigerator. Counts remained unchanged over a storage period of several months. The use of spore suspensions of known count considerably facilitated the tests which followed.

The development of the basal medium

Yolk saline (1:1) was prepared as described in the appendix (p.i). Concentrations of yolk between 0.5 and 5% (v/v) were tested for opacity in the presence of sterile milk (1 ml./plate), using nutrient agar as the basal medium. With all strains, optimum contrast with the background was obtained with 2.5% yolk. This concentration was adopted in all subsequent work.

Preliminary experiments with agar containing 2.5% yolk and varying concentrations of vitamin-free casamino acids soon revealed that sufficient nutrients were being supplied by the milk inoculum to satisfy the requirements of even the most fastidious organisms. Surface inoculation of dried plates using 0.1 ml. inocula spread over the agar did little to improve the situation. In all cases, growth of other

organisms was so abundant that the colonies of B. cereus (inoculum of known size) were completely obscured. This obscuring effect was due partly to crowding and partly to masking of the opaque reaction by opacity due to other causes, such as casein precipitation.

The growth of B. cereus strains was now compared on nutrient agar, casamino acids agar and water agar all containing yolk. Eventually nutrient agar with 2.5% yolk (yolk agar) was adopted as the basal medium because it gave the best growth of the organism together with the most favourable colour for observing opacity changes. Future modifications of this basal medium were designed to improve the contrast between lecithinase-caused opacity and other sorts of opacity, and to inhibit the growth of other organisms relative to that of B. cereus.

Selection of optimal incubation conditions

Smith, Gordon & Clark (1952) found that while all their strains of B. cereus grew at 28°, 33° and 37°, about half were unable to grow at 45°. Thus, it was not practicable to use a relatively high temperature for selection of B. cereus from other milk organisms.

A series of plates containing raw milks inoculated with B. cereus was set up at 22°, 26° and 30°. Using a short incubation time - 18 hr. - the test strains outgrew other organisms best at their optimum temperature for growth, 30°. Although at 22°, Streptococcus lactis grew poorly, Gram-negative

organisms rapidly outgrew B. cereus in many cases.

The incubation period used in future work was, therefore, overnight at 30°.

Modifications of the basal medium

1. Attempts to improve differentiation of the opaque reaction

It was thought that it might be possible to incorporate an indicator into the medium for the purpose of staining the substance responsible for the opaque reaction. This was shown to be fatty in nature by Macfarlane et al. (1941), and for this reason some fat stain was sought. Victoria Blue, whose base is red and fat-soluble was used successfully for the detection of lipolysis by Paton & Gibson (1953), and this was the indicator tried here. The red base was suspended in a small amount of nutrient agar by autoclaving and from this a number of plates were poured, yolk saline being incorporated at the time of pouring the plate. Spot inoculations of the test strains were made on to these plates. It was hoped that the opaque areas produced by lecithinase action would take up the suspended dye and stain a deep red colour. It was found after 24 hr. that the colonies themselves were stained deep blue but the opaque zone remained quite white. It is interesting that the blue-staining colonies were shown to be non-lipolytic on Paton & Gibson's medium. Presumably the colour resulted from the combination of red base with an acid and in this case was possibly due to long chain fatty acids liberated from the lecithin molecule by the action of the lecithinase.

However, this method was abandoned since the opaque zone was not stained and there was no means of distinguishing these blue-staining colonies from those formed by lipolytic organisms.

The possible use of fat solvents for dissolving the opaque zone was now considered. Alcohol, ether and benzol were applied to the opaque zones, but none of these solvents penetrated the agar.

Attention was now turned to methods of reducing the opacity of the inoculated medium relative to that of the yolk reaction. Brown & Howe (1922) described a method of rendering milk transparent by the addition of citrate. This was of particular interest because most of the opacity of the inoculated medium was due to the milk inoculum. Concentrations of sodium citrate of 0.5, 1 and 2% (w/v) allowed growth of B. cereus and gave marked clearing of both yolk and milk. There was slight reduction in size of the B. cereus colonies in the presence of 2% citrate, so that in order to allow a safety margin for inhibition, 0.5% was the concentration used in the medium. Sodium chloride also gave clearing but was more inhibitory to B. cereus than sodium citrate and the latter was therefore preferred.

2. Attempts to inhibit the growth of other organisms

a) Initial attempts to inhibit lactic acid bacteria

It was assumed that most trouble both with crowding and with casein precipitation was likely to result from the growth of streptococci. Since it was not possible to take advantage of the relative nutritional fastidiousness of this group,

some utilizable point of difference from the aerobic spore-formers was sought. One possibility was the production of certain enzymes by B. cereus. Firstly, the fact that it produces a catalase whereas streptococci do not, suggested the use of hydrogen peroxide. Secondly, some strains at least of B. cereus are known to produce a penicillinase (Pollock, 1950) and although the ability to produce this enzyme is not necessarily accompanied by penicillin resistance (Luria, 1946), there was some likelihood that a selective effect might operate. This possibility was strengthened by the work of Curran & Evans (1945b) who found that vegetative cells of two strains of B. cereus could multiply in the presence of 5 i.u./ml. sodium penicillin G. The success of both these methods depended on the initial advantage possessed by the enzyme-producing organism in the presence of the inhibiting substance, allowing it to grow away from other organisms, for inevitably the destruction of the substrate by the enzyme would provide conditions suitable for the growth of the inhibited organisms.

The effect of hydrogen peroxide. A range of concentrations were tried and it was found that all concentrations of hydrogen peroxide not actually inhibitory to the test strains, growth of other organisms was so prolific that the lecithinase-producing colonies were obscured.

The effect of penicillin. Penicillin concentrations of 0 - 30 i.u./ml. were tried (sodium penicillin G). All strains

TABLE 1.

The effect of penicillin on the growth of a strain of *B. cereus* in yolk agar.

Penicillin (i.u./ml.)	*B. cereus* (count/ml.)	
	Series 1	Series 2
0	24	26
3.0	22	24
4.5	17	-
6.0	24	-
7.5	23	20
9.0	19	-
15.0	-	12
30.0	-	0

grew well at concentrations up to 15 i.u./ml. (see Table I). It is reported, on the other hand, that most milk streptococci are inhibited at 1 i.u./ml. (Overby, 1954).

However, when penicillin was tried in plates inoculated with raw milks, prolific growth was obtained in many cases and again the opaque zones were obscured. This was found to be due to organisms which were Gram-negative and thus resistant to the drug. Since most of this growth appeared to be on the surface and seemed to cause relatively little clouding of the medium, it was thought that it might be possible to avoid the use of another inhibitor by merely inducing the formation of discrete colonies. This was attempted by means of a cover of water agar.

The effect of a cover of water agar. Different amounts of water agar were poured over the surface of 5 and 10 ml. volumes of inoculated yolk agar containing penicillin. On the whole, there was a definite reduction in crowding causing a general clearing of the medium, the best results being obtained with 10 ml. of medium covered by 5 ml. of water agar. However, there were still a number of cases in which there was too much growth to allow the opaque reaction to be seen clearly.

For this reason, various inhibitors for the Gram-negative group of organisms were tried; at the same time, there seemed to be sufficient reason for retaining the agar cover and this was done in subsequent trials.

b) Attempts to inhibit Gram-negative organisms.

There was little information available on the effect of

the commonly used inhibitors of Gram-negative organisms on B. cereus. Thus, for want of a better starting point, two classical inhibitors of Gram-negative organisms were tried, viz. potassium tellurite and sodium azide.

The effect of potassium tellurite. This substance has been used for many years, particularly as a selective inhibitor in the isolation of diphtheria organisms. (e.g. Allison & Ayling, 1929; Anderson, Happold, McLeod & Thomson, 1931). In spite of this fact, its action is still a matter for conjecture. Fleming (1932), Smith, Morton & Leberman (1950) and Morton & Lecce (1953) have reported that Gram-negative organisms are mainly inhibited at a concentration of 1/50,000, while Gram-positive organisms - especially streptococci and staphylococci - show a remarkable degree of resistance. No information could be found concerning the resistance of B. cereus, but Fleming (1932) reported that B. anthracis was inhibited by 1/2 00,000 and B. subtilis by 1/20,000. Concentrations of from 1/1,000 to 1/1,000,000 were all inhibitory to the test strains, and thus this inhibitor was discarded.

The effect of sodium azide. Sodium azide was first used as a general inhibitor of Gram-negative organisms by Snyder & Lichstein (1940). It is known to be an inhibitor of a number of enzymes, including catalase (Keilin & Hartree, 1934). Again, information relevant to the resistance of B. cereus was scant. However, Edward (1947) reported that 0.005% did not inhibit B. subtilis and Johansson (1953) used

0.02% to inhibit Bacillus species in his medium for clostridia. On the other hand, complete inhibition of coliforms, salmonellae and the spreading growth of Proteus species occurs at 0.01% (Snyder & Lichstein, 1940).

Concentrations between 0.1% and 0.005% (w/v) all completely inhibited the test strains. Growth did occur at 0.0005% but this was of no use, since Gram-negative organisms were not inhibited.

Other inhibitors. Eurich & Hewlett (1930) in a discussion of the haemolytic powers of B. anthracis, noted that the addition of bile to blood plates did not affect the growth of the organism. In view of the close relationship of this organism to B. cereus, it was decided to test the effect of bile salts on the latter. Concentrations of sodium taurocholate up to 1.5%(w/v) were tested and it was found that concentrations inhibitory to Gram-negative organisms were also inhibitory to the strains of B. cereus.

In his tween-acetate agar for selective isolation of small numbers of lactobacilli from silage, Keddie (1951) occasionally found spore-formers capable of growth in the presence of the concentration of sodium acetate used to inhibit the majority of silage organisms. For this reason, the effect of sodium acetate on B. cereus was tried. Growth occurred in the presence of all concentrations between 0.1% and 2% (w/v), whereas Keddie (1954) had reported inhibition of Gram-negative organisms at 0.5% (w/v). However, there was a

reduction in colony size of B. cereus, quite marked at concentrations of 1% and above. Thus, another method was sought in the hope that a wider inhibition margin might be found.

Sodium metabisulphite which had shown some indication of selection of aerobic spore-formers when used in silage (Stirling, 1956), was also of no use, concentrations of 0.5 and 1% (w/v) being inhibitory to B. cereus.

Other substances tried and discarded were sodium carbonate and borax, inhibitory concentrations for B. cereus being found to be 0.5 and 1% (w/v) respectively.

The effect of polymyxin. Attention was now turned to the possibility of the use of some antibiotic for selective inhibition of Gram-negative organisms. B. cereus or related species has been found to be susceptible to most of the 'broad spectrum' antibiotics, e.g. aureomycin (Price, Randall & Welch, 1948), streptomycin (Kavanagh, 1947), chloramphenicol (McLean, Schwab, Hillegas & Schlingman, 1949) terramycin (Hobby, Dougherty, Linert, Hudders & Kiseluk, 1950) and neomycin (Waksman, Katz & Lechevalier, 1950). However, of the antibiotics of narrower range, polymyxin presented some possibility. This antibiotic is remarkable for the specificity of its action against Gram-negative organisms, being 10-1,000 times more powerful against this group than against Gram-positive organisms (Jawetz, 1956); and while many examples could be quoted of the high degree of susceptibility of pseudomonads and coliforms (e.g. Sherwood, Delage & Herman, 1953; Frank, Wilcox & Finland, 1950a,b), Stansly, Shepherd

TABLE 2.

The effect of polymyxin on the growth of a strain of *B. cereus* in yolk agar.

Polymyxin (i.u./ml.)	*B. cereus* (count/ml.)
0	24
50	27
100	34
200	13
300	22
400	16

TABLE 3.

The effect of penicillin and polymyxin on the recovery from raw milk of a strain of *B. cereus*, using yolk agar.

Antibiotic(s) present	Growth of *B. cereus*
Penicillin (9 i.u./ml.)	Obscured
Polymyxin (25 i.u./ml.)	Obscured
Penicillin (9): Polymyxin (5 i.u./ml.)	Obscured
Penicillin (9): Polymyxin (25 i.u./ml.)	Clearly distinguishable

TABLE 4.

The effect of penicillin and polymyxin on the recovery from sterile milk of a strain of *B. cereus*, using yolk agar.

Penicillin (i.u./ml.)	*B. cereus* (count/ml.)	
	With polymyxin (50 i.u./ml.)	Without polymyxin
0	31	26
1.5	18	34
3.0	19	24
7.5	23	20
15.0	7	12
30.0	0	0

TABLE 5.

The effect of penicillin and polymyxin on the recovery from raw milk samples of a strain of *B. cereus* using yolk agar.

Milk	*B. cereus* (count/ml.)	
	With inhibitors*	Without inhibitors
1	25	34
2	23	26
3	12	28
4	13	35
5	18	27
Sterile (control)	15	32

* (5 i.u./ml. penicillin.
(50 i.u./ml. polymyxin.

& White (1947) showed that members of the genus Bacillus (including B. mycoides) have a high degree of resistance to the drug. Concentrations of polymyxin B sulphate up to 500 i.u./ml. of basal medium had no effect on either the count or the colony size of the B. cereus strains (see Table 2). When the antibiotic was incorporated into plates inoculated with raw milks and the known suspension of the strain of B. cereus under test, a considerable reduction in count of milk organisms occurred, but except in milks having abnormally low counts, there was still too much growth to permit certain recognition of the B. cereus colonies. When penicillin was also included in the plates, however, a much more promising picture was obtained (see Table 3).

Counts were now done on the test strains using sterile milk and varying the concentrations of the antibiotics. As seen in Table 4, growth of B. cereus was satisfactory over the range of combinations involving 0 - 7.5 i.u./ml. penicillin and 50 i.u./ml. polymyxin. For further tests, the combination of 5 i.u./ml. sodium penicillin G with 50 i.u./ml. polymyxin B sulphate was adopted. This combination was now tested with five raw milks having counts sufficiently low to allow a control series without inhibitors to be set up for comparison with the inhibitor medium. The results appear in Table 5. There is some suggestion from these results that polymyxin and penicillin have a synergistic action against B. cereus. This finding throws considerable doubt on the validity of the

method and thus, possible alternative combinations were sought.

c) Further attempts to inhibit lactic acid bacteria

Polymyxin was retained in the medium and various other means of inhibiting the lactic acid group were tried with the idea of replacing penicillin.

The effect of gelatin. An attempt was made to reduce the effects of acid production by the incorporation of a buffering substance into the medium. Small concentrations of gelatin were tested, but the effect was negligible.

The effect of sodium fluoride. This substance has been used successfully as an inhibitor of lactic acid producing organisms (Wright, 1937). It operates through inhibition of certain enzymes in the glycolytic reaction sequence. Concentrations of 0.01M to 0.2M were tested in combination with polymyxin and it was found that 0.05M was inhibitory to the growth of most milk organisms. However, B. cereus was also inhibited at this concentration. At lower concentrations where its growth was possible, many other organisms could also grow.

The effect of triphenyltetrazolium chloride. Weinberg (1953) reported that this substance, used quite extensively as an indicator of dehydrogenase activity, at a concentration of 0.05% (w/v), exerted a selective inhibitory effect against certain Gram-positive bacteria. Tests with the B.cereus strains showed them to be no more resistant than the lactic acid bacteria.

TABLE 6.

The effect of sulfanilamide alone, with polymyxin and with penicillin on the recovery from raw milk of a strain of *B. cereus* using yolk agar.

Inhibitors present	Growth of *B. cereus*
None	Obscured
Sulfanilamide (1 mg. %)	Colonies faint and small
Sulfanilamide + polymyxin (50 i.u./ml.)	Colonies faint and small
Sulfanilimide + penicillin (5 i.u./ml.)	Colonies clear, but low count

The effect of surface tension depressants. The anionic surface active agent, di-octyl sodium sulphosuccinate has been used with success by Crosse and Bennett (1955) and by Paton (1956) in selective media for Pseudomonas species at concentrations of 0.01% and 0.05% respectively. It was found that B. cereus grew satisfactorily in the presence of 0.005%, 0.01% and 0.05% (w/v) in the basal medium. However, if the yolk were omitted from the basal medium, all these concentrations were found to be inhibitory to the test strains. Thus, the presence of the yolk has considerably affected the activity of the anionic detergent, rendering it useless for the present purpose.

The effect of sulphanilamide Waksman (1945) reported a high degree of resistance of species of Bacillus to sulphanilamide. Preliminary trials with the test strains showed that concentrations of up to 10 mg.% permitted good growth of the organism. Since this drug is not a specific inhibitor of organisms of a particular Gram reaction, it was tried in combination with penicillin as well as with polymyxin. The results are summarized in Table 6. It was felt that the combination sulphanilamide-penicillin showed some promise and it was not discarded at this stage.

The effect of lithium ion. MacLeod (1951, 1954) and MacLeod & Snell (1947) described the toxic effect of lithium ion against certain species of streptococci. This work provided the suggestion that lithium salts might be of use in the present case. Up to 2% (w/v) lithium chloride was included

TABLE 7.

The effect of lithium chloride on the growth of *B. cereus* on yolk agar in the presence of *S. lactis*.

Lithium chloride (%)	Growth of *B. cereus*
0	None visible due to crowding or acid inhibition
0.25	None visible due to crowding or acid inhibition
0.5	100% recovery; no inhibition
1.0	100% recovery; no inhibition
2.0	No growth

TABLE 8.

The effect of various inhibitors on recovery of *B. cereus* from raw milk samples, using yolk agar.

Inhibitors present	*B. cereus* (count/ml.)				Appearance of medium
	Milk 1	Milk 2	Milk 3	Mean	
None	14	15	17	15	Cloudy
S:Pen.	15	11	18	14	Cloudy
L:C:S	11	3	7	7	Good clearing
L:C:S + cover	6	11	9	9	Good clearing
L:C:Poly.	19	9	12	13	Very good clearing
L:C:Poly. + cover	21	19	22	21	Very good clearing

S = Sulfanilamide (10 mg. %)
Pen. = Penicillin (5 i.u./ml.)
Poly. = Polymyxin (50 i.u./ml.)
L = Lithium chloride (0.5%)
C = Sodium citrate (0.5%)
Cover = 5 ml. water agar over the surface of the solidified medium.

TABLE 9.

Recovery of *B. cereus* from raw milk samples having high total counts by means of yolk inhibitor agar.

Milk	*B. cereus* (count/ml.)		
	Inoculated		Uninoculated
	With inhibitors	Without inhibitors	
Sterile (control)	-	17, 10	-
Raw 1	16	0	0
Raw 2	16	0	0
Raw 3	18	0	0
Raw 4	10	0	0
Raw 5	20	0	1
Raw 6	13	0	0

in the basal medium and it was found that there was no inhibition of the test strains at concentrations up to 1%. A series of tests were now carried out using a mixed inoculum of a strain of Streptococcus lactis with each of the strains of B. cereus. From Table 7, it can be seen that concentrations of 0.5 and 1% lithium chloride, although not completely inhibiting growth of the streptococcus, did inhibit casein precipitation. The colonies of B. cereus could thus be clearly distinguished without interference. To allow a margin of safety for inhibition of B. cereus, a concentration of 0.5% lithium chloride was used in the test medium.

A number of different combinations were now compared, using three raw milks of fairly low count. (see Table 8). It was found that lithium chloride exerted a clearing effect additional to that due to the presence of citrate, and that, of the inhibitor combinations tried, the basal medium incorporating lithium chloride, sodium citrate and polymyxin with an agar cover gave not only the best recovery of the organism but also the clearest medium.

It remained now to test this inhibitor medium in poor quality milks, since these would provide rigorous test conditions. The results with a number of milks of this kind appear in Table 9, and it can be seen that the recovery of B. cereus in the inhibitor agar was quite satisfactory.

Composition of the final medium

The medium finally adopted (yolk inhibitor agar) had the following composition:-

TABLE 10.

Comparison of the growth of B. cereus on yolk agar and yolk inhibitor agar.

Strain	B. cereus (count/ml.)		Spot inoculations			
	Yolk agar	Yolk inhibitor agar	Yolk agar		Yolk inhibitor agar	
			Colony diam.*	Opacity diam.*	Colony diam.*	Opacity diam.*
C.01	117	117	6	11	4	11
C.03	56	48	4	11	4	11
C.04	103	76	4	12	4	11
C.05	114	169	9	14	3	11
C.06	160	117	4	12	4	9
C.10	93	79	8	12	7	12
C.11	88	91	5	11	6	12
C.12	161	118	5	11	4	11
C.13	352	401	5	11	5	11
C.14	189	195	5	12	5	12
C.15	183	209	5	12	4	10
Mean	147	147	5	12	4	11

* expressed in inches x 10^{-1}

0.5% (w/v) peptone

0.5% (w/v) beef extract (lab. lemco)

added individually to the medium at the time of pouring:

- 0.5% (w/v) lithium chloride
- 0.5% (w/v) sodium citrate
- 50 i.u./ml. polymyxin B sulphate
- 2.5% (v/v) egg yolk (1:1 saline, 0.5 ml./plate)

1.5% (w/v) agar

1,000 ml. distilled water

pH was approximately 7.0 and a cover of 5 ml. water agar was poured over the medium in each plate after it had solidified. Incubation was at 30° for 18-24 hr..

Tests of the final medium

Eleven strains of B. cereus isolated from milks showing bitty cream were now used to test the medium further. Results appear in Table 10. It can be seen that counts of all strains were comparable with and without inhibitors. With some strains, there was a slight reduction in colony size in the presence of the inhibitors, but this disadvantage was thought to be outweighed by the advantages of the medium in raw milk platings.

SECTION II

The incidence of *Bacillus cereus* in Scottish milk supplies and its relationship to the development of bitty cream

REVIEW OF LITERATURE

Early investigations on outbreaks of bitty cream in southern England (Davis, 1940; Stone & Rowlands, 1952) have emphasized the complex nature of the conditions under which they occur.

The work of Stone & Rowlands (1952) suggested that few milks are free from contamination by B. cereus. In an examination of a number of samples from a local dairy, they detected the organism in 79% of raw milks and 100% of pasteurized milks. (It must be remembered when considering these results that this work was done in an area where bitty cream had been occurring for a number of years, so that they may not be representative of all parts of the country.) However, the presence of B. cereus does not mean that bitty cream will necessarily develop. Stone (1952b) detected the organism in all samples examined of pasteurized milk from two creameries, but at only one of these creameries did bitty cream cause trouble.

Assuming that B. cereus is present in a milk, the results of previous work suggest that whether or not bitty cream develops depends on a number of factors, amongst which one or more of the following may be included: the extent of the original contamination by B. cereus, the temperature at which the milk is stored, the number and types of other organisms present and whether or not the milk has been pasteurized.

The influence of extent of initial contamination

It was assumed by previous workers that the vegetative cell

was unimportant as an initial contaminant of milks developing bitty cream. Therefore the work mentioned here is only concerned with spores. It is generally accepted that freshly produced or freshly pasteurized milk contains very few spores (e.g. Abd-el-Malek, 1943; Egdell & Bird, 1950). Thus, Stone & Rowlands (1952) took the point of view that initial numbers of B. cereus in any milk will be so small that the actual size of the inoculum will have no bearing on the development of the fault. However, later Stone (1952b) found that if raw and pasteurized milks were inoculated with 25 or 50 spores/ml., bitty cream developed more rapidly than if inocula of 1 or 10 spores/ml. were used. Further, using a plating method to estimate actual numbers of spores in positive samples of pasteurized milk, she discovered that initial numbers were rarely less than 10/ml. and sometimes as high as 100/ml.. Thus, there was some suggestion that the extent of initial contamination could be important in determining the development of bitty cream.

The influence of temperature of storage

Davis (1940) and Stone & Rowlands (1952) reported that outbreaks of bitty cream were confined to warmer weather, although the latter authors showed that the organism was still present in the milks during the winter months. Galesloot (1953) in experiments on the deterioration of pasteurized milks at different temperatures, found that in uncontaminated milks B. cereus frequently dominated the flora after one or two days at 20 or 27°, but not at other temperatures. Stone (1952b),

in fact, has suggested that once a milk has been infected with B. cereus spores, the rate at which bitty cream develops depends primarily on the temperature of storage, 22° being particularly favourable. As mentioned earlier, she was able to isolate the organism from two creameries which were in the same district and thus presumably subject to approximately the same weather conditions. Yet, an outbreak of bitty cream only occurred at one of them. There was, however, another point of difference between the two creameries, and this brings us to the next influencing factor.

The influence of the numbers and types of other organisms present

Stone (1952b) observed that the total numbers of organisms in samples from the creamery where no bitty cream occurred were on an average greater than at the creamery where the fault did occur. Davis (1940) found that bitty cream was particularly associated with milks having low counts and this was confirmed by Stone & Rowlands (1952) who found that the initial colony counts of raw milks which developed bitty cream rarely exceeded 5,000/ml., while the corresponding figure for pasteurized milks was 150/ml.. It is not clear whether this effect is attributable to competition for nutrients by a large and rapidly growing population of other organisms, or whether there is some direct inhibition of the growth of B. cereus by a particular group or groups of the flora. Davis (1940) suggested that the appearance of bitty cream is retarded by the presence of large numbers of acid producers. It is interesting in this connection to note a report of Orcutt (1942)

that an increase in occurrence of Bacillus albolactis (the lactose-fermenting variant of B. cereus) accompanied the gradual replacement of normal Streptococcus lactis by a slow acid producing variety in Virginia milk supplies. On the other hand Garvie & Stone (1952) grew B. cereus in the presence of varying inocula of S. lactis and found that the development of bitty cream proceeded independently of the numbers of S. lactis present. The possibility of inhibition by Pseudomonas species in raw milks is suggested by reports of similar effects in soil (Lewis, 1929).

The influence of pasteurization

Stone & Rowlands (1952) reported bitty cream in unheated milks, but it is generally agreed that pasteurization stimulates development of the fault. It is not clear whether this is due to the removal of competing or inhibiting organisms, as suggested by Davis (1940), or due to stimulation of spore germination as a result of the heat treatment (see Section IV) or both, or whether some other factor, as yet undefined, contributes to the effect.

Taking into consideration the foregoing literature review, the following aims for this section of the work were formulated:

a) To determine the incidence of B. cereus in raw and pasteurized milk samples in an area where no outbreak of bitty cream had been reported. Edinburgh and the surrounding districts seemed suitable for this purpose. At the same time to

assess the importance of contamination by vegetative cells in causing bitty cream in raw milks.

b) To determine whether high counts of the organism could be associated with particular farms or creameries and to relate this to handling methods.

c) To determine the relationship between the size of the original inoculum of B. cereus and the subsequent development of bitty cream.

d) To determine the relationship between the numbers and types of other bacteria present and the development of bitty cream.

e) To attempt to isolate from raw milks organisms able to inhibit B. cereus.

METHODS

Source of Samples. The majority of the samples examined in this section of the work were drawn from two creameries, referred to here as A and B.

At Creamery A, about 17,000 gal. of milk were pasteurized and bottled each day, the milk being drawn from about 200 suppliers, supplemented by up to 9,000 gal. daily from country creameries. The milk from the country creameries was transported to the central creamery in insulated bulk road tankers, distances of up to 100 miles. The precise source of the country creamery milks varied according to the allocation of the Milk Marketing Board, which was in turn determined by the surplus available at each country creamery and the demand at the central creameries from day to day.

Creamery B handled approximately 7,000 gal./day, the source of which was about 60 suppliers and country creamery tanker milks as above.

Occasional samples were also taken from a third creamery, C, of comparable size to A.

In addition, samples were taken direct from several country creameries and from individual farms, but where this occurred specific mention has been made in the text.

From creameries A and B, three main types of sample were taken:-

(a) milk from individual farms, which had been transported to the creamery in cans. This was sampled from the

weigh tank by means of sterile dippers. In this way, it was hoped to obtain a sample representative of both the morning's and the previous evening's milk.

(b) milk from the bulk road tankers transported from the country creameries. This was sampled direct from the tanker by means of a special long-handled dipper, before the milk was pumped into the creamery storage tank.

(c) milk from the creamery plant. This was sampled wherever possible by means of a sterile dipper, or where this was impossible, by directing the flow of milk through a loosened joint or cock into a sterile sample bottle.

Treatment of Samples. All samples were taken into sterile bottles of approximately 150 ml. capacity. The volume of milk sampled was usually about 100 ml., but sometimes circumstances forced the taking of smaller volumes. Samples were transported to the laboratory with as little delay as possible and held in a refrigerator until they could be tested. The length of time in the refrigerator generally did not exceed a few hours but sometimes overnight storage was necessary. It was not expected that the results would be seriously affected by this storage period.

Ten ml. sub-samples of each milk were taken into sterile 6 x $\frac{5}{8}$" test tubes. These were transferred to a water bath, thermostatically controlled at 63° (±0.5°) and laboratory pasteurization was carried out by holding the tubes with the level of the milk below that of the water for 35 min.. At the

end of this time, the tubes were withdrawn from the bath and rapidly cooled by means of running tap water.

Testing of Samples.

(a) Count of B. cereus: One ml. volumes of raw milk were plated out using the yolk inhibitor agar described in the previous section. Incubation was for 18 hr. at 30°. Laboratory pasteurized samples were also plated out, this time using plain yolk agar, the inhibitors being omitted as an additional check on the inhibitor agar.

(b) Development of bitty cream: Stone & Rowlands (1952) stored milk in 150 ml. conical flasks. After the incubation period, the milks were examined by gently swirling the flasks, causing breaking up of the cream layer. In the case of bitty cream, this failed to re-emulsify in the milk and the typical bitty appearance was obtained. In this laboratory, considerable difficulty was found in distinguishing between true bittiness and other types of change by this method. Therefore, the following method was used. 5 ml. quantities of milk were stored in 6 x $\frac{5}{8}$" test tubes and held at 22° for 48 hr.. At the end of this time a number of sub-samples had developed a condition in the cream layer which might conceivably be described as 'bitty'. To distinguish cases of true 'bittiness', a drop of cream was withdrawn from each tube and touched on to the surface of a yolk agar plate (without inhibitors). This plate was incubated at 30° overnight. In cases of true bitty cream, a surface growth having the typical mealy appearance of

B. cereus or the unmistakable rhizoid appearance of B mycoides had developed, surrounded by a zone of opacity. The identity of the organism was checked by examination of a Gram-stained smear.

The tubes were replaced in the incubator for a further 24-48 hr.. It was found that in every case recorded as 'positive bitty cream' by the spot plate test, a clot with marked proteolysis at the surface subsequently developed. Since this is quite characteristic of the growth of B. cereus in milk, it was considered to be sufficient confirmatory evidence that B. cereus had dominated the population at the earlier stage. One therefore felt justified in using the spot plate method as a test for bitty cream.

The storage period of 22° for 48 hr. was used in accordance with the method of Stone & Rowlands (1952) to simulate conditions in the average household during the warmer months of the year when outbreaks of bitty cream appear.

The above tests were applied to all milks, but in addition the following tests were applied in some cases.

(c) Total count: In accordance with the Scottish Milk Regulations, the milks were plated on yeastrel milk agar (see appendix, p.i) and incubated for 48 hr. at 37°. It is realized that this method may estimate only a small proportion of the total population, but it has been used in this case because this is the routine method of grading milk and it thus allows comparison with existing standards.

(d) Laboratory pasteurized count: There is no official method of carrying out this test and no standard for grading on this basis. Therefore, in this case, the aim was to use a method giving as good as possible an estimate of the actual population which it claims to measure. Thus, lactose-tryptone-beef extract agar (see appendix, p.i) was used with incubation at 30° for 5 days. As was to be expected, the laboratory pasteurized count occasionally greatly exceeded the total count, which emphasizes the futility of any attempt to compare the two sets of results.

(e) Count of aerobic spore-formers growing at 45°: This figure was obtained from raw and pasteurized samples using lactose-tryptone-beef extract agar and incubating for 48 hr. at 45°. Spore-formers could be readily distinguished from other organisms by their colonial appearance and could be counted with ease, since spreading growth is limited at this temperature. The use of 45° incubation excluded strains of B. cereus from the count.

(f) Total count at 45° (omitting spore-formers): This was carried out in the same way as the estimate of aerobic spore-formers, except that in this case only raw samples were used. The majority of colonies growing under these conditions proved to be those of thermoduric streptococci, so that the method gave a rough estimate of this group.

Note: The reason for the use of raw samples only for this count was that it was found that the S. faecalis group did not

survive under these conditions after laboratory pasteurization (see Part II of this thesis, p.83).

TABLE 11.

The incidence of *B. cereus* in milk from farms, country creameries and creamery plant.

Source of Milk	Total Samples	Samples with *B. cereus* in 1 ml.					
		Raw		Laboratory pasteurised		Raw and/or Lab. pasteurised	
		No.	%	No.	%	No.	%
Farms	280	21	8	12	4	27	10
Country creameries	62	10	16	11	18	14	23
Creamery plant	84	15	18	2	2	15	18
Total	426	46	11	25	6	56	13

TABLE 12.

The incidence of *B. cereus* in milk samples from two creameries.

Source of milk	Creamery A			Creamery B		
	Total Samples	*B. cereus* in 1 ml. No.	%	Total Samples	*B. cereus* in 1 ml. No.	%
Farms	61	13	21	219	14	6
Country creameries	43	10	23	4	0	-
Creamery plant	60	13	22	24	2	8
Total	164	36	22	247	16	7

RESULTS

The incidence of B. cereus in milk samples from different sources

Four hundred and twenty-six samples from individual farms, country creameries and creamery plant were examined for the presence of B. cereus in 1 ml. sub-samples, both raw and after laboratory pasteurization. The results are summarized in Table 11.

It can be seen from this table that 13% of these samples showed 1 organism or more per ml.. Of these 82% were detected in the raw sample and 44% in the laboratory pasteurized sample. However, the counts obtained were so low (see Table 20) that the discrepancy between the two treatments is likely to be within the error of the method and does not prove that contamination by vegetative cells occurred more commonly than contamination by spores.

Association of B. cereus with milk samples from particular sources

From Table 11, it appears that a smaller percentage of farm samples were contaminated than plant samples. However, if these figures are examined in greater detail, it can be seen that this is merely a reflection of the proportion of samples coming from each of the two central creameries, a higher incidence of the organism occurring in all samples taken at creamery A than those taken at creamery B (see Table 12). On the other hand, the agreement between results for different

TABLE 13.

Monthly variation in incidence of *B. cereus* in farm samples from two creameries.

Month	Creamery A			Creamery B		
	Total Samples	Samples with *B. cereus* in 1ml. No.	%	Total Samples	Samples with *B. cereus* in 1ml. No.	%
January	11	6	55	0	-	-
February	36	5	14	47	1	2
March	14	2	14	47	3	6
April	0	-	-	43	2	5
May	0	-	-	60	7	12
June	0	-	-	22	0	-
Total	61	13	21	219	13	6

TABLE 14.

Frequency of occurrence of *B. cereus* in milk from particular country creameries.

Country creamery	Occasions of sampling	Samples with *B. cereus* in 1 ml.		
		No.	%	Mean count
YB	5	1	20	10
XC	6	1	17	2
WD	9	5	56	4
TG	5	1	20	1
PL	20	3	15	13
HS	4	2	50	2
GS	5	1	20	1
Total	54	14	26	5

types of samples from the same creamery is good, suggesting some influencing factor associated with that creamery. In Table 13, the samples are further sub-divided according to the month when they were taken. The highest incidence of the organism occurred in January at creamery A and in May at creamery B. No particular seasonal effect seems to be operating.

Samples from each source were now examined in greater detail.

Samples from country creameries. Sixty two samples were taken from 11 country creameries, the number of occasions of sampling varying from 1 to 20. Of these creameries, 4 gave negative results on every occasion. Results for the remaining 7 creameries are summarized in Table 14. Twenty six percent of samples from these creameries showed the organism in 1 ml. compared with 23% of all samples. Three creameries - WD, PL and HS - account for 10 or 71% of the positive results, and 30% of all samples from these creameries were positive. There is thus a slight tendency for the organism to be associated with particular country creameries. It is worth noting at this stage that of the 15 cases of bitty cream obtained in these samples, 14 occurred at creameries WD, PL and XC. Moreover, 40% of the total samples taken from these three creameries developed bitty cream compared with a figure of 24% for all creameries.

Samples from individual farms. The 60 farm samples taken at creamery A were from 48 producers on 6 occasions, so that only

TABLE 15.

Frequency of occurrence of *B. cereus* in milk from particular farms.

Farm	Occasions of sampling	Samples with *B. cereus* in 1 ml.		
		No.	%	Mean count
1A	5	3	60	1
3B	3	1	33	1
11B	3	1	33	1
30F	5	2	40	2.5
47M	3	1	33	1
62S	3	1	33	1
68S	4	2	50	26
77W	4	1	25	1
37G	3	1	33	1
73W	2	1	50	1
Total	35	14	40	5

8 farms were sampled more than once. Of these, 7 were negative on all occasions of sampling and the other 1 gave a count of 1/ml. on one occasion and < 1/ml. on the other.

The 255 farm samples taken at creamery B were from 63 producers on 22 occasions. Of these, 53 were negative on every occasion of sampling. The results for the other 10 producers are summarized in Table 15. It can be seen that 40% of samples taken from these 10 producers were positive, compared with 13% for all producers. This suggests a tendency for the organism to be associated with particular farms. It must be remembered that the negative results do not signify that the organism was entirely absent from a milk sample, but merely that it was not detected in 1 ml. of that sample. Thus, it would be more correct to suggest that there is a tendency for relatively high counts of the organism to be associated with particular farms. It was felt that detailed investigation of the methods in use on farms showing such results was warranted.

During a spell of particularly warm weather in the summer of 1955, samples from 17 producers supplying a country creamery, T, were examined for the presence of B. cereus in 1 ml.. (It should be mentioned in passing that samples from all these farms were giving total counts of 200,000/ml.. Of these, 4 or 25% - farms L, M, B and H - showed the presence of the organism, with counts of 13, 4, 3 and 4/ml. respectively. Visits were made to farms L, M and B, and, for purposes of comparison, to two other farms, S and W, for which counts of <1/ml. B. cereus had been obtained. Their equipment was

TABLE 16.

The occurrence of B. cereus on the equipment of selected farms

Source of sample	Nature of sample	B. cereus (count/ml.)				
		Farm B	Farm L	Farm M	Farm S	Farm W
Cans at creamery T	Bulk milk	3	13	4	<1	<1
Clusters	500 ml. rinse	<1, <1, <1, <1	<1, <1, <1, <1	3, <1	-	<1, <1, <1, <1
Unit cans	500 ml. rinse	<1, <1, <1	<1, <1, <1, <1	<1, <1, <1	-	<1, <1, <1, <1
Milk lift	500 ml. rinse	<1	-	-	-	-
Cooler	Swab	<1	<1	<1	-	<1
Cans	500 ml. rinse	118, 142, <1, <1, <1, <1	>200, <1	46, 1, 3	<1, <1	<1, <1, <1, <1
Can brushes	Rinse	9, <1	51	1, <1	5, 150	<1, <1
Can rag	Rinse	38+	>200	17	<1	-
Water for cleaning cans	-	<1	94, 2	1	-	-
Steaming stool	Swab	200	-	-	-	-
Unit cans	1st milk into	<1, <1, <1	<1, <1, <1, <1	<1, <1	-	<1
Cooler	1st milk over	<1	-	<1	1	<1
Cans	Bulk milk	<1, <1	<1, 1	1	-	<1
Cans	Bulk milk (stored overnight)	-	30	<1	<1	<1

TABLE 17.

The occurrence of *B. cereus* in milk samples taken from different parts of the plant of a creamery.

Source of milk	Occasions of Sampling	Samples with *B. cereus* in 1 ml.		
		No.	%	Mean count
Weigh tank	5	0	-	-
Before 1st pump	6	1	17	1
After 1st pump	7	1	14	6
Storage tanks	13	6	46	2
Before 2nd pump	4	1	25	1
After 2nd pump	8	1	13	3
Balance tank	9	3	33	2
Cooler exit	8	0	-	-
Can residues[*]	80	38	48	26,000
Can rinses (500 ml.)[Ø]	22	14	64	16,000[θ]

* = Before washing
Ø = After 'sterilization'
θ = Count per can

examined in detail by means of rinses, swabs, etc.. Results for these five farms appear in Table 16.

High counts of the organism were found to be particularly associated with cans and materials used in cleaning them. These cans receive only a cold water rinse at the creamery, the actual sterilization being left to the farmer. Those arriving at the creamery on a particular day are tipped, rinsed and stand overnight on the platform. The following day, they are returned to the farmer. Samples of milky water were taken from 8 such cans after they had stood overnight. Four of these showed high counts of B. cereus.

The fact that high counts were found on the farms in rinses of cans which had been steamed - even though inadequately - suggested that the organism was present in the spore stage.

Samples from creamery plant. As mentioned previously, all samples taken from creamery A showed a higher incidence of B. cereus than those taken from creamery B. The results of detailed examination of the plant of creamery A are to be found in Table 17 (upper part only). In all cases, the organism seemed to be distributed in a random sort of way through the plant and there was nothing to suggest build-up at any particular point. It seemed to be significant that the higher incidence of the organism from creamery A was reflected in samples from the individual farms as well as from the creamery plant itself. This suggested some influencing factor

peculiar to the creamery. However, each producer sent his milk to the creamery in his own cans and these were returned to him after washing. Thus the only treatment common to all farms supplying the creamery and peculiar to that creamery was the can washing. This was done by means of a mechanical can washer. It was found that apart from the producers' cans certain others owned by the creamery were also put through this washer. In these cans, bulked pasteurized milk is sent out to institutions such as schools, hotels and restaurants. The creamery, as owner of the cans, is responsible for cleaning them. Thus, at the particular institution the majority are simply rinsed out after use and may be standing for days before being collected and returned to the creamery for cleaning. Eighty samples of watery residues from these cans were taken on their return to the creamery. As may be observed from Table 17 (lower part), almost half of these residues showed large numbers of the organism. It remained now to demonstrate the presence of the organism in the cans after they had passed through the washer and were assumed to be sterile. Rinses were taken of 22 of these cans. Fourteen were found to contain B. cereus in numbers varying between 250 and 75,000 per can. Thus, the sterilization process which the cans had undergone in the washer was obviously not adequate for the destruction of B. cereus spores. It was felt that contamination of producers' cans was likely to occur from these cans in the washer although there is no direct evidence

TABLE 18.

The proportion of milk samples from farms, country creameries and creamery plant developing bitty cream after storage at 22 for 48 hr.

Source of milk	Total samples	Samples developing bitty cream											
		Creamery A*				Creamery B*				All samples			
		Raw		Lab. past.≠		Raw		Lab. past.≠		Raw		Lab. past.≠	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Farms	280	0	-	10	16	2	<1	18	8	2	<1	28	10
Country creameries	62	0	-	15	35	0	-	0	-	0	-	15	24
Creamery plant	84	0	-	18	30	0	-	2	8	0	-	20	24
Total	426	0	-	43	25	2	<1	20	8	2	<1	63	15

* Creamery where samples taken

≠ Laboratory pasteurized

TABLE 19.

The relationship between the presence of B. cereus in 1 ml. of a milk sample and the development of bitty cream after storage at 22° for 48 hr.

Results of tests	Creamery A*		Creamery B*		Other creameries*		Total	
	No.	%	No.	%	No.	%	No.	%
Bitty cream + (all)	43	25	20	8	0	-	63	15
Bitty cream +; B. cereus +	16	10	4	2	0	-	20	5
Bitty cream +; B. cereus -	27	16	16	6	0	-	43	10
B. cereus + (all)	36	22	12	5	4	26	52	12
B. cereus +; bitty cream -	20	12	8	3	4	26	32	8
B. cereus -; bitty cream -	101	62	219	89	11	74	331	77
Total samples	164	-	247	-	15	-	426	-

* Creamery where samples were taken

TABLE 20.

The relationship between the average no./ml. *B. cereus* in milk samples and the development of bitty cream after storage at 22° for 48 hr.

	B. cereus (mean count/ml.)								
	Creamery A*			Creamery B*			All samples		
	B+	B-	All	B+	B-	All	B+	B-	All
Farms	3	1	2	2	11	6	3	6	4
Country creameries	9	3	5	-	-	2	9	3	4
Creamery plant	3	2	2	-	1	1	3	2	2
All samples	5	2	3	2	8	5	5	3	4

* Creamery where samples were taken

B+ signifies samples in which bitty cream developed

B- signifies samples in which bitty cream did not develop.

that this is so.

It was discovered later that it is the practice at this creamery to re-pasteurize bottled milk returned from the previous day. This milk is tipped into the cans mentioned above on the night when it is returned to the creamery and stands in them until the following day on the creamery platform during which time spore contamination from the sides of the can would be possible. The quantity of milk treated in this way varies but up to 900 gal. may be involved, allowing considerable contamination of the plant if conditions are favourable.

Relationship between the size of the original inoculum of B. cereus and the development of bitty cream

Table 18 shows that of the 426 samples examined, 15% developed bitty cream in the laboratory pasteurized sample on storage, whereas considerably less than 1% developed the fault in the corresponding raw sample. A higher incidence of the fault occurred at creamery A than at creamery B (25% compared with 8%). This can be associated with the observation that a higher degree of contamination by B. cereus occurred at creamery A than at creamery B.

In Table 19, the relationship between the presence of B. cereus in 1 ml. and the development of bitty cream has been examined. Of the 63 samples in which bitty cream developed, only 20 or 32% were associated with B. cereus detectable in 1 ml. of the original sample. The average counts for each type of sample are given in Table 20. It can be seen that

TABLE 21.

Comparison between monthly mean total 37° counts and laboratory pasteurized 30° counts of those samples which developed bitty cream and those, known to contain B. cereus, which did not develop the fault

Month	No. samples			Total 37° count/ml.			Lab. past. 37°count/ml.		
	Total	B+	C+	All	B+	C+	All	B+	C+
February	47	3	1	23,000	29,000	18,000	7,000	160	200
March	47	4	2	102,000	18,000	54,000	4,000	700	110
April	43	1	2	46,000	11,000	6,000	9,000	20	320
May	60	10	4	47,000	59,000	204,000	25,000	83,000	100,000
June	22	1	0	74,000	28,000	-	46,000	2,000	-
Total	219	19	9	56,000	43,000	106,000	13,000	44,000	47,000

B+ signifies samples in which bitty cream developed.

C+ signifies samples known to contain B. cereus in which bitty cream did not develop.

counts were uniformly low. It seems that the development of bitty cream is determined at least in part by factors other than the size of the original inoculum.

The relationship between the number and types of other bacteria present and the development of bitty cream

Total count and laboratory pasteurized count. In the case of creamery B, laboratory pasteurized 30° counts and total 37° counts were carried out on all farm samples. In Table 21, monthly averages for these tests are given for all samples and for samples showing bitty cream, or B. cereus in 1 ml. but no bitty cream. Considering first total 37° counts, there was very little difference between the average count for all samples and the average for those developing bitty cream after pasteurization. On the other hand, the one milk developing bitty cream before pasteurization had a total count of 2,000/ml., which was considerably below the average. Further, in the 9 cases in which B. cereus was detected in the raw milk, but in which bitty cream did not develop, the mean count was 106,000/ml.. However, individual counts ranged from 800-790,000/ml., suggesting that even if the presence of large numbers of other organisms was exerting an important deterrent effect to the development of bitty cream in some cases, there was certainly some other factor operating in other cases. Considering now the laboratory pasteurized 30° count in the different groups of samples, again no consistent relationship emerges. For example, although the samples showing B. cereus in 1 ml. but

TABLE 22.

Comparison between counts of spore-formers at 45° of those samples which developed bitty cream and those, known to contain B. cereus, which did not develop the fault

Samples	No. Samples				Spore-formers at 45° (count/ml.)			
	All	B+	C+	Clear	All	B+	C+	Clear
Farms	61	10	6	45	330	270	350	350
Creamery plant	64	17	11	36	80	63	148	65
Total	125	27	17	81	200	140	220	220

B+ Signifies samples in which bitty cream developed.

C+ Signifies samples known to contain B. cereus in which bitty cream did not develop.

TABLE 23.

Comparison between counts of thermoduric streptococci at 45° of those samples which developed bitty cream and those, known to contain *B. cereus*, which did not develop the fault.

Source of Samples	No. Samples				Streptococci at 45° (count/ml.)			
	All	B+	C+	Clear	All	B+	C+	Clear
Farms	61	10	6	45	2,000	3,000	1,400	200
Creamery plant	64	17	11	36	37,000	71,000	37,000	68,000
Total	125	27	17	81	19,000	45,000	25,000	9,000

B+ signifies samples in which bitty cream developed.

C+ signifies samples known to contain *B. cereus* in which bitty cread did not develop.

no bitty cream had average counts of 47,000/ml., individual counts varied from <10/ml. up to > 400,000/ml., and 6 of the 9 samples had 200/ml. or less.

Estimate of aerobic spore-formers growing at 45°. Estimates of numbers of aerobic spore-formers growing at 45° were done on all samples at creamery A. The results for farms and plant samples appear in Table 22. Very little difference can be seen between the average count for all samples in which bitty cream developed and those in which it did not.

Estimates of thermoduric streptococci. Samples from creamery A were tested for the presence of streptococci able to grow at 45°, i.e. the thermoduric group. From Table 23, it can be seen that there is no relationship between numbers of thermoduric streptococci and the development of bitty cream.

Inhibitors of the growth of B. cereus in raw milks.

Attempts were made to isolate from raw milks organisms able to inhibit B. cereus. The method used was that of double layer plates. The lower layer was seeded heavily with a strain of B. cereus and the upper was inoculated with an appropriate dilution of the milk under test. It was expected that a cleared zone would appear underneath the colonies able to inhibit the test strain. Both spores and vegetative cells of B. cereus were tested in this way. Several raw milks were used and from a total of about 200 colonies, 2 able to cause clearing of the lower layer were isolated. No other colony showed any visible inhibition. The two inhibiting colonies were tested

T A B L E 24.

Inhibition of *B. cereus* by isolates from milk.

Isolate	Inhibition of *B. cereus* (4 strains)
1	+
2	+
3-18	-
19	+
Y1	+
Y2	-
S. lactis (stock)	+
Ps. fluorescens (stock)	+

TABLE 25

Inhibition of *B. cereus* by *S. lactis* in yeastrel milk agar and nutrient agar containing varying amounts of milk

Medium	Inhibition of *B. cereus* (10^{-1} in.)				
	Strain 12	Strain 806	Strain 479	Strain 490	Mean
Yeastrel milk agar	0.5	2.5	1.5	1.0	1.5
Nutrient agar (N.A.)	3.5	6.0	2.0	4.5	4.0
N.A. + 20% milk	0.5	0.5	0.5	0.5	0.5
N.A. + 10% milk	1.0	5.0	1.0	2.0	2.25
N.A. + 5% milk	1.0	6.5	0.25	2.5	2.5
N.A. + 1% milk	3.0	9.0	1.0	5.0	4.5

further by streaking on to yeastrel milk agar, incubating for 24 hr. at 30° and then streaking four test strains of B. cereus at right angles to the line of growth. The degree of inhibition was roughly assessed from the width of the zone of no growth between test organism and the strain of B. cereus.

Another method used to isolate inhibiting organisms was to pick colonies at random from a total count plate and to test these by the streak method described above. Seventeen isolates were tested in this way and the results for these as well as for the two original isolates obtained by the other method are given in Table 24. Also tested were two contaminants from yolk which appeared to have an inhibitory effect against the B. cereus strains. The inhibitory organisms 1, 2, 19 and Y1 were all found to be Gram-negative rods, producing a fluorescent pigment. On this basis they were regarded as members of the genus Pseudomonas. A very rough classification of the other isolates on the basis of litmus reaction and morphology indicated that the majority were streptococci. As can be seen from the table, the stock strains both of Pseudomonas fluorescens and of Streptococcus lactis also showed marked inhibition of the four strains of B. cereus tested. The S. lactis strain was a suspected nisin producer. This probably explains its ability to inhibit B. cereus.

Further studies with the stock strain of S. lactis. In Table 25, the effect of different media on the ability of the S. lactis strain to inhibit B. cereus can be seen. Relative

FIG.1

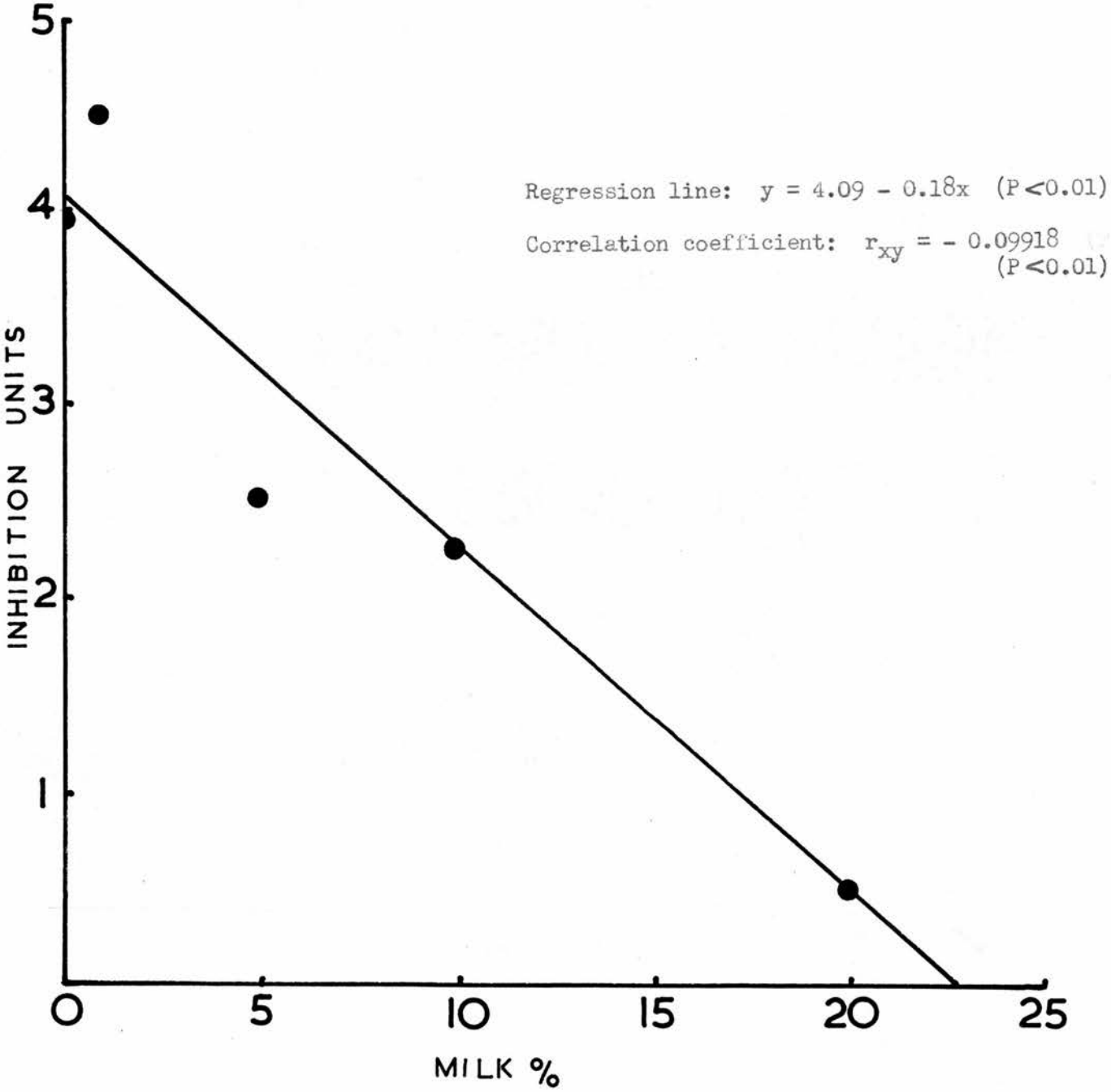


Fig. 1. The inhibition of B. cereus by a strain of S. lactis in the presence of varying concentrations of milk.

TABLE 26

Inhibition of *B. cereus* by *S. lactis* at different incubation temperatures.

Incubation temperature (°C)		Inhibition of *B. cereus* (10^{-1} in.)			
S. lactis	*B. cereus*	Strain 12	Strain 806	Strain 479	Strain 490
22	22	6	7	5	7
22	30	3	4	1	3
30	22	6	5	4.5	6
30	30	2.5	2.5	1.5	4

to nutrient agar (with or without additions of milk) inhibition on yeastrel agar is poor. Now the results given in Table 24 were based on tests carried out on yeastrel milk agar. They were therefore repeated using nutrient agar, but the 15 isolates which were negative previously remained negative. It may be noted from Table 25, that as the concentration of milk in the agar increases, so does the degree of inhibition decline (see also Fig. 1). It is assumed that the milk absorbs the inhibiting substance and thus reduces its effectiveness.

The effect of temperature on inhibition by S. lactis was tested by growing the inhibiting organism on agar for 24 hr. at 22 and 30° and then inoculating the plates with B. cereus and incubating at 22 and 30°.

From Table 26, it can be seen that for all strains, inhibition is less at the optimum temperature for growth of B. cereus than at 22°.

A note on the action on milk of certain lecithinase-producing organisms other than B. cereus

The spot plate method described earlier (p.28) for the detection of bitty cream occasionally revealed the presence of lecithinase-producing organisms other than B. cereus. These were found only in raw milk samples and were invariably Gram-negative rods. Although their presence was not associated with changes characteristic of bitty cream in the stored sample, it seemed of interest to determine whether

these organisms could induce such changes when grown in pure culture.

Six isolates were examined further and all were placed in the genus Pseudomonas using the criteria of Paton (1956). Three strains were used for the tests, one belonging to the Ps. aeruginosa group and two to the Ps. fluorescens group. These strains were inoculated in pure culture into aseptically drawn raw milk and incubated at suitable temperatures to determine whether bitty cream developed. For comparison, two strains of B. cereus and an uninoculated control series were set up at the same time.

For detecting bitty cream, two methods were used. The first was that of Stone & Rowlands (1952) involving the use of a small inoculum (a straight wire) and long incubation (24 hr.) at a relatively low temperature (22°). Twenty ml. milk were held in 150 ml. conical flasks and, after the storage period, the presence of bitty cream could be detected by gently swishing the milk in the flask. The second involved the use of a large inoculum (2 drops from a Pasteur pipette) and short incubation (the end point was usually reached within 12 hr.) at a relatively high temperature (30°). A series of tubes containing 5 ml. milk was set up for each strain and a whole tube removed every 2 hr. until some change in the milk was observed. A little cream was withdrawn and mixed with water on a black surface. The presence of small pieces of cream floating in the water signified the presence of the fault.

By both methods it was found that the Pseudomonas strains all produced clotting without preliminary breaking up of the cream layer. Thus, one must assume that the strains did not possess the enzyme or enzymes, additional to the lecithinase, which are involved in the development of bitty cream.

DISCUSSION

Taking the presence of one or more cells in 1 ml. of raw milk as an arbitrary index of contamination by B. cereus, a relatively small proportion of samples (13%) were found to be positive. However, there was a marked tendency for the organism to occur in milk from a particular creamery and from particular farms. This was found to be associated at least with improper sterilization of cans, and probably also with the history of the cans prior to sterilization. The fact that the organism is able to survive the sterilization of cans strongly suggests that it is in the spore stage (for the vegetative cell is relatively heat susceptible, being destroyed within a few minutes at 63°). Moreover, the importance of the previous history of the can may be in providing an opportunity for spore formation.

Of 426 milk samples tested, both raw and laboratory pasteurized, bitty cream appeared in 2 raw sub-samples, while 63 laboratory pasteurized sub-samples developed the fault. Again this finding focuses attention on the spore. Since the temperature of storage was the same for all samples, this can be eliminated as a factor in the non-development of the fault in raw milks. There remain, then, two possibilities: Firstly, that spore germination proceeded at the same rate in both types of milk, but that vegetativedevelopment was prevented from reaching a high enough level in the raw samples either due to competition from other organisms present or due to

inhibition by a particular group; secondly, that spore germination was somehow retarded in the raw samples relative to that in the pasteurized samples. The evidence from the foregoing work favours the second possibility, for bitty cream did not develop in raw milks of low total count nor could any relationship be shown with the numbers of any particular bacterial group. Further, of several hundred organisms from raw milks examined for inhibition of B. cereus, only 3 were found to be capable of it. All were Pseudomonas species, and since this group is not normally dominant in raw milk populations, it is felt that this type of inhibition, if it ever occurs in practice, will only do so rarely. In addition the stock S. lactis which was isolated from milk showed inhibition, but this was probably due to nisin production. It is interesting to note that in the studies of this S. lactis strain, the antibiotic effect was diminished in the presence of milk and at the optimum temperature for growth of B. cereus.

Finally, no relationship could be seen between the extent of initial contamination and the development of bitty cream. The fault developed in a number of milks containing before storage fewer B. cereus than 1/ml. and, on the other hand, it did not develop in a number of cases where more than 1/ml. was present.

All the evidence obtained in this section of the work has emphasized the importance of the spore. It seemed logical, therefore, to proceed to an examination of some of the factors

inducing sporulation of B. cereus with special reference to the conditions obtaining in milk cans. This was followed by an examination of some of the factors stimulating germination of B. cereus spores in milk with special reference to pasteurization.

SECTION III

Studies of some of the factors influencing the spore formation of *Bacillus cereus* in milk

REVIEW OF LITERATURE

The large volume of information which has accumulated on spore formation in the genus Bacillus has emphasized the extremely complex nature of the process. (For a general summary of all aspects of the subject, the review of Murrell (1955) is recommended.). The factors which may influence it, both physical and chemical, are many. Moreover, there is such variation in behaviour within the genus, that it is virtually impossible to predict the response of a certain species to a given stimulus from results with even closely related species under the same conditions.

Although few results are available on the subject of sporulation in milk, it is generally accepted that most species normally form few spores in that medium (e.g. Grinstead & Clegg, 1955). It is thus significant that B. cereus appears to form spores in milk cans at some stage before they are cleaned (see previous section). A number of possible reasons are suggested by the literature.

Firstly, it seems possible that metal contamination from the cans might stimulate sporulation. Before milk comes into contact with metal surfaces, it is very low in heavy metals (Waite, 1947). Thus any prolonged contact with a corrosible surface is likely to result in considerable contamination. At the present time, most milk cans are of tinned steel (Meanwell, 1956), which is susceptible to corrosion by milk acids, especially in the presence of oxygen. Thus, when sour

milk is allowed to stand for a long time in a shallow layer in the bottom of a can, conditions will be ideal for the passing of metal ions into solution. Iron and tin seem the most likely contaminants from tinned steel. Curran & Evans (1954) found marked stimulation of the sporulation of B. subtilis in milk when trace amounts of iron or manganese were added. However, there was no response to any other metal ion tested including tin, and other species, including strains of B. cereus, did not even respond to iron or manganese. Weinberg (1955) also demonstrated stimulation of B. subtilis sporulation by iron, this time in nutrient broth, but he attributed this response to the presence of trace amounts of manganese in the iron salts. The manganese effect with B. subtilis was also recorded by Charney, Fisher & Hegarty (1951). The only reported case of metal stimulation of sporulation in B. cereus of which one is aware was by potassium ion in certain deficient media, the response being of the order of a thousand fold (Foster & Heiligman, 1949).

Secondly, increased sporulation in cans might occur in response to increased oxygen availability. This would occur when milk was allowed to lie for long periods in shallow layers in the bottom of cans. It is generally agreed that an abundant supply of oxygen is essential for maximum sporulation of all species of aerobic spore-formers (Leifson, 1931; Brunstetter & Magoon, 1932; Knaysi, 1945; Roth & Lively, 1956, etc.). Thus, if other conditions were favourable, aeration

would be likely to be suitable for sporulation.

Thirdly, it seems that sporulation might occur in response to dilution of milk. This would be the situation when cans were rinsed out with cold water after use and allowed to stand for long periods before cleaning. Williams (1930) observed that sporulation of B. subtilis in peptone solutions increased with dilution of the medium. This effect was confirmed with B. mycoides (Brunstetter & Magoon, 1932; Knaysi, 1945) and with Bacillus megatherium (Bayne-Jones & Petrilli, 1933). On the other hand, Schmidt (1950) observed increasing sporulation with increasing concentration of peptone in the case of a number of flat-sour thermophiles.

The examination under suitable aeration conditions of the effects of dilution of milk and of the addition to it of certain cations on the sporulation of B. cereus therefore provided a starting point in this section of the work.

METHODS

Organisms. The test organisms were three strains of B. cereus (C.12, C.14, C.15) which were isolated from milk during the earlier part of the work.

Preparation of inoculum. Some workers (e.g. Curran & Evans, 1954) have favoured the use of spore inocula. A vegetative inoculum was preferred here because it was felt that with spore inocula irregularities in germination rates could introduce an unnecessary complication. Grelet (1951) has also noted that cell division from spore inocula occurs less rapidly and regularly than from vegetative inocula.

Because of the inhibitory effect of glucose on sporulation (Knaysi, 1945; Grelet, 1951), glucose peptone water (see appendix, p.i) was used to obtain the maximum number of cells in the vegetative condition. A liquid medium was used for the same reason. Transfers through glucose peptone water were made on at least 4 successive days; incubation was at 22° in order to slow down growth. On the fifth day, when plate counts showed the presence of less than one spore per ml., the inoculum was prepared. The cells were centrifuged down and washed 4 times in sterile glass-distilled water. Finally, they were re-suspended in sterile glass distilled water and used for inoculation of the test medium as soon as possible. Inoculation was at the rate of 1 ml. of suspension of the required dilution to 9 ml. of test medium.

Conditions under which spore formation was examined. It was

desirable to provide optimum conditions of aeration in order to minimise the limiting effect of this factor. The method most commonly used for studies of this kind has been that of shaking relatively small volumes of the test medium in flasks throughout the incubation period and withdrawing sub-samples for testing at the required time intervals. There were certain objections to the use of that method in this laboratory, primarily the difficulty of maintaining a constant incubation temperature throughout the shaking period. Thus another method was sought. Various ways were tried and eventually it was found that satisfactory aeration, i.e. giving results comparable to the shaken flask, was obtained in the following way. The inoculated medium was pipetted in 1 ml. amounts into a series of sterile 6 x $\frac{5}{8}$" test tubes. Each tube was then incubated in the horizontal position, so that the medium spread out in a thin film over the wall of the tube. At each time interval one or two tubes per treatment were withdrawn and the entire contents used for testing. Thus sampling error was eliminated and likewise the possibility of contamination of the rest of the medium during sampling. Initially, there was some difficulty due to drying out of the medium during long incubation periods. For this reason, rubber bungs were used in the tubes instead of cotton wool plugs. It was found that the volume of air in the tubes relative to the volume of medium was sufficient to allow this change without alteration in the results obtained.

In all experiments, incubation was at 30°. Except where

otherwise stated, the test medium was sterile milk.

Bacteriological tests. Each 1 ml. sample was diluted 1 in 10 and thoroughly shaken before testing. It was then divided into two parts. One portion was held at 65° for 30 min., a treatment which was found to destroy the vegetative cells, and then plated out for a spore count. The other was plated for a total viable count. In both cases, the medium used was nutrient agar with 0.1% soluble starch to remove substances inhibitory to spore germination (Olsen & Scott, 1946) and incubation was for 24 hr. at 30°. In addition, estimates of percentage sporulation were made on smears stained with dilute methylene blue, a stain which showed up both spores and vegetative cells quite satisfactorily.

Expression of Results. To allow for differences in total growths in the different treatments, results were expressed as percentage of spores rather than as total spores. The weakness of this method, as Knaysi (1945) has pointed out, is that autolysis of vegetative cells sometimes occurs after prolonged incubation periods. Where this has occurred in the results about to be presented no estimate of sporulation was made and reference has been made to it in the ~~text~~ figures.

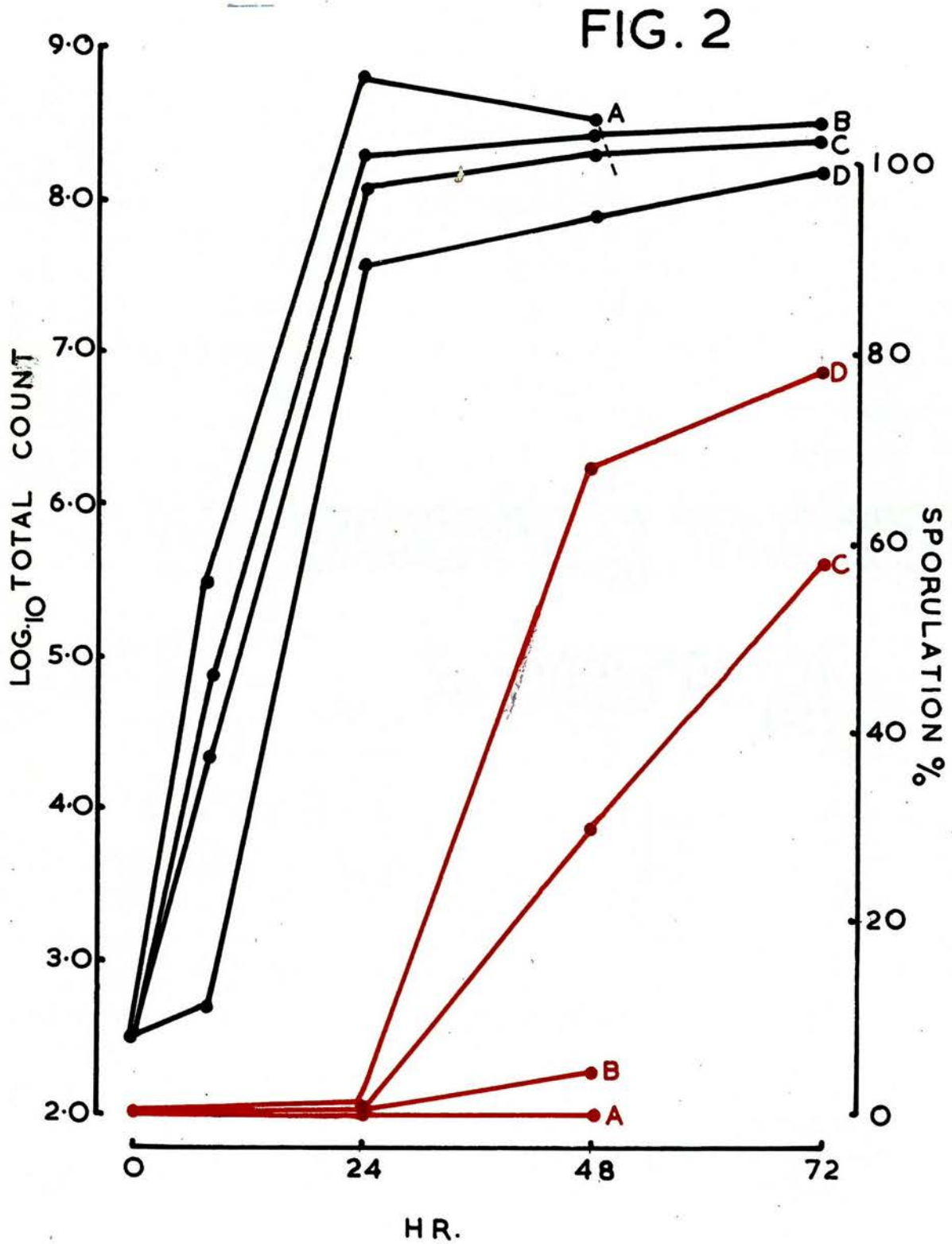


Fig. 2. The effect of dilution of milk on % sporulation and total growth of B. cereus (strain C.14). (A = undiluted; B = 1/5; C = 1/10; D = 1/50; —— sporulation; —— total growth).– – – autolysis

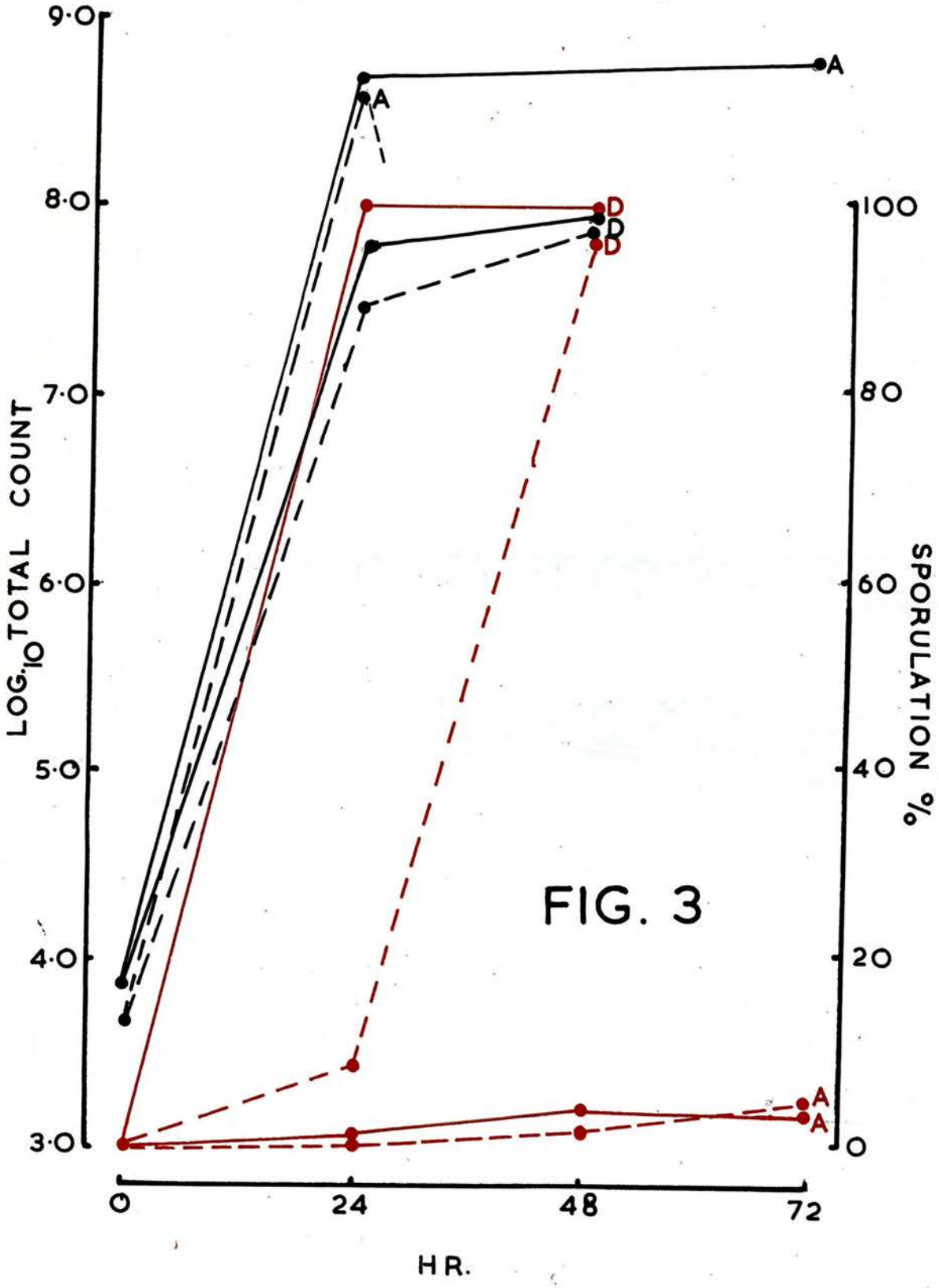


Fig. 3. The effect of dilution of milk on % sporulation and total growth of B. cereus (═══ strain C.12; ═══ strain C.15; ═══ sporulation; ═══ growth). ---- autolysis

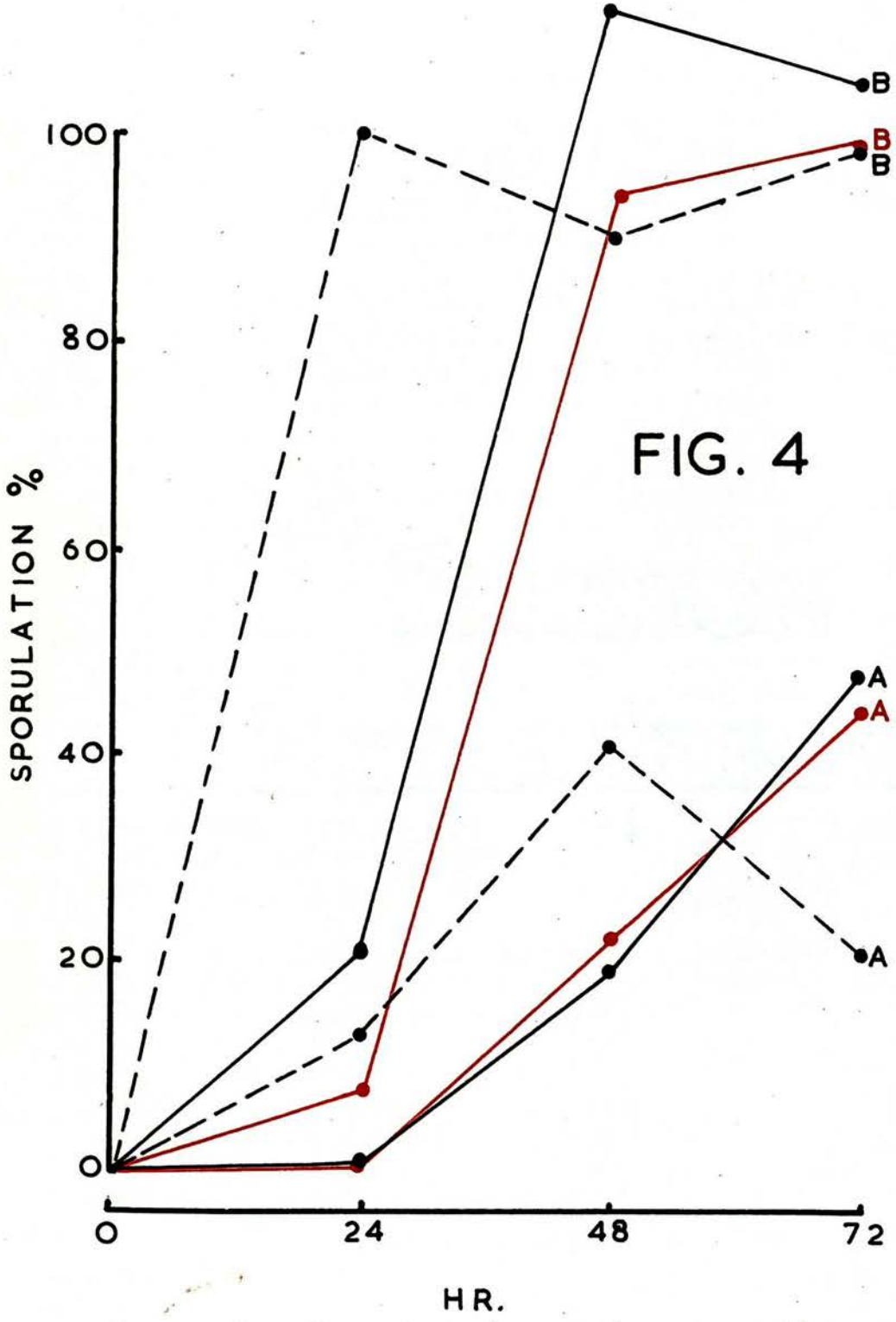


FIG. 4

Fig. 4. The effect of dilution of 5% peptone, 1% nutrient broth and 1% casamino acids on % sporulation of B. cereus. (A = undiluted; B = 1/10; —— peptone; —— casamino acids; --- nutrient broth).

RESULTS

The effect of dilution

A preliminary experiment was carried out using strain C.14 and the following four media:-

A - sterile milk

B - sterile milk, diluted 1/5)
C - sterile milk, diluted 1/10) with glass-distilled water
D - sterile milk, diluted 1/50)

The results are presented graphically in Fig. 2. It can be seen that both the rate of sporulation and the final percentage of spores formed increased with dilution. Strains C.12 and C.15 were now tested in media A and D. Again, as may be seen in Fig. 3, a marked stimulation in sporulation occurred in response to dilution. Ten other strains of *B. cereus* from milk were found to behave in the same way, 80-100% sporulation occurring within 48 hr. at 30° in milk diluted 1/50.

The literature suggested that the dilution effect was a fairly general one. Thus, a number of other media were tried, using strain C.14. It was found that sporulation was stimulated by dilution of all three media tested, namely 1% casamino acids solution, 5% peptone and a nutrient broth containing 1% beef extract and 1% peptone (see Fig. 4).

Attempts to explain the effect of dilution

It seemed reasonable to suppose that the effect of dilution on sporulation was attributable to one or more of the following:- reduction in concentration of nutrients or of substances

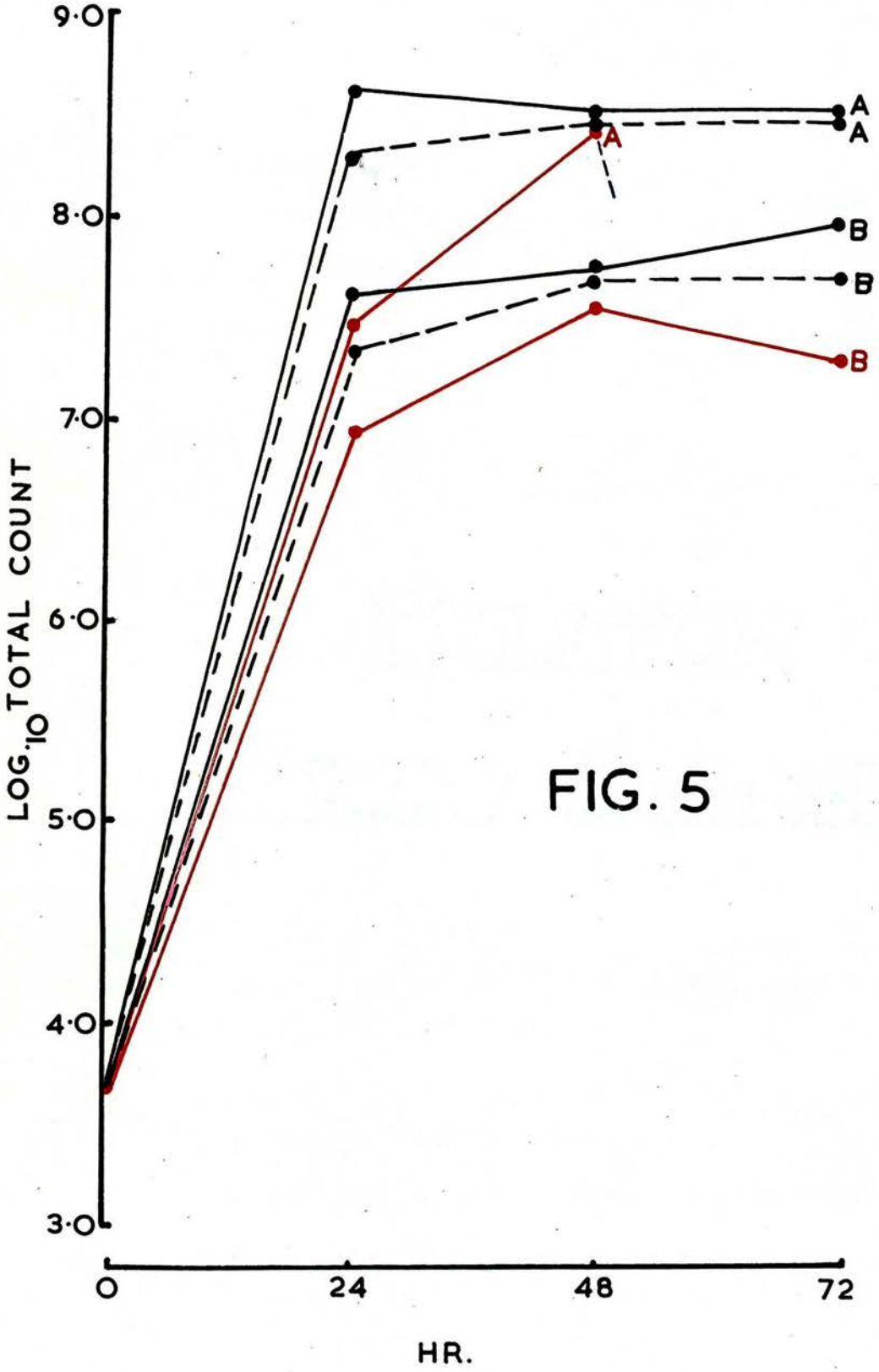


FIG. 5

Fig. 5. The effect of dilution of 5% peptone, 1% nutrient broth and 1% casamino acids on growth of B. cereus (A = undiluted; B = 1/10; —— peptone; ▬ casamino acids; – – – nutrient broth). – – – autolysis

inhibitory to sporulation, or increase in oxygen availability due to reduction in cell density. Experiments were carried out with the intention of determining which of these factors was operating. The design of these experiments was, however, such that the desired information could not in fact be extracted from the results. The reason for this was that no account was taken of differences in total growth of the organism under the different conditions. For example, Monod (1949) has shown that the total population attained by a given organism in a given medium decreases with dilution. Now, in Fig. 2, 3 and 5 rough growth curves for the different media and treatments have been constructed. Although in every case, the final population levels do decline with increasing dilution, in Fig. 2 this is less marked than had been expected. This is especially evident if the comparison is made with the decline in population levels with dilution seen in Fig. 3 and 5, and also in Fig. 6 where the behaviour of strain C.14 in dilutions greater than those in Fig. 2 are given. This has been interpreted as meaning that, at least with strain C.14, growth has been brought to a halt in undiluted milk due to some factor other than nutrient depletion, i.e. a factor not operating in the diluted media. Whether this be accumulation of toxic products of metabolism, depletion of oxygen or some other effect, it is clear that the mechanism of sporulation cannot be studied under these conditions without regard to total growth. Unfortunately the experimental work about to be described had been carried out

FIG. 6

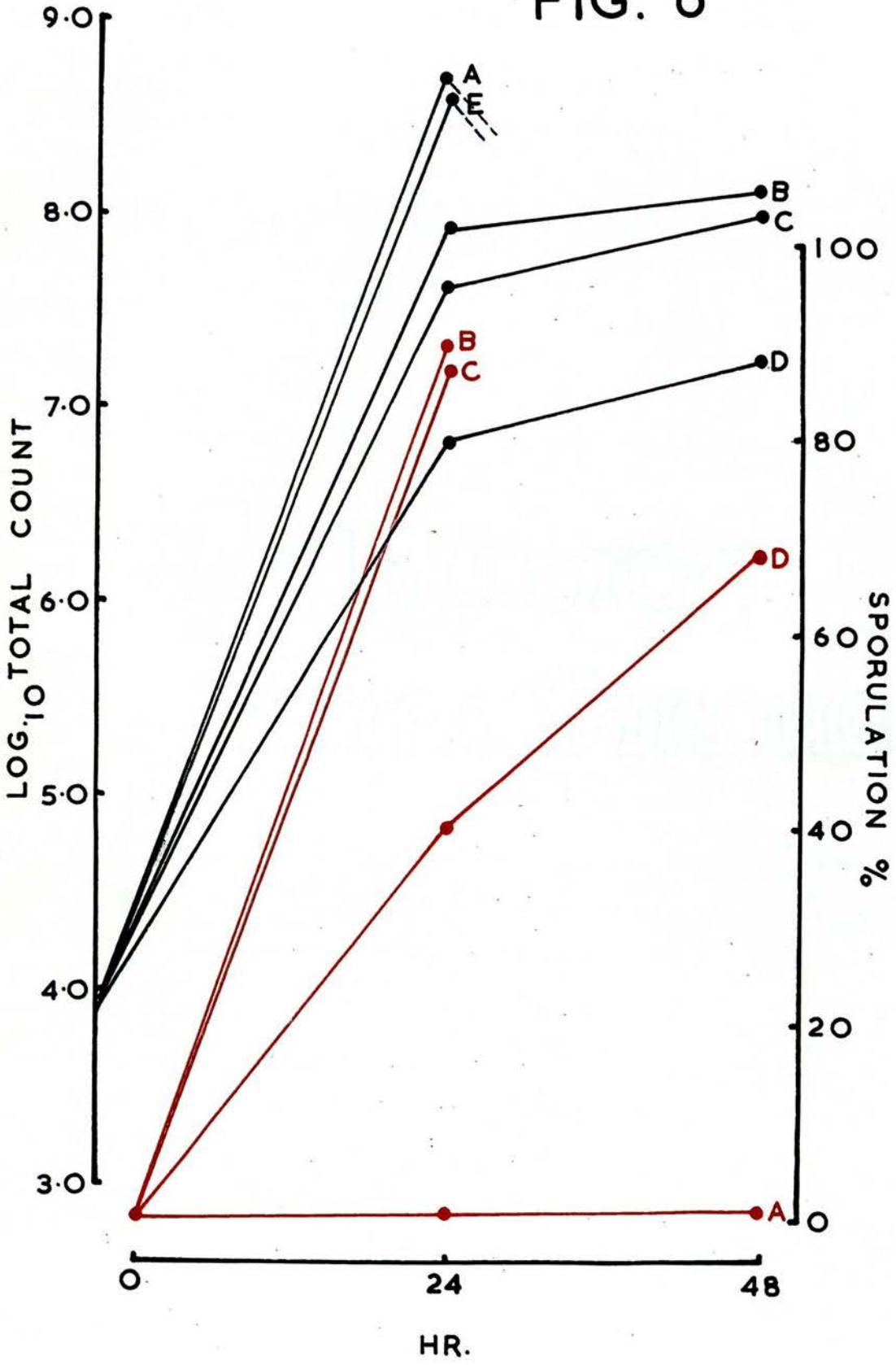


Fig. 6. The effect of dilution of milk up to 1/500 on % sporulation and total growth of B. cereus. (A = undiluted; B = 1/50; C = 1/100; D = 1/500; E = 1/2; —— sporulation; —— growth). --- autolysis

before this was realised and there was no time to do any further work. Thus, the results obtained will only be mentioned briefly.

Reduction in concentration of nutrients. Knaysi (1945) observed that sporulation of some species can occur in the complete absence of nutrients. This finding, together with the fact sporulation in certain media increases as nutrient concentration declines (Williams, 1930; Brunstetter & Magoon, 1932; Knaysi, 1945), led him to suggest that sporulation is the response of healthy cells to starvation conditions (Knaysi, 1948). Thus, according to this theory, the stimulatory effect of dilution on sporulation of B. cereus in milk may be attributed to reduction in nutrient concentration, leading to earlier and more complete nutrient exhaustion. Poor sporulation in undiluted milk will then be explainable in terms of the halting of growth by some factor other than nutrient depletion. (Some evidence for this has already been given.). The results in Fig. 2 provide further support for the theory, the rate of sporulation and the final level attained increasing with dilution. However, it was reasoned that if the starvation theory held, further dilution should result in further stimulation of sporulation. Therefore, strain C.14 was tested in milk diluted 1/50, 1/100 and 1/500. The results are given in Fig. 6. At first, these data were regarded as constituting evidence against the theory of Knaysi, for it can be seen that the level of sporulation at 48 hr. and the rate of sporulation are lower in medium D (1/500) than in media B (1/50) and C (1/100). However, if these

TABLE 27

The effect of additions of small amounts of glucose to milk on sporulation of *B. cereus*.

Condition of milk	Glucose addition (%)	Estimated sporulation (%)
Diluted 1/50	0	80-100
Diluted 1/50	0.05	80-100
Diluted 1/50	0.1	80-100
Diluted 1/50	0.2	80-100
Diluted 1/50	0.3	< 1
Undiluted	0	< 1
Undiluted	0.3	< 1

results are considered in relation to growth, an entirely different interpretation may be placed on them. It might be reasoned that the length of the lag phase of growth of the organism may be prolonged in very dilute media, for example due to the need for the cell to synthesize some essential metabolite (Monod, 1949). (There is some suggestion of such a lag in growth curve D, Fig. 2). Further, since one may assume that growth rates in the logarithmic phase will be independent of dilution (Monod, 1949), there will be a delay in the onset of the stationary phase corresponding to the delay in the onset of the logarithmic phase. This will in turn be reflected in the time of onset of sporulation, for it has been shown that sporulation does not occur until vegetative growth has virtually ceased (Bayne-Jones & Petrilli, 1933; Knaysi, 1946; Hardwick & Foster, 1952). Moreover from the shape of the sporulation curve in medium D (Fig. 6), it seems very likely that sporulation would have gone to completion with further incubation.

The next approach to be tried was to determine the effect of the addition of an energy source to diluted media. Grelet (1951), with B. megatherium in a salts solution, and Hardwick & Foster (1952), with B. mycoides in distilled water, have reported inhibition of sporulation by addition of small amounts of glucose, an effect which these authors have shown to be independent of pH. From Table 27, it can be seen that a sharp fall in the sporulation of strain C.14 in diluted

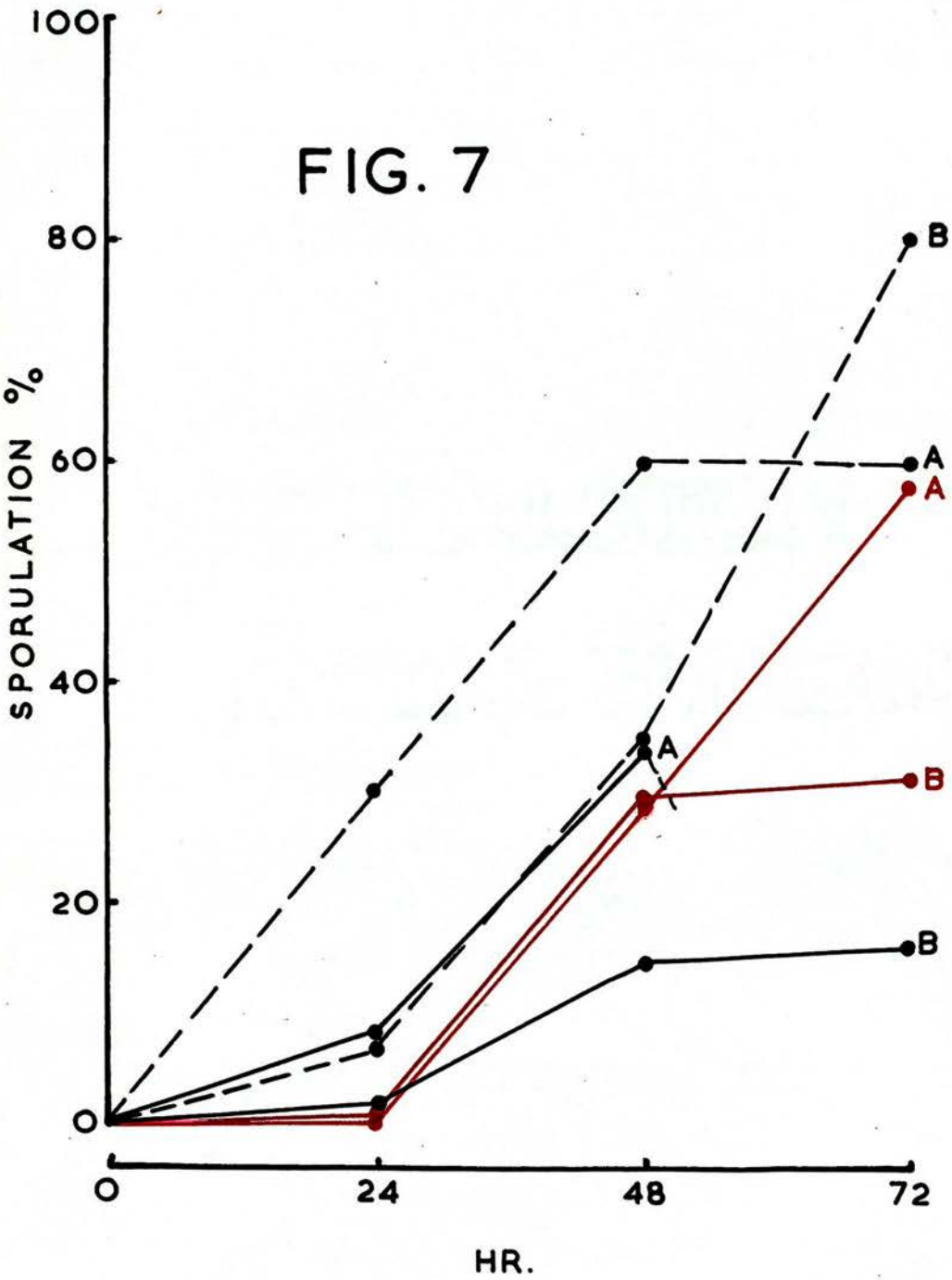


Fig 7. The effect of the addition of 0.15% glucose to 5% peptone, 1% casamino acids and 1% nutrient broth on % sporulation of B. cereus. (A = without glucose; B = with glucose; —— peptone; —— casamino acids; — — nutrient broth). --- autolysis

milk to less than 1% occurred in the presence of 0.3% glucose. This could conceivably have been a pH effect. Unfortunately, there are no data on this point. Lactose concentrations up to 5% - the approximate concentration in undiluted milk - were without effect on sporulation in milk diluted 1/50. In Fig. 7 is recorded the effect on sporulation of incorporation of 0.15% glucose into casamino acids, nutrient broth and peptone. The results are somewhat variable, but on the whole glucose seems to have had an inhibitory effect.

Removal of substances inhibitory to sporulation. Roberts & Baldwin (1942) found that the sporulation of B. subtilis in concentrated peptone solutions was stimulated by the addition of activated charcoal. Foster, Hardwick & Guirard (1950) observed a similar effect on Bacillus larvae in certain media with both activated charcoal and soluble starch. This was interpreted as being due to the removal of substances inhibitory to sporulation - so-called 'anti-sporulation factors'. It seemed possible that the favourable effect of dilution on sporulation of B. cereus in milk might be due to dilution of such factors. Neither the incorporation of 1% soluble starch into milk nor shaking with activated charcoal had any effect on the percentage sporulation of any of the strains. Thus, the 'anti-sporulation factors' which have been found in peptone media were not detected in milk under these conditions.

Increase in oxygen availability. Bayne-Jones & Petrilli (1933) from their work with B. megatherium in peptone solutions,

TABLE 28

Sporulation of B. cereus in duluted and undiluted milk under conditions of relatively very good, good and bad aeration (24hr. at 30°).

Treatment	Aeration		Condition of milk	Total count/ml.	Spore count/ml.	% Spores	D/U*
	Description	Method					
A	Very good	3ml. medium shaken in 150 ml. conical flask	Diluted Undiluted	60,000,000 930,000,000	68,000,000 1,700	>100 0.0002	565,000
B	Bad	5ml. medium incubated vertically in 6x⅝" tube	Diluted Undiluted	11,000,000 118,000,000	79,000 1,200	7 0.001	7,000
C	Good	1ml. medium incubated horizontally in 6x⅝" tube	Diluted Undiluted	35,000,000 820,000,000	37,000,000 179,000	>100 0.02	3,000

* D/U = ratio of % spores in diluted and undiluted milks.

suggested that the stimulating effect of dilution on sporulation was due to the fact that in concentrated media growth reached a level where sufficient oxygen was removed to limit sporulation. It was thought that if this were the factor operating in this case then the stimulating effect of dilution would be the greatest under conditions of relatively poor aeration and least under conditions of relatively good aeration, assuming that all other factors were equal. An experiment designed to test this was set up and the results appear in Table 28. The ratio between percentage sporulation in diluted and undiluted milk was taken as an index of the stimulatory effect of dilution, and in this way, it appeared that - contrary to expectations - the greatest stimulatory effect occurred under the best aeration conditions. The weakness of this experiment lay in the assumption that all factors other than that under test were virtually equal in the different media. As pointed out previously, this is not a valid assumption. Two points of interest may, however, be noted from Table 28. Firstly, the effect of oxygen on sporulation may be seen by comparing the diluted milks. Secondly, it was of interest that while there was virtually no difference in total counts in treatments A and C, there was an hundredfold difference in spore counts. Perhaps this could be attributed to some mechanical effect of the shaking.

The effect of metal ions

The interpretation of the results recorded in this section

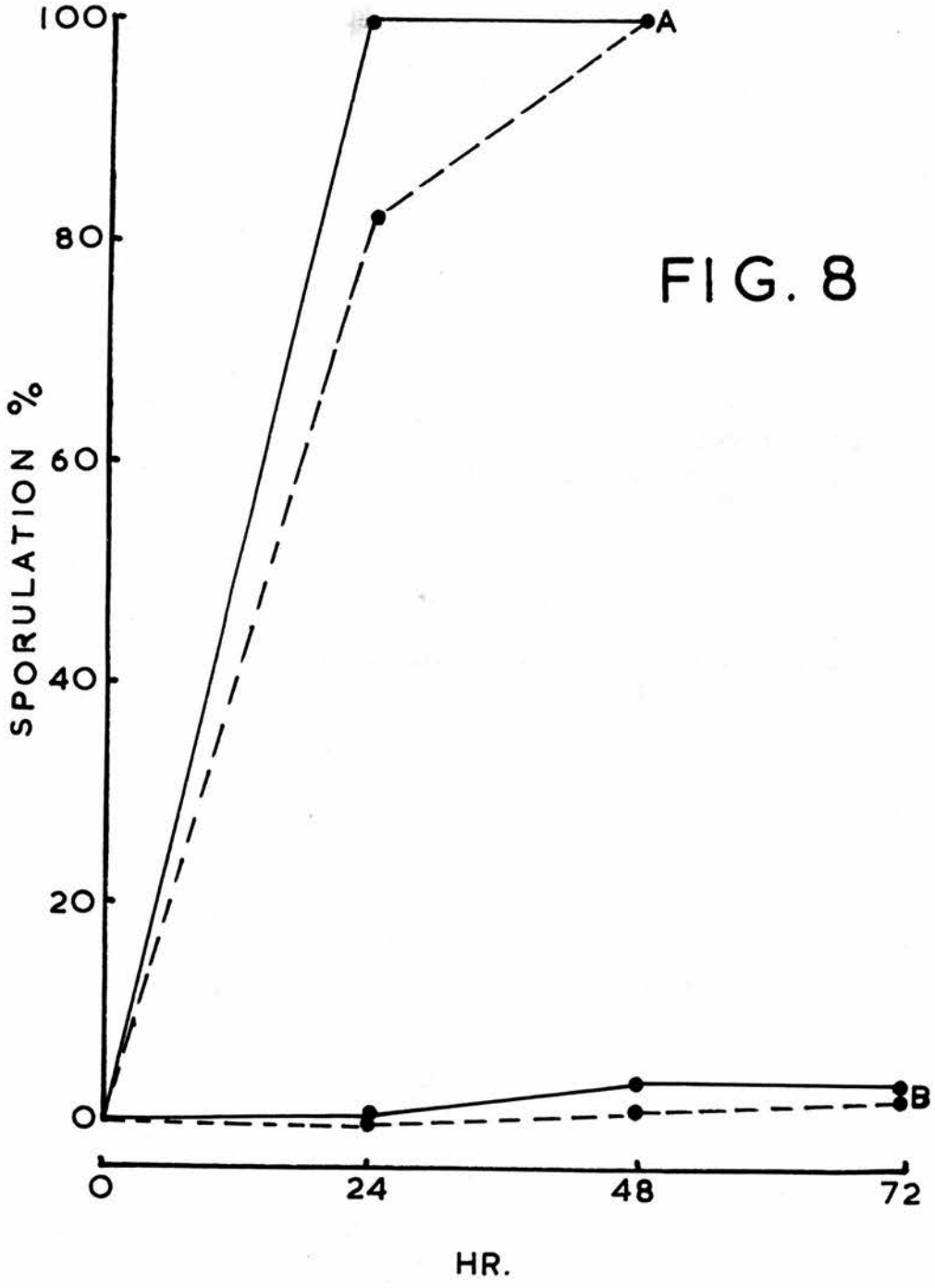


FIG. 8

Fig. 8. The sporulation of B. cereus (strain C.12) in milk with and without added potassium. (A = diluted; B = undiluted; --- with potassium; —— without potassium).

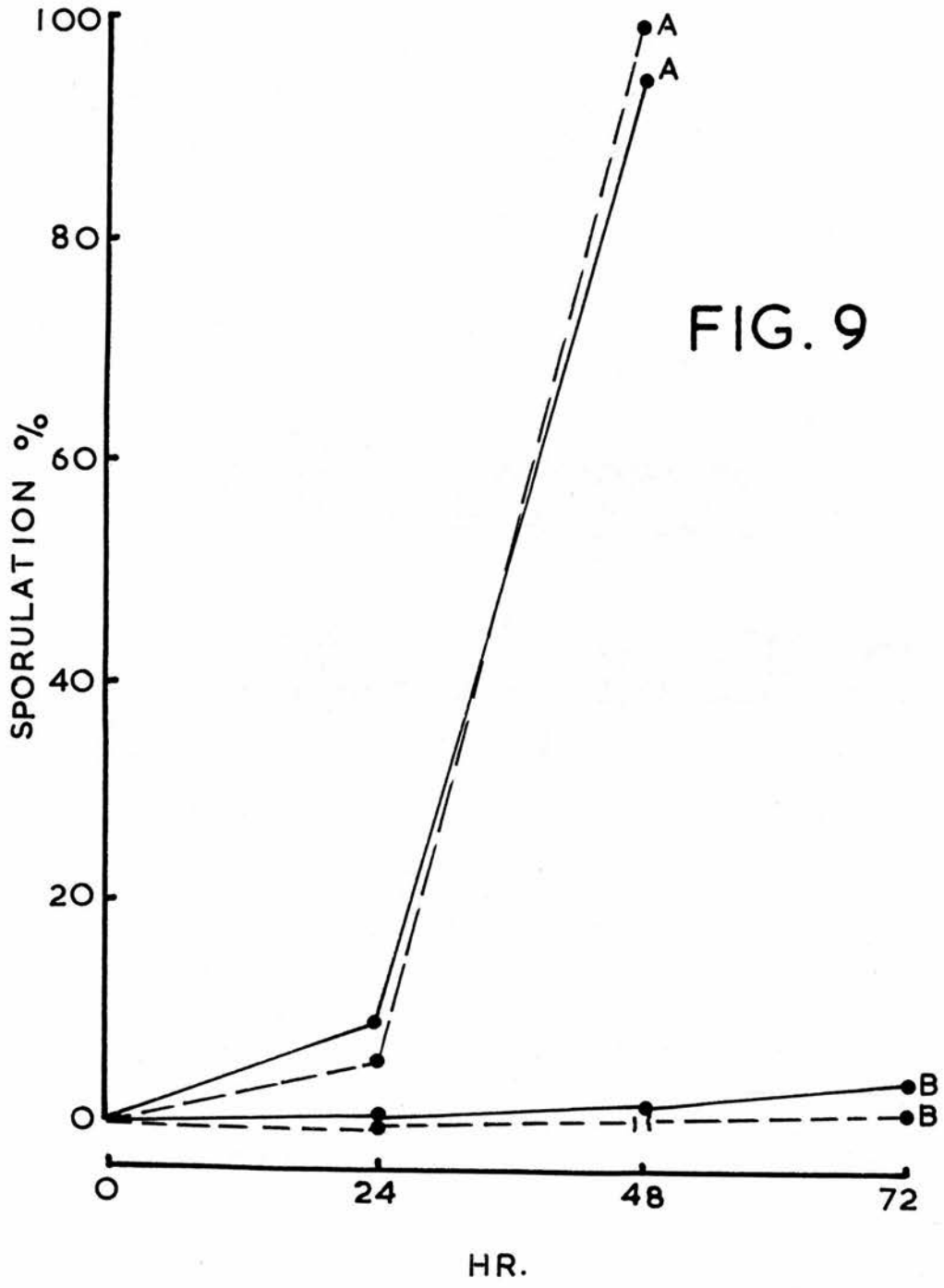


FIG. 9

Fig. 9. The sporulation of B. cereus (strain C.15) in milk with and without added potassium. (A = diluted; B = undiluted; --- with potassium; —— without potassium).

is subject to the same limitations as those outlined in the previous section. However, they are included because they do have a certain negative value.

Manganese and potassium were the only ions tried. Manganese was selected for examination partly because it seems to be a fairly general promoter of spore formation and partly because there is recent evidence that the stimulatory effect attributed to iron by several workers was due to contamination of the test salt with manganese (Weinberg, 1955). Potassium was selected because it is the only ion yet reported as having a stimulatory effect on sporulation of B. cereus (Foster & Heiligman, 1949).

The effect of manganese. Manganese ion was added as $MnSO_4 \cdot 4$ to $5H_2O$, giving a final concentration of 2×10^{-3}%. Strains of C.12 and C.15 were used, and Fig. 8 and 9 show that again no stimulatory effect was observed.

These findings are in agreement with those of Curran & Evans (1954) for B. cereus in milk, but since these authors took no account of the total growth of the organism in milk, the interpretation of their findings is probably subject to the same limitations as those mentioned above. The only valid conclusion which can be drawn is that under the conditions of the test, which can not be clearly defined, B. cereus was not stimulated to form spores in milk by the addition of either manganese or potassium.

DISCUSSION

The foregoing results indicate that B. cereus forms spores rapidly and more or less completely in diluted milk, provided that sufficient oxygen is present. From the practical point of view, the significance of this is, clearly, that shallow layers of watery residues lying about on dairy equipment provide ideal conditions for the sporulation of the organism. Thus, it can be understood why rinsed cans may often be a considerable source of contamination. It seems unlikely that metal contamination from cans is of any importance, for it is difficult to see how dilution in clean glassware could bring about 100% sporulation if the process were inhibited in undiluted milk due to a metal deficiency.

Beyond this, little can with any confidence be claimed from the results presented in this section. The process of sporulation is complex and very sensitive to changes in environment; thus, strict control of experimental conditions is essential in any attempt to elucidate the mechanisms involved. Most studies of sporogenesis, including the present work, have failed to take account of the total growth of the organism and consequently have been carried out under uncontrolled conditions. A considerable advance was made by Hardwick & Foster (1952) when they developed a method of examining sporulation in complete isolation from growth by the use of washed cell suspensions in distilled water. In this way they have developed a theory of sporogenesis, with good evidence to support it, which provides

a reasonable explanation for the dilution effect. They postulate a dynamic equilibrium in the cell between the processes of sporogenesis and vegetative protein synthesis, both types of synthesis utilizing a common intra-cellular pool of low molecular weight nitrogenous compounds. Vegetative synthesis dominates as long as a readily utilizable exogenous energy source is present, but when it is exhausted spore synthesis dominates. This explains why energy depletion has been associated with sporulation, and to this extent, they are in agreement with Knaysi (1948). However, in addition they suggest that an endogenous source of energy and nitrogen is essential before sporulation will take place, the evidence for this being that cells grown on media of low protein content or in the absence of glucose will not sporulate in distilled water. To summarize, it may be said that while it is still uncertain how the dilution effect operates, it is almost certain that energy depletion is involved (Knaysi, 1945; Grelet, 1951; Hardwick & Foster, 1952) and it is probable that the medium on which the organism has been grown will play an important part. Other factors such as oxygen availability may well be involved also.

SECTION IV

Studies of some of the factors influencing spore germination of *Bacillus cereus* in milk

REVIEW OF LITERATURE

The influence of heat treatment on spores has received a great deal of attention, principally because of its implications for the canning industry. Eckelmann (1917), as a result of her studies on the effect of fractional sterilization on certain aerobic spore-formers from soil, suggested that in some cases, heat might stimulate spore germination; and Allen (1923) reported that the generation time of Bacillus subtilis in milk was reduced as a result of pasteurization. However, the bulk of the early work suggested that the germination of spores of both aerobes and anaerobes was either retarded or unaffected by heat (Burke, 1923; Dickson, Burke, Beck & Johnston, 1925; Esty & Williams, 1924; Williams, 1929; Sommer, 1930, etc.). The weakness of this work was that the assumption was made that growth following germination was a valid measure of germination. What seemed to be a reasonable assumption has since been shown to be a fallacy, for germination often occurs under conditions not favourable to vegetative growth (Knaysi, 1945; Curran & Evans, 1945b; Knaysi & Baker, 1947; Wynne & Harrell, 1951; Wynne, 1952; Sacks, 1955, etc.).

When in later years, more valid criteria were used, a very different picture emerged. Cook (1931) demonstrated stimulation in oxygen uptake by spores of Bacillus subtilis in glucose solution as a result of holding the spores at 100° for 10 min.. Tarr (1933) confirmed this effect with heating periods of 30 min. at 60 to 90°, the maximum response being obtained at 80°. The changes in heat resistance and in permeability to

stains of the germinating spore have been used to show that heat stimulation of germination is very widespread among the spore-formers, both aerobic (Evans & Curran, 1943; Levinson & Sevag, 1953; Levinson & Hyatt, 1955) and anaerobic (Reynolds & Lichstein, 1949).

In the case of Bacillus cereus, Evans & Curran (1943) observed stimulation of their strains in response to heating at temperatures between 65 and 90° in sterile milk but not in glucose extract broth. Stone (1952b), with 2 strains from milk, reported 96 and 100% germination 2 hr. after pasteurization compared with 48 and 84% respectively in the corresponding unheated milks.

METHODS

Organisms. Two strains of B. cereus (C.12 and C.15) isolated from milk during the earlier part of the work were the test organisms.

Preparation of inoculum. Inoculation was from spore suspensions. The strains were grown for 2 weeks at 30° on slopes of weak nutrient agar (see appendix, p. i). At the end of this time, the growth was washed off with sterile glass-distilled water, and the suspension thoroughly shaken and centrifuged. The washing process was repeated three times. The cells were finally re-suspended in sterile glass-distilled water, held at 65° for 30 min. to destroy vegetative cells and stored in a sterile vial in a refrigerator until needed. Counts carried out on these suspensions from time to time showed little variation for several months. For the examination of the effect of laboratory pasteurization on unheated spores, suspensions were prepared as above but the heat treatment was omitted.

Conditions of the test. The test medium was inoculated at the rate of 1 ml. spore suspension at the appropriate dilution to 9 ml. test medium in a sterile 6 x $\frac{5}{8}$" test tube with a rubber stopper. The inoculated medium was then thoroughly shaken and pipetted in 1 ml. amounts into a series of sterile 6 x $\frac{5}{8}$" tubes as in the case of the sporulation experiments. However, since it has been shown that oxygen is not critical for germination (Roth & Lively, 1956), incubation was in the vertical position. Except where the effect of temperature was being studied,

incubation was at 30°. A thermostatically controlled water bath was preferred to an incubator for these experiments because readings were made at short intervals, making the greater accuracy desirable. At each time interval, three tubes were withdrawn and the entire contents used for testing. Thus all results are based on triplicate readings.

Determination of germination. The heat lability of germinating spores and their increased permeability to dyes are the two properties which have been utilized in recent studies on germination of spores under different conditions. Although, these methods are much more accurate than those utilizing growth subsequent to germination, even they are somewhat arbitrary, for increase in sensitivity to lethal agents may precede any cytological change or change in stainability of the spore (Mefferd & Wyss, 1951). On the other hand, cytological changes characteristic of germination may precede loss of heat resistance (Curran & Evans, 1937; 1945b). Nevertheless, there seems to be fairly good correlation between results obtained by the two methods (Wynne & Foster, 1948a).

Powell (1951) estimated percentage germination in B. subtilis by staining smears with hot carbol fuchsin and aqueous methylene blue. Levinson & Sevag (1953) with Bacillus megatherium obtained satisfactory results with methylene blue only. Both these methods were tried, and it was found, in agreement with Levison & Sevag, that more satisfactory results were obtained without carbol fuchsin. This is

especially the case where the test medium is milk, for a great deal of difficulty is encountered due to staining of the background by carbol fuchsin. Eventually, however, staining methods were abandoned in favour of heat lability because enormous initial spore inocula were required to give countable fields and because as the vegetative cells increased in number the estimate of percentage germination became less and less accurate.

As each tube was withdrawn from the water bath, it was held at 65° for 15 min. to destroy all vegetative cells. Residual spores were then estimated by plating using nutrient agar with 0.1% soluble starch. Counts were done after 24 hr. at 30°.

Where the effect of pasteurization was being examined, one series of tubes was held at 65° for 30 min. at the beginning of the experiment, while the other series was placed in the 30° water bath straight away. However to allow for any possible changes during the actual heating period, the unheated milks were held for 45 min. at 65° at the end of the experiment instead of for 15 min.. In this way all tubes received a total heat treatment of 45 min. In other experiments, the heat activation at the beginning of the experiment was carried out as a routine.

Expression of results. For each treatment spore counts were made before the incubation period began. These counts at 0 hr. were used to calculate residual spores at any time interval and from that percentage germination was calculated.

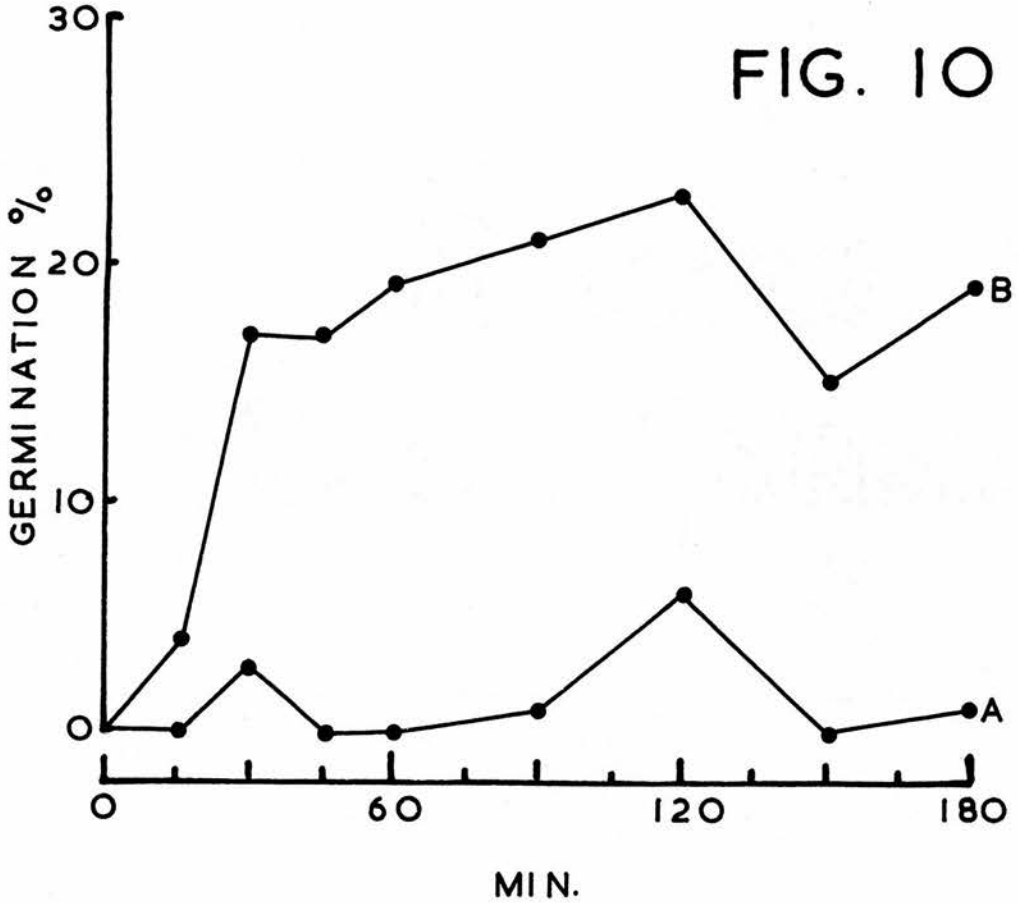


Fig. 10. The effect of laboratory pasteurization on the germination of unheated spores of B. cereus (strain C.12). (A - unheated; B = laboratory pasteurized).

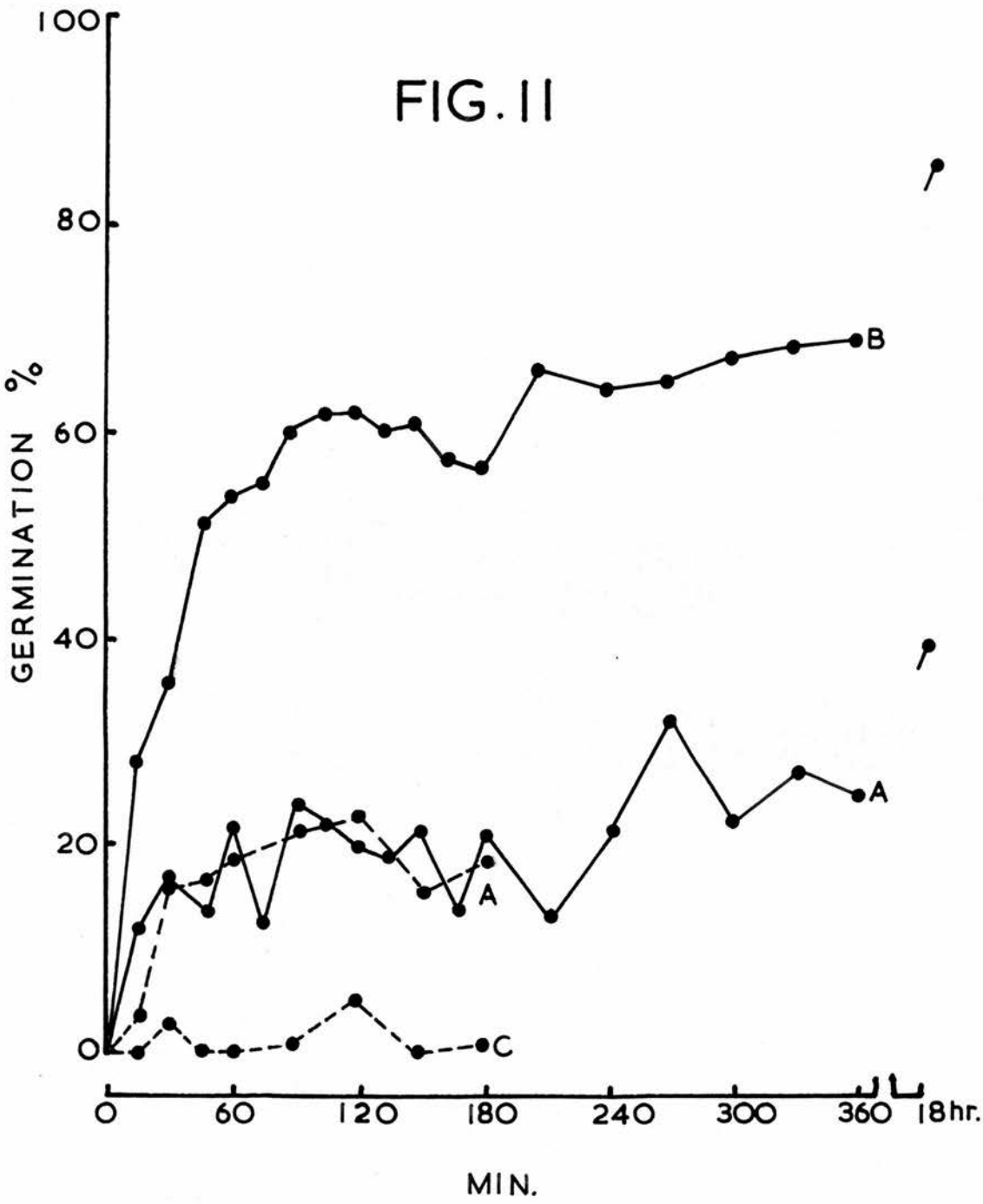


Fig. 11. The effect of laboratory pasteurization on the germination of heated spores of B. cereus (strain C.12). (A = once-heated; B = twice-heated). C = unheated

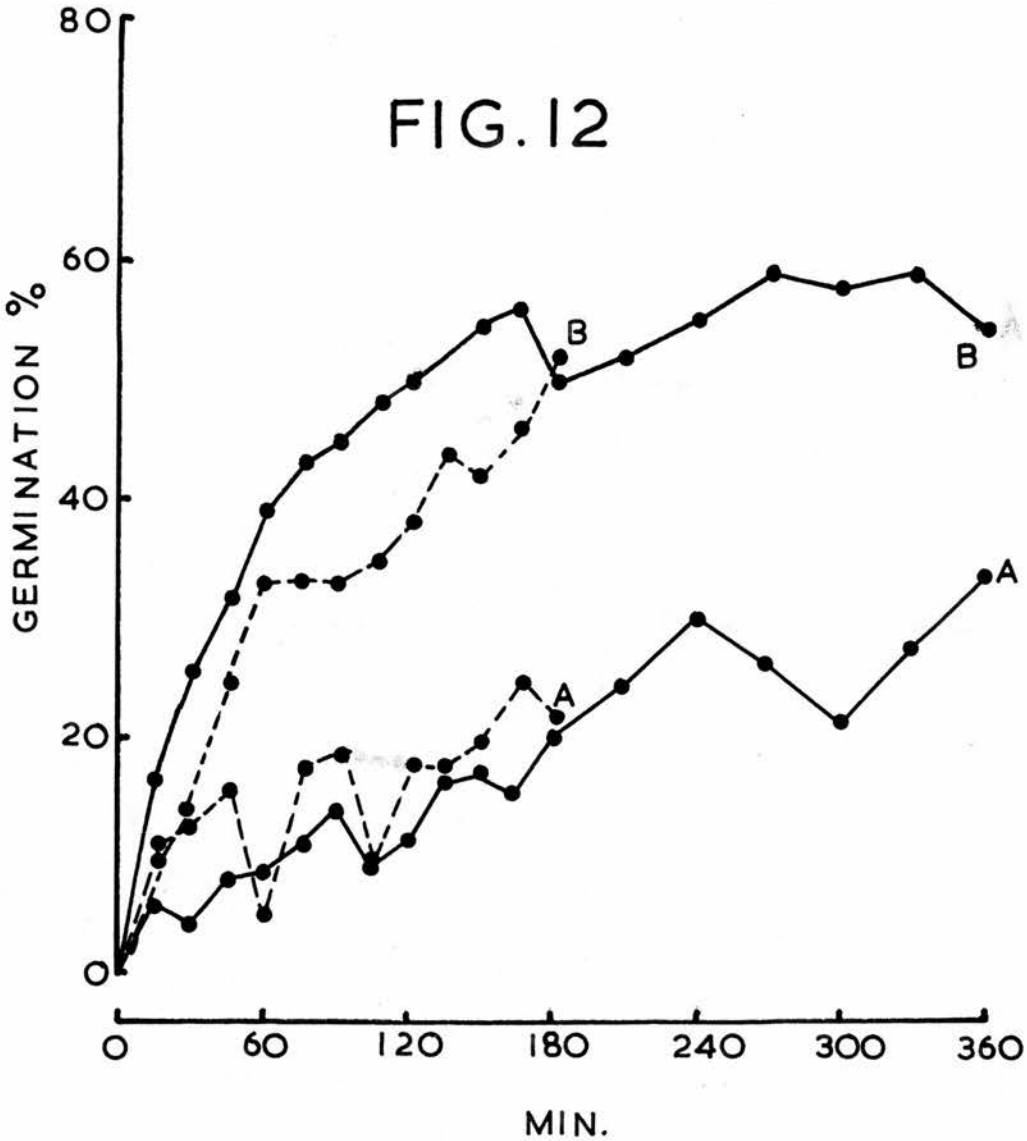


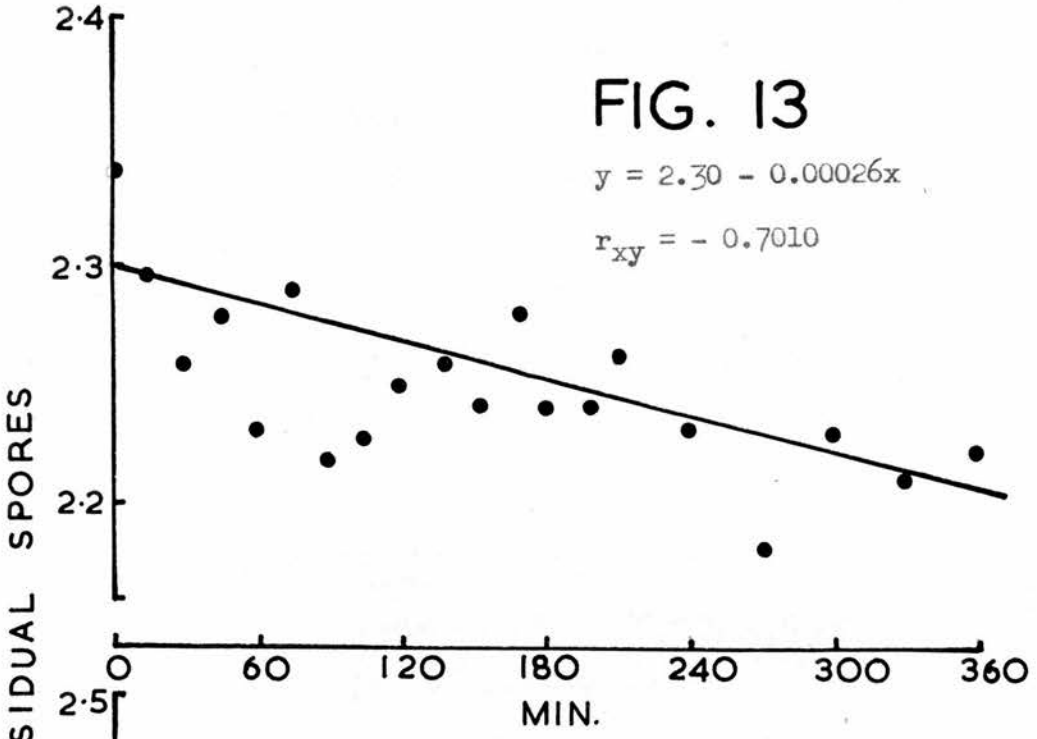
Fig.12. The effect of laboratory pasteurization on the germination of heated spores of B. cereus (strain C.15). (A = once-heated; B = twice-heated).

RESULTS

The effect of laboratory pasteurization

Unheated spores. In Fig. 10, the effect of laboratory pasteurization on germination of spores of strain C.12 may be seen. These results show that the heat treatment has caused a definite stimulation in germination. In the case of the unheated spores even after 180 min. at 30°, there was little tendency to germinate.

Heated spores. The literature suggested that the activating effect of heat on germination might persist for some time when spores are stored under conditions unfavourable to germination (e.g. Curran & Evans, 1945a). Thus, the finding that contamination of milk by B. cereus often occurred from inadequately sterilized cans led one to suspect that often the spores might already be heat-activated at the time of pasteurization. It, therefore, seemed of importance to examine the effect of pasteurization on the germination of spores which had received a previous heat treatment followed by a storage period under conditions unfavourable to germination. The spore suspensions which had been heat treated during their preparation to destroy vegetative cells were thus suitable for the purpose (p.64). Fig.11 and 12 record findings with these suspensions using strains C.12 and C.15. In these figures, the graphs "A" represent the results for spores which had been held at 65° for 30 min. during the preparation of their suspensions, i.e. 1-3 weeks before the experiment was carried out, but to which no



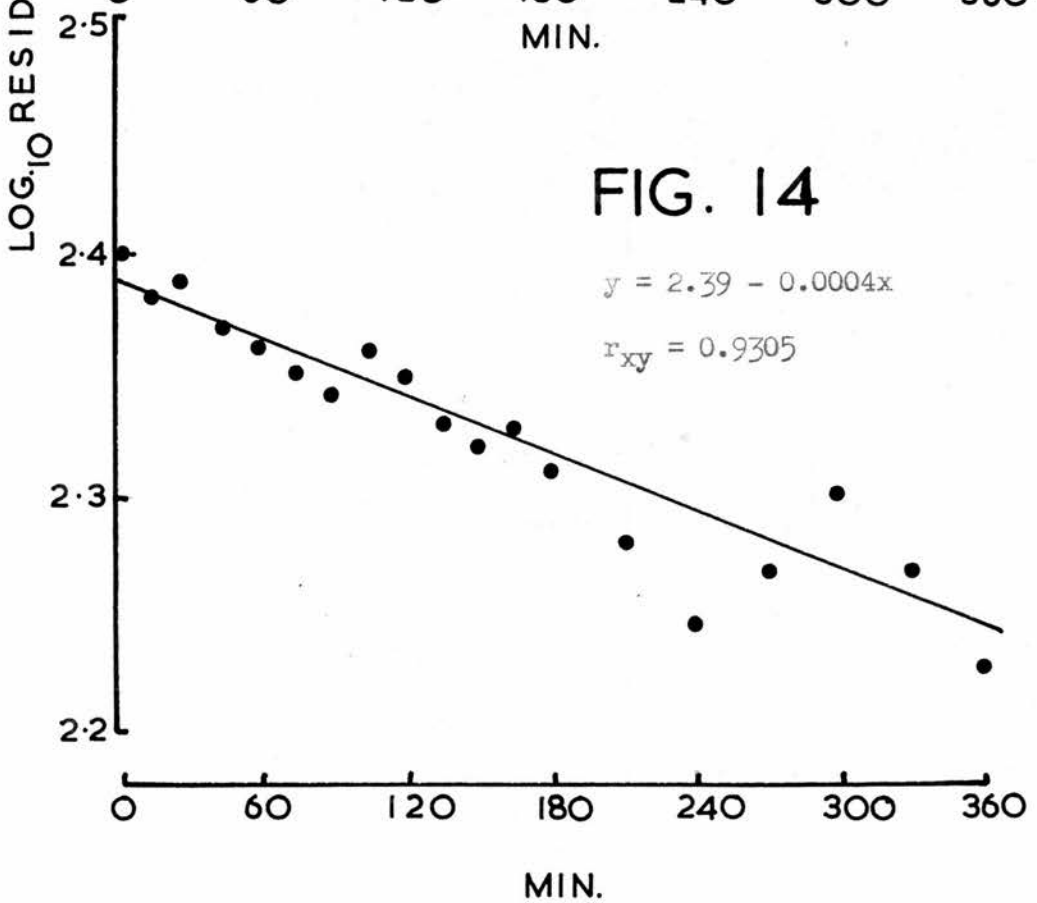


Fig. 13. Regression of $\log_{10}$ residual spores on time in the case of once-heated spores of B. cereus (strain C.12).

Fig. 14. Regression of $\log_{10}$ residual spores on time in the case of once-heated spores of B. cereus (strain C.15).

further heat treatment was applied. The graphs "B" show the response of spores to an additional heat treatment of 65° for 30 min. applied at the beginning of the experiment. For comparison, the graphs in Fig.10 have been replotted in Fig. 11. It can be seen that there was good agreement between the results for the 2 series of once-heated spores, i.e. those heated some time before the experiment, and those in which the heat treatment was received at the beginning of the experiment. It can be seen, in addition, that a very marked stimulation in germination occurred in response to the second heat treatment. For example, in the case of strain C.15, the once-heated spores showed 11% germination at the end of 120 min. compared with 50% in the twice-heated series. The corresponding figures for C.12 are 20% and 62%. Further, after 18 hr., while the twice-heated spores had reached a level of 85% germination, only 43% of once-heated spores had germinated. Thus, more spores had germinated after 120 min. following two heatings than after 18 hr. following one heating.

Wynne & Foster (1948b) found a linear relationship (following an initial lag period) between $\log_{10}$ residual spores and time with heated spores of Clostridium botulinum in brain heart infusion broth. It was of interest to determine whether such a relationship held with spores of B. cereus in milk. In Fig. 13 and 14, the results for once-heated spores of strains C.12 and C.15 have been recorded. The points were in both cases found to conform significantly ($P < 0.01$) to simple linear

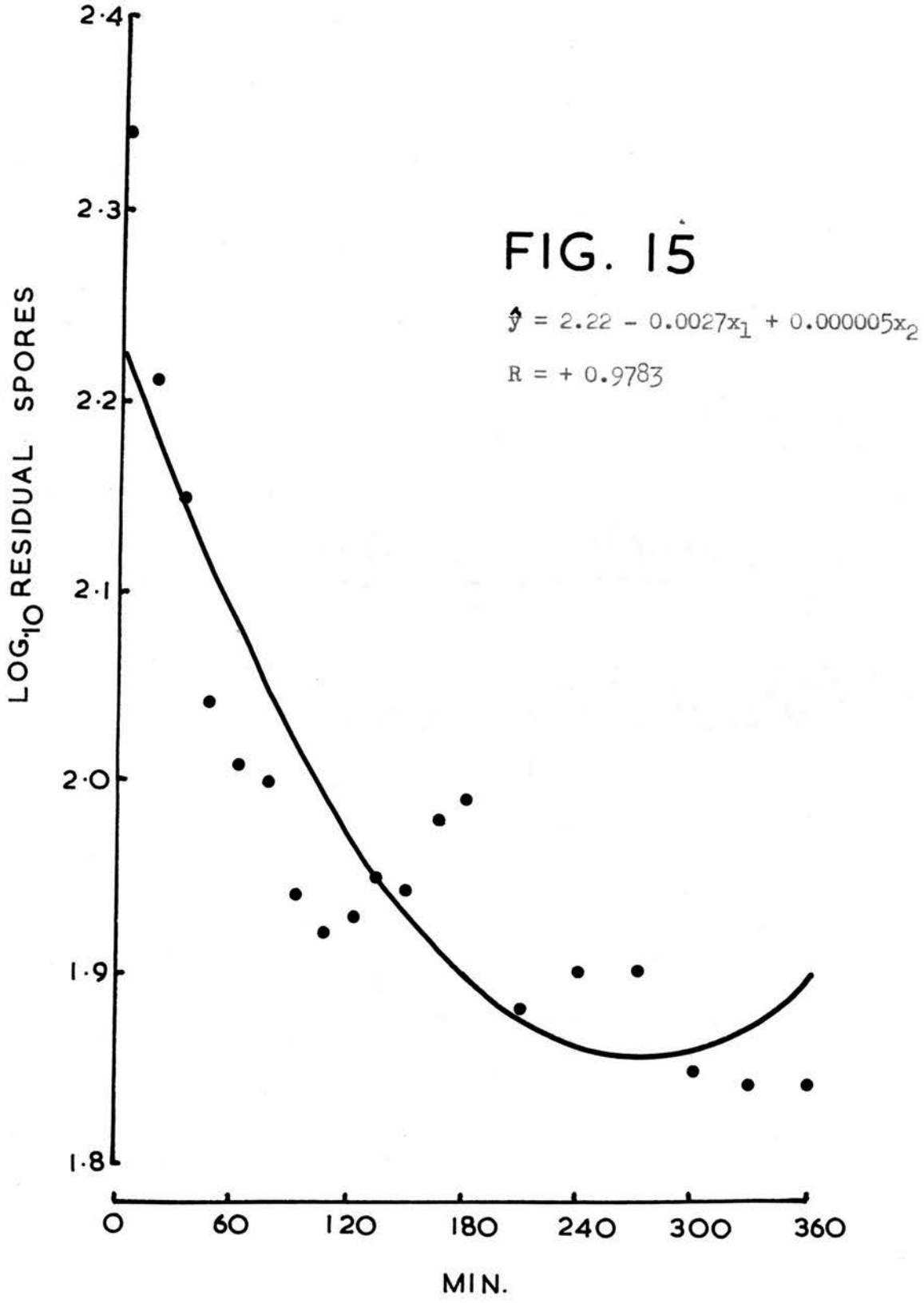


Fig. 15. Regression of $\log_{10}$ residual spores on time in the case of twice-heated spores of B. cereus (strain C.12).

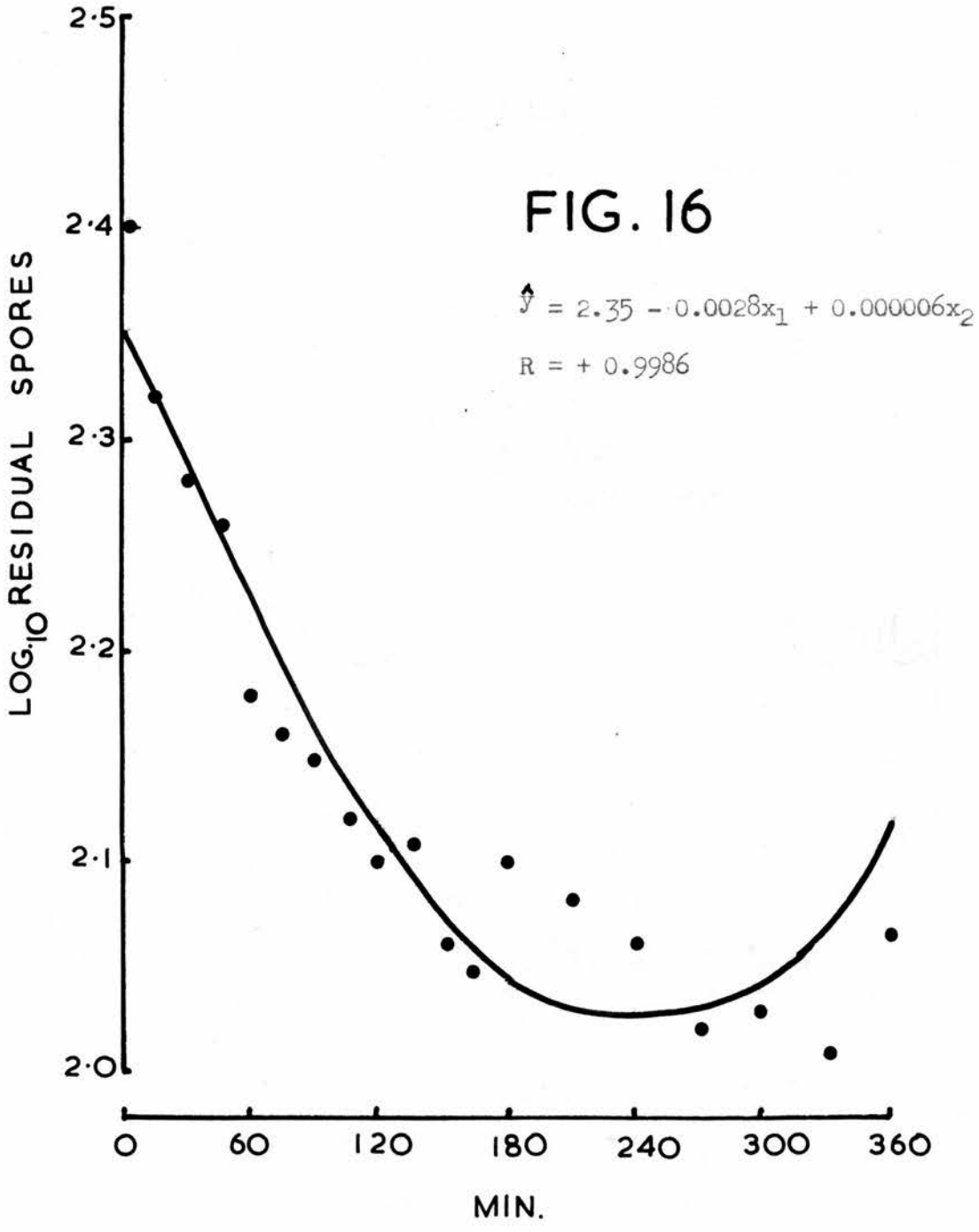


Fig. 16. Regression of $\log_{10}$ residual spores on time in the case of twice-heated spores of B. cereus (strain C.15).

regressions described by the equations given in the figures. The correlation coefficients recorded were also found to be highly significant at the 1% level of probability. These results are substantially in agreement with those of Wynne & Foster except that no initial lag period preceded germination.

In Fig. 15 and 16 $\log_{10}$ residual spores has been plotted against time for the twice-heated spores of each strain. In both cases, the points conformed significantly ($P < 0.01$) to second order regression curves described by the equations given in the figures. The multiple correlation coefficients given were highly significant ($P < 0.01$). Details of the calculations involved in preparing Fig. 13, 14, 15 and 16, and the relevant analyses of variance are given in the appendix (p.ii). The binomial regressions obtained in the case of the twice-heated spores were suggestive of heterogeneous spore populations. The fact that linear regressions held for the once-heated spores indicates that this was due to differences in the degree of activation of the spores by the first heat treatment.

Twice-heated spores were used in the following experiments in order that the effects of different treatments be readily seen.

The effect of post-pasteurization storage temperature

With P.A. 3679, Mehl & Wynne (1951) found germination rate to be a function of temperature over the range 20-45°. Again, Williams & Reed (1942) found that in the range 24-37°, the lower temperatures of incubation were most favourable for the survival of Cl. botulinum.

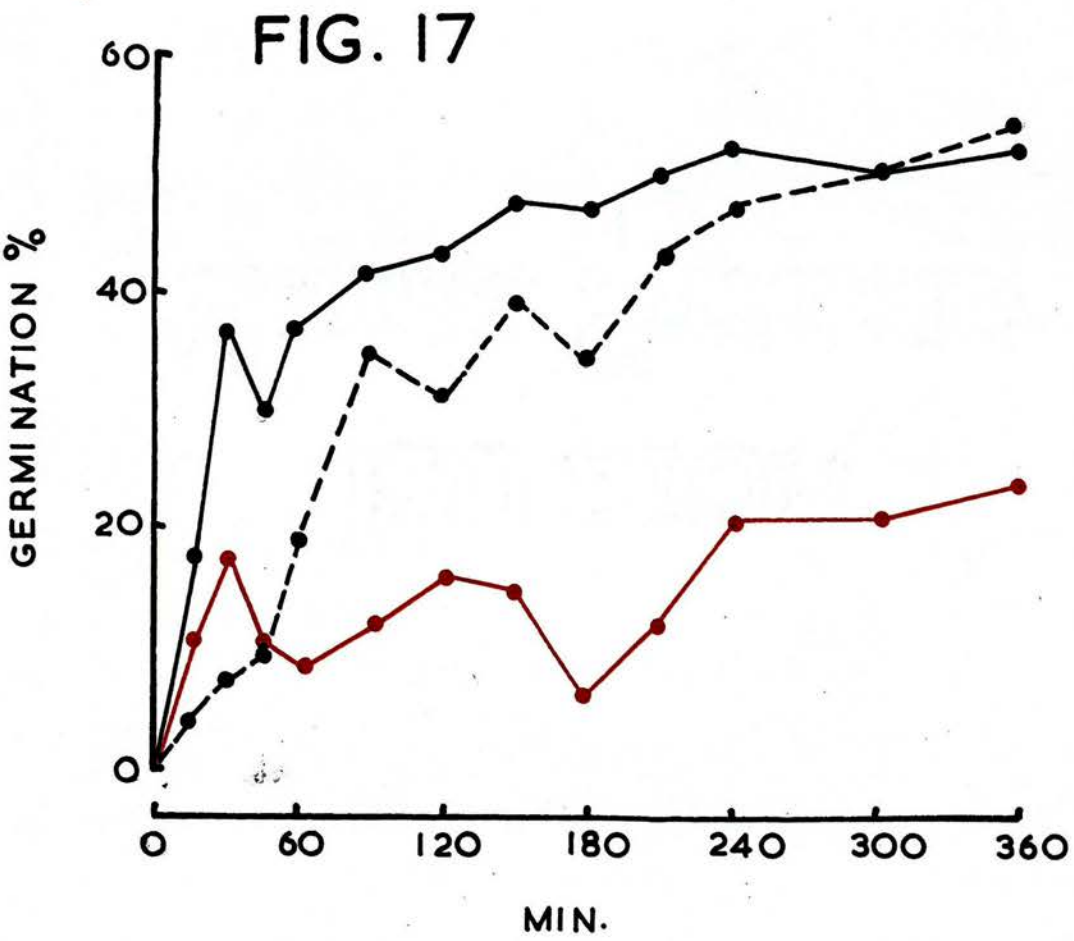


Fig. 17. The effect of post-pasteurization storage temperature on the germination of spores of B. cereus (strain C.15). (—— 30°; - - - 22.5°; —— 15°).

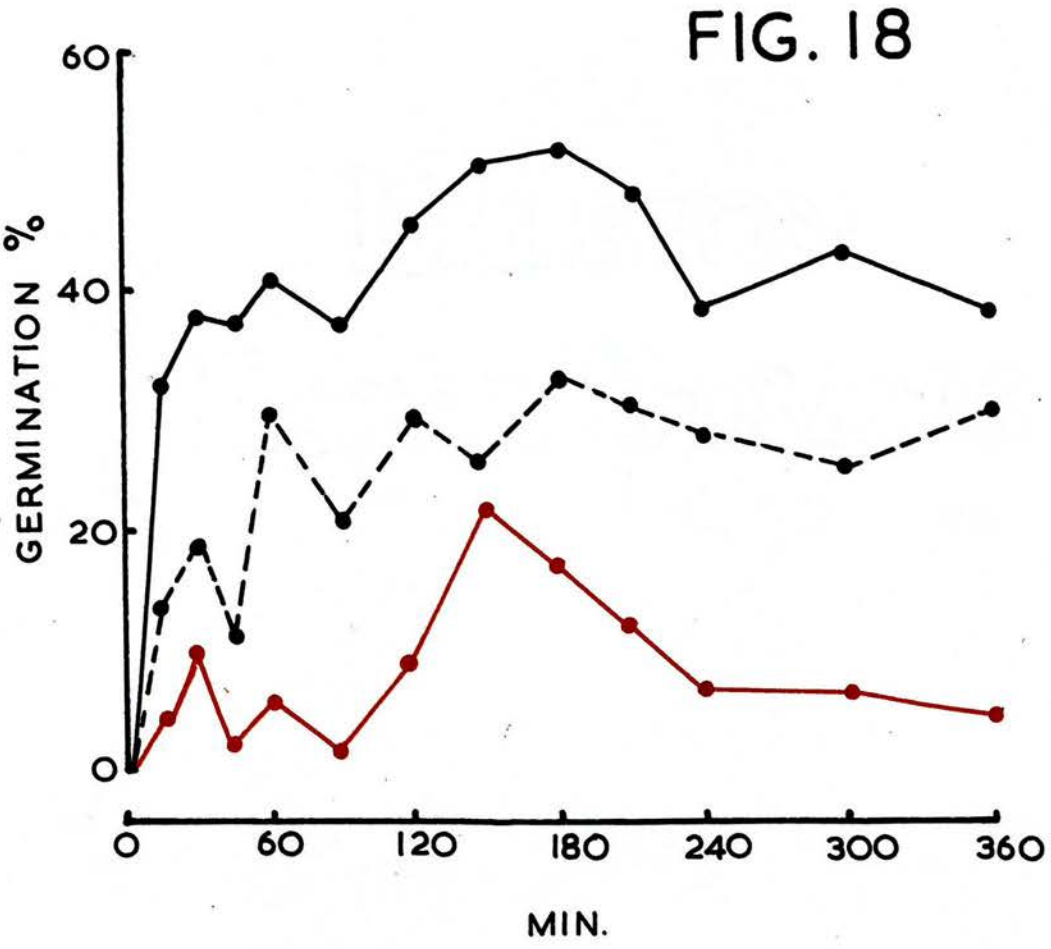


Fig. 18. The effect of post- pasteurization storage temperature on the germination of spores of B. cereus (strain C.12). (—— 30°; - - - 22.5°; —— 15°).

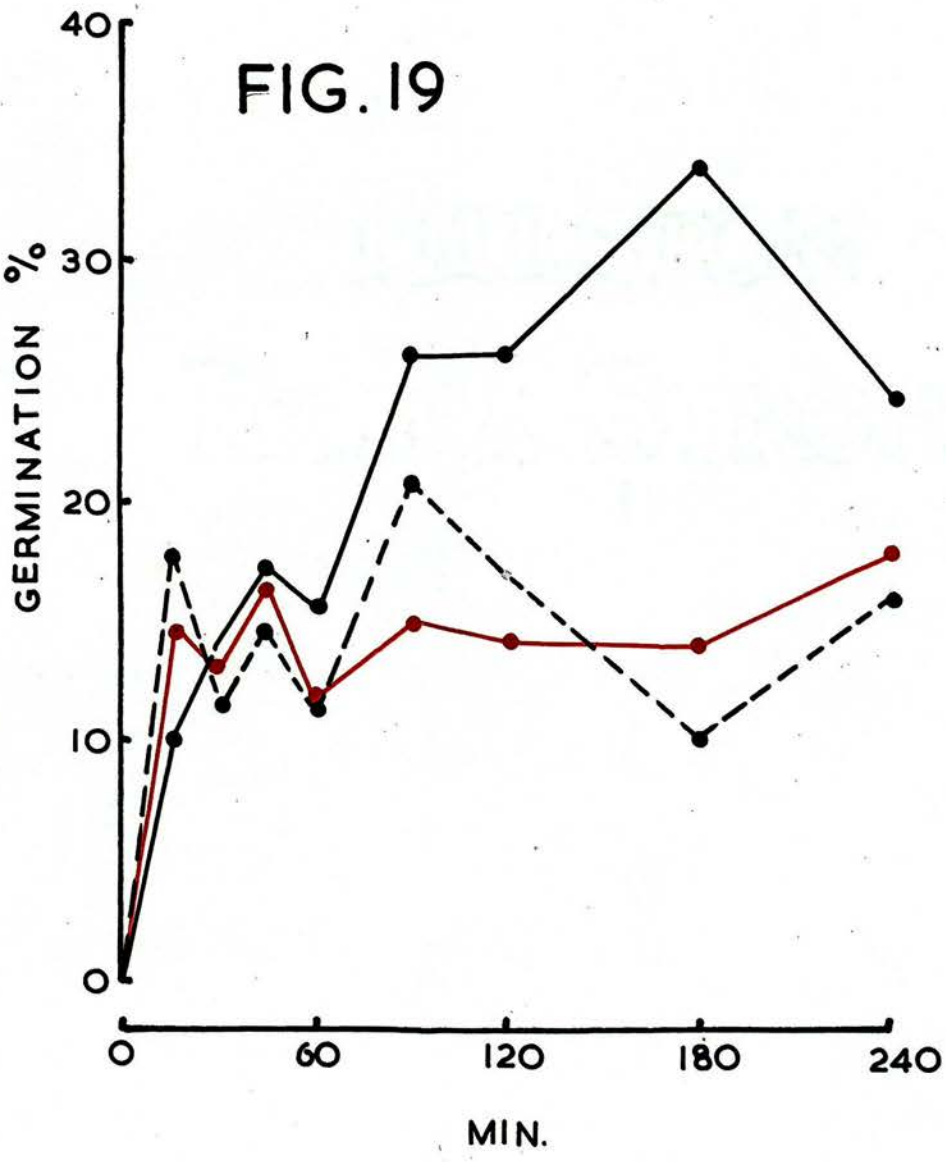


Fig. 19. The germination of heated spores of B. cereus (strain C.15) in milk and diluted milk. (—— undiluted; - - - 1/10; —— 1/50).

Using three temperatures of storage - 15, 22 and 30° - germination rates for the two strains were determined. Results appear in Fig. 17 and 18. Both strains show considerably diminished percentage germination at 15° compared with 30°. The importance of refrigeration in retarding germination is thus apparent. However, keeping in mind the possibility of the persistence of the activating effect of heat, it is probable that germination will proceed at the increased rate as soon as temperature conditions become favourable.

The effect of dilution

This experiment was to determine the probable behaviour of spores in milk residues. Twice-heated spores of C.12 were incubated at 30° in undiluted milk (A), milk diluted 1/10 with sterile distilled water (B) and milk diluted 1/50 with sterile distilled water (C). The results appear in Fig. 19. In agreement with the observations of Curran (1931) with B. mycoides in peptone solutions, it was found that dilution had a slight retarding effect on germination.

DISCUSSION

The results presented in this section of the thesis provide evidence, in addition to that of other authors, that pasteurization has a stimulatory effect on the germination of spores of B. cereus and consequently on the development of bitty cream. Moreover, it is probable that the higher temperature used in commercial pasteurization, by the H.T.S.T. process, will result in an even greater stimulation than that observed here - although the importance of heating time is not known.

While it is uncertain how long the activating effect of sub-lethal heat on spore germination persists, the results of Curran & Evans (1945a) together with those reported here, indicate that it will be at least a matter of weeks if conditions are unfavourable to germination. Thus, where spore contamination occurs from inadequately sterilized cans, the 'sterilization' process probably causes an initial heat activation of spores which persists at the time of pasteurization. This will result in rapid germination after pasteurization. However, if 'sterilization' stimulates germination after pasteurization, it will also stimulate germination before pasteurization, although to a much smaller extent as these results show. Thus, the temperature at which the milk is stored prior to pasteurization may be of some importance. Efficient cooling on the farm, for example, may actually increase the possibility of the appearance of bitty cream after pasteurization by delaying germination before pasteurization. The retarding effect of low temperatures on the appearance of bitty cream additional to that attribut-

able to retardation of vegetative growth reported by Stone (1952b) is also probably due to delayed germination.

The retarding effect of dilution of milk on spore germination may be of significance in that milk residues will be more unfavourable for germination than milk itself. Thus if the spore forms in milk residues,it may remain ungerminated for long periods until 'sterilized' and re-inoculated into milk.

Conclusions from Part I of this thesis

CONCLUSIONS

The main aim of this part of the thesis was described as an attempt to elucidate the conditions leading to outbreaks of bitty cream. From the results recorded here together with those of other authors, the following conclusions are drawn.

Firstly, the causative organism, B. cereus, is present both in areas where bitty cream has been a source of trouble and where it has not. Moreover, a heavy initial contamination is not necessary before the fault will develop, e.g. a number of samples containing < 1/ml. B. cereus were found to develop bitty cream if other conditions were favourable.

Secondly, outbreaks of bitty cream seem to be especially associated with warm weather. Not only do the higher temperatures stimulate vegetative growth, but they probably also stimulate spore germination.

Thirdly, bitty cream is almost entirely confined to pasteurized milks. Other authors have stressed the importance of competition from and inhibition by other organisms in raw milk in preventing the appearance of the fault. The present work suggests, in addition, that delayed germination of spores in raw compared with pasteurized milks may be of considerable importance in allowing the competing flora to dominate the population. On the other hand, the destruction of a large proportion of the actively growing raw milk flora together with the stimulation in germination rate caused by pasteurization are likely to result in the dominance of B. cereus in pasteurized milk.

Fourthly, since the fault is mainly confined to pasteurized milks and the vegetative cell is known to be rapidly destroyed at pasteurization temperatures, it is concluded that bitty cream develops mainly from spore contaminants. An important source of contamination of milk by B. cereus spores has been shown to be inadequately sterilized cans. The organism, though producing few spores in milk, will sporulate prolifically in milky water, which is often left lying for long periods in cans. In practice the 'sterilization' of cans both at creameries and farms is often inadequate for destruction of spores.

From the above conclusions, it seems that control of the fault might be attempted in at least three ways - by preventing sporulation on equipment, by destroying spores on equipment before they have an opportunity to recontaminate milk, and by inducing spores which do enter milk to germinate before the pasteurization process is applied.

One obvious way to avoid prolific sporulation on equipment, especially cans, would be to eliminate the delay between rinsing, and cleaning and sterilizing of cans. This would mean transferring the onus for the last two operations from the individual farmer to the creamery. However, one is immediately confronted with an economic problem here, for it does not pay the smaller creamery to do this.

The second way of attacking bitty cream probably presents greater practical possibilities. Adequate steaming of all equipment, especially cans, preferably following an acid rinse, should destroy most spores of B. cereus.

The problem of inducing spore germination in raw milk before pasteurization is a major one in itself. The practice of re-pasteurization of surplus milk carried on at some creameries during the summer, although designed for a different purpose, very probably reduces the incidence of bitty cream in this way.

These suggestions are only indications of the lines along which a control programme might be developed, and are made because they seemed to follow from the results reported here.

PART II

STUDIES OF THE OCCURRENCE AND BEHAVIOUR OF STREPTOCOCCUS THERMOPHILUS IN MILK

INTRODUCTION

The importance of Streptococcus thermophilus in milk and dairy products has been recognised for many years. Long before the species was named and described (Orla-Jensen, 1919), its properties of rapid growth and acid production at relatively high temperatures were being utilized in the preparation of certain cheeses and soured milks. Following the introduction of pasteurization to the dairy industry, reports appeared of the common occurrence of the organism in freshly pasteurized milk. Later, when the low temperature process was in general use, it attracted a considerable amount of attention due to its ability to multiply during pasteurization.

In spite of the undoubted importance of the organism little has been added to our knowledge of it in the thirty seven years which have elapsed since the first description was made. Its position within the genus Streptococcus, for example, remains uncertain. While in its heat resistance and ability to grow at relatively high temperatures, it resembles the enterococci, on biochemical or serological grounds its inclusion within this group is not at present possible. Furthermore, the species delineation still rests on the insecure basis of negative reactions to most of the conventional biochemical tests. The unsatisfactory nature of this situation is emphasized when one considers the vigorous growth of the organism in milk.

It is perhaps significant, in view of the mystery that surrounds the biochemical activities of the organism that its

source has not yet been determined. It was towards this end that the work undertaken in this part of the thesis was directed.

Initially, some time was spent in an attempt to evolve a medium selective for S. thermophilus. This was not entirely successful, but a fairly satisfactory method was worked out as described in Section I. Section II is devoted to a general survey of different types of milk and an investigation of a particular creamery where an outbreak of S. thermophilus occurred, in an attempt to trace the organism to its source. Section III deals with the characterization of strains isolated during the work.

SECTION I

The development of a method for detecting *Streptococcus thermophilus* in milk

REVIEW OF LITERATURE

Previous investigations of S. thermophilus in milk have been incidental to more general population studies. Abd-el-Malek & Gibson (1948), studying the total flora of milk, used a non-selective, direct plating method, picking random colonies for identification. Obviously, this method is not suitable for the study of a single species. Sherman & Stark (1931), studying streptococci able to grow at high temperatures, preceded the plating of each milk sample by a period of enrichment incubation at 45°. However, in the present work, the aim was to detect differences in the extent of contamination under different conditions, so that a quantitative method was to be preferred. The most satisfactory method for this purpose seemed to be a direct plating technique using a medium selective for S. thermophilus. The first step was therefore the determination of a suitable basal medium.

S. thermophilus is generally quoted as producing 'pin-point' colonies on ordinary milk count agars, and thus has acquired a reputation for nutritional fastidiousness. This is primarily due to the fact that the organism will not grow in the absence of a fermentable carbohydrate (Orla-Jensen, 1919; Wright, 1936a). Moreover, growth of many strains is more satisfactory in the presence of the disaccharides sucrose and lactose than where the energy source is glucose (Wright, 1936b). Some authors have reported slightly better growth in the presence of sucrose than lactose (Hucker, 1928; Wright, 1936a).

Provided that a suitable carbon source is present, the nitrogen requirements of the organism may be easily met; for example, by casein hydrolysate (Orla-Jensen, 1919), beef extract and peptone (Hucker, 1928; Wright, 1936a) or tryptone (Guss & Delwiche, 1954). Yeast extract has been used to fulfil any additional growth factor requirements (Guss & Delwiche, 1954). In a study of the nutritional requirements of a number of strains, the latter authors found growth to be as satisfactory on a medium containing 0.5% tryptone, 0.5% sucrose, 0.5% yeast extract and 0.5% K_2HPO_4 as in the presence of a whole range of amino acids and growth factors.

The property which stood out as most likely to be of value in the selection of S. thermophilus from the majority of other milk organisms was its ability to grow at relatively high temperatures. Orla-Jensen (1919) found that his strains grew best above 40° and were still capable of growth in milk at 50°, an observation confirmed by Sherman & Stark (1931) with their strains. It was felt that the only other species likely to occur in significant numbers at 45° were S. bovis and members of the S. faecalis group.

EXPERIMENTAL AND RESULTS

Five isolates from Edam cheese (2) and from pasteurized milk (3) were used as the test strains. In the preliminary work, incubation was always at 37° for 24 hr..

Development of a basal medium

At first, a medium containing 0.5% (w/v) lactose, 1% (w/v) tryptone, 1% (w/v) beef extract (lab. lemco) and 0.5% (w/v) yeastrel with pH about 7 was tried. Good growth of all the test strains was obtained. The importance of each constituent was now examined.

The effect of tryptone and beef extract. Reduction of the quantities of these constituents to 0.5% resulted in considerable reduction in colony size.

The effect of yeastrel. Omission of yeastrel from the medium caused a slight reduction in colony size.

Comparison between lactose, sucrose and milk. The substitution of 1% (v/v) milk or 0.5 and 1% (w/v) sucrose for 0.5% lactose gave no visible improvement in growth.

In an attempt to stimulate growth further, the effect of certain other additions to the basal medium was now tried.

The effect of oleate. This growth factor is essential for a number of lactic acid bacteria. It was possible that S. thermophilus might be amongst them. Tween 80, as used by Keddie (1951) in his medium for lactobacilli, was incorporated at the rate of 0.005 and 0.05% (v/v). No stimulation was obtained in the case of any strain.

The effect of egg yolk. The incorporation of 2.5% (v/v) yolk

into the medium as an additional source of growth factors resulted in slight stimulation of growth. However, when 45° incubation was subsequently used, this supplement was abandoned owing to the coagulation of the yolk proteins at this temperature.

The effect of manganese dioxide. Since many strains of S. thermophilus produce slight greening on blood agar, it was thought possible that colony size might be limited due to growth inhibition by hydrogen peroxide production. The manganese dioxide plate method of Kneteman (1947) was used to test this. A layer of basal medium inoculated with the particular test strain was poured into a petri dish and allowed to solidify. This was then covered by a second layer of basal medium containing a suspension of pyrolusite (finely powdered manganese dioxide) in a concentration sufficient to produce a black appearance through the plate but insufficient substantially to reduce opacity. Colonies producing hydrogen peroxide were expected to clear the manganese dioxide. For comparison, a peroxide-producing strain of Streptococcus lactis was tested at the same time. This strain produced marked clearing and increase in colony size, but none of the S. thermophilus strains gave any detectable response. No further attempt was made to improve the basal medium.

Development of a method for selective culture of S. thermophilus.

The effect of 45° incubation. Incubation at 45° was obtained by means of an electric, water-jacketed incubator. Preliminary tests were carried out to determine the variation in temperature

TABLE 29.

Growth of five strains of *S. thermophilus* on the basal medium at 37° and 45°

Strain	37° Count/ml.	45° Count/ml.
1	443	455
2	100	119
3	350	261
5	420	521
6	500	461
Mean	363	363

in different parts of the incubator. This was found to be negligible. In practice, the incubator itself was adjusted to approximately 46° in order to attain as close as possible the required temperature in the petri dish. At the same time, it was realized that adequate control of temperatures in an agar plate is not possible, at least where high temperatures are being used and the fact that an organism was isolated at 45° under these conditions was not taken as an indication of its ability to grow at 45°. Thus "growth at 45°" where used here refers to ability to grow in an agar plate in a 46° incubator.

The growth of the test strains in the basal medium at 37° and 45° was compared. The results appear in Table 29. It can be seen from this table that there was no marked difference in count at the two temperatures. Similarly, no visible difference in colony size could be detected.

A number of raw and pasteurized milks were now plated out, using the basal medium and incubation at 45° for 48 hr.. Representative isolations from these plates soon showed that the majority of bacteria growing under these conditions - other than spore formers, which were readily distinguishable - were thermoduric streptococci, while micrococci and lactobacilli were very occasionally observed. Members of the S. faecalis group occurred with much greater frequency than had been expected from the results of Abd-el-Malek & Gibson (1948) and, furthermore, could not be distinguished with certainty from S. bovis and S. thermophilus on the basis of colonial morphology.

TABLE 30.

Comparison of counts of '*S. thermophilus* type' organisms in pasteurized milk incubated at 45° with and without re-pasteurization

Milk Sample	Count/ml.		Percentage of isolates *S. thermophilus*	
	Raw	Pasteurized	Raw	Pasteurized
1	2,600,000	2,500,000	100	100
2	540,000	291,000	100	100
3	29,000	46,000	100	100
4	2,000	1,400	100	100
5	138,000	118,000	100	100
6	620,000	970,000	100	100
7	9,000	7,000	100	100
8	480,000	274,000	100	100
Mean	552,000	526,000	100	100

It was observed, however, in the course of this work, that while the S. faecalis group were frequently dominant on plates prepared from raw milks, a streptococcus capable of hydrolysing arginine was never isolated from a pasteurized milk incubated at 45°.

The effect of laboratory pasteurization followed by 45° incubation. A number of raw milks known to have high counts of the S. faecalis type were held at 63° for 30 min. before plating. One series was incubated at 45° and one at 37°. A control, unheated series was also set up at the same time and incubated at 45°. In every case, heating followed by 45° incubation completely eliminated enterococci from the population. Counts of unheated milk at 45° were comparable to the heated series incubated at 37°. It is assumed that the heat treatment caused an increase in the nutritional requirements of the S. faecalis group, which became apparent when the organisms were subsequently incubated at temperatures close to their normal growth limits. Effects of this kind have been noted on a number of occasions with other organisms (Nelson, 1942; Borek & Waelsch, 1951; Ware, 1952; Heinmets, Taylor & Lehman, 1954, etc.).

Although this treatment satisfactorily eliminated the S. faecalis group, it was not certain whether S. thermophilus would completely survive. Since the organism had not yet been isolated from any of the raw milks plated, it was necessary to obtain this information from milks which had already been heat treated. Results for a number of milks before and after re-pasteurization are given in Table 30. There is an overall

TABLE 31.

Growth of strains of S. thermophilus and S. bovis in the presence of different concentrations of potassium tellurite

Organism	Strain	Growth in the presence of tellurite	
		1/5,000	1/3,200
S. thermophilus	3T	+	+
	5T	+	+
	6T	+	+
	7T	+	-
	8T	+	+
S. bovis	4B	-	-
	10B	-	-
	12B	+	-
	13B	+	-

slight reduction in count due to the re-pasteurization, but it was felt that the difference was insufficient to warrant discarding the method. The test strains were not affected by the treatment, but it must be remembered that these strains had been all isolated at 45° and thus may have been specially adapted to high temperatures.

Up to this point, no mention has been made of S. bovis. It was found that while the majority of strains of this organism were unable to grow at 45° following pasteurization, a few strains were able to do so. Thus, one was forced to the conclusion that the method as it stood gave a true estimate of neither the S. thermophilus-S. bovis group as a whole, nor of S. thermophilus alone. Thus, some method of excluding the relatively heat resistant strains of S. bovis was sought. No information could be found in the literature with regard to possible inhibitors with the exception of potassium tellurite. The effect of potassium tellurite. Anderson, Meanwell & Wright (1949) referred briefly to their finding that certain strains of S. thermophilus were relatively resistant to tellurite. On the other hand, Bornstein (1940) and Skadhauge (1950) describe S. bovis as relatively susceptible among the streptococci, although neither of these authors studied S. thermophilus. It seemed worthwhile investigating the resistance of strains of the two species. Five strains of S. thermophilus and four of S. bovis were tested for growth in lactose broth containing varying concentrations of potassium tellurite. Results for the relevant concentrations appear in Table 31. It can be seen

that the strains of S. thermophilus are relatively more resistant than those of S. bovis , but the margin is too narrow to allow its use for the present purpose.

The effect of sodium acetate and polymyxin. Keddie (1951) in his medium for the isolation of lactobacilli from silage observed that certain heterofermentative streptococci were able to survive concentrations of sodium acetate inhibitory to other organisms. For this reason, its selective action against members of the S. thermophilus group seemed worth examining. 0.01M and 0.1M sodium acetate in the basal medium were found to be completely inhibitory to the test strains.

The incorporation into the medium of polymyxin B sulphate (50 i.u./ml.) resulted in complete inhibition of the test strains. The close biochemical relationship between the two species (Abd-el-Malek & Gibson, 1948) made the chances of finding an inhibitor suitable for our purpose slight. Thus, it was decided that no useful purpose would be served by further studies on inhibitors. The method - unsatisfactory though it was - was adopted without further modification at this stage. It was to be used for a preliminary screening of milks for the presence of S. thermophilus after which the characterization of random isolates from plates was to be used for the differentiation of S. thermophilus from the relatively heat resistant S. bovis strains. Because of the limitations of the method as it stood, a control series of unheated milks was set up throughout the work and random isolations were made from

these. In this way, it was hoped that any gross inaccuracies in the method would be revealed. In practice, high counts of S. thermophilus were never found in association with high counts of S. faecalis, so that there was generally good agreement between pasteurized and unpasteurized counts where high counts of S. thermophilus occurred. Moreover, it was found as the work progressed that S. bovis could be distinguished from S. faecalis quite easily on the basis of colonial appearance if plates were held at room temperature for a day following the 45° incubation. In this time a considerable increase in the size of the S. faecalis colonies relative to those of S. bovis occurred.

In practice, the method proved to be of greatest value for relative counts in the study of the outbreak in the creamery plant described in the next section. For other purposes there was always the possibility that relatively heat susceptible strains of the organism were being overlooked.

Final method adopted for detection of S. thermophilus in milk.

Ten ml. sub-samples of each milk were held at 63° for 30 min.. Following cooling, 1 and 0.1 ml. amounts (or the appropriate dilution) were plated out using a medium having the following composition:

0.5% (w/v) lactose
1.0% (w/v) tryptone
1.0% (w/v) beef extract (lab. lemco)
0.5% (w/v) yeastrel
1,000 ml. distilled water

pH was adjusted to approximately 7.

Incubation was for 2 days at 45°.

SECTION II

The occurrence of *Streptococcus thermophilus* in different types of milk

REVIEW OF LITERATURE

Streptococcus thermophilus has rarely been reported from any source other than milk or milk products. Orla-Jensen (1931) states that it may occur in the udder of the cow, but gives no detail of the work. Rodenkirchen (1939) reported it from cow faeces, and Olsen (1949) succeeded in isolating it from the intestines of infants. Certainly, as Sherman (1937) points out, its growth temperature range is consistent with an intestinal origin. However, the bulk of evidence, including its inhibition by bile, is against this. For example, of 294 isolates of high temperature streptococci from raw milk, ice cream, human faeces, cow faeces and the mouths of cows, Sherman & Stark (1931) identified 76 as S. thermophilus and all of these were from raw milk or ice cream.

The reports of the occurrence of S. thermophilus in milk and milk products are many, but only the literature concerning whole milk is considered here. The organism has been isolated from raw milk , either by direct plating (e.g. Hucker, 1928; Galesloot, 1951a) or after a period of selective culture at 40-50° (Orla-Jensen, 1919; Sherman & Stark, 1931) and, more commonly, from freshly pasteurized milks by direct plating (Orla-Jensen, 1919; Sherman, 1937; Abd-el-Malek & Gibson, 1948; Galesloot, 1951b). Outbreaks of so-called 'pin-point' organisms in association with pasteurizing plants at the time when the Holder process found common acceptance (Yates, 1923; Harding, 1923; Taylor, 1924; Swenarton, 1925) were almost certainly due to S. thermophilus, although no description of the causative

organism was given until Johnson & Exworthy (1925) examined a number of isolates during such an outbreak. They found that in every case, the organism was a thermoduric streptococcus having a growth range of 25-50°. Further, Galesloot (1951b) found S. thermophilus often to be responsible for high counts of milk pasteurized by the H.T.S.T. process.

It seemed logical as a starting point in an investigation of the source of S. thermophilus to investigate its occurrence in different milks to determine whether this could be associated with any particular stage in production or with handling methods.

METHODS

Source of Samples. The samples were taken at the two creameries, A and B, of which details appear on p.26, and, in addition, at certain individual farms where mentioned in the text. The creamery samples were from individual farms, bulk road tankers and creamery plant, taken according to the methods described on p.26.

Treatment of Samples. See p.27.

Testing of Samples.

(a) Count of S. thermophilus and closely related organisms: This was carried out according to the method described in Section I.

(b) Total count at 45°: This was carried out using the method described in Section I but omitting the preliminary heat treatment.

(c) Characterization of isolates: This will be dealt with in detail in Section III, but it is necessary to anticipate the results recorded there to some extent in this section. Therefore, it should be mentioned that the preliminary placing of strains of streptococci in the S. thermophilus-S. bovis group was made according to the following criteria - growth at 45°, no action on arginine, acid with or without clot, followed by little or no reduction in litmus milk, and inhibition of growth by 0.1% methylene blue. The distinction between the two species was made on the following bases - preferential utilization of disaccharides and survival of 65° for 30 min. by S. thermophilus; growth in the presence of 40% bile, hydrolysis

of starch and fermentation of maltose by S. bovis. In conjunction with these main features, the action of the particular strain against a range of sugars, its salt tolerance and a number of other properties were taken into consideration. Full details may be found on Pp.117-124.

TABLE 32.

The incidence of '*S. thermophilus* type' organisms in milk samples from different sources

Source of milk	Creamery A				Creamery B				All samples			
	Total Samples	'*S. thermophilus* type'+		Mean count/ml.	Total Samples	'*S. thermophilus* type'+		Mean count/ml.	Total Samples	'*S. thermophilus* type'+		Mean count/ml.
		No.	%			No.	%			No.	%	
Farms	61	6	10	2,400	219	13	6	2,600	280	19	7	2,500
Country creameries	29	24	83	450	4	0	-	-	40	32	80	400
Creamery plant	68	50	74	650	25	13	50	170	93	76	82	440
Total	138	80	58	1,000	248	26	10	1,400	413	127	31	730

TABLE 33.

Frequency of occurrence of '*S. thermophilus* type' organisms on particular farms

Farm	Occasions of Sampling	'*S. thermophilus* type' + Samples		
		No.	%	Count/ml.
11B	5	2	40	2,000
18C	2	2	100	1,000
20D	3	1	33	1,000
25D	3	2	67	5,000
32G	4	3	75	4,000
59S	3	1	33	700
64S	4	2	50	600
Total	24	13	54	3,000

RESULTS

Preliminary general survey of milks from different sources

The results recorded here were obtained during the period January-August, 1955.

A total of 386 samples from individual farms, country creameries and creamery plant were screened for the presence of S. thermophilus. The results are recorded in Table 32. It can be seen that 69% of all samples examined showed <10/ml. S. thermophilus or closely related organism. Considering the different types of milk, 93% of all farm samples, but only 20% of country creamery samples and 18% of creamery plant samples were clear. On the other hand, the average count of positive farm milks was higher than in the case of the other two types of milk. The picture for the two creameries was very similar, namely that a certain low level of contamination persisted in the majority of samples examined. This is consistent with the observations of Abd-el-Malek & Gibson (1948).

The results for samples recorded as positive in this preliminary survey were examined in greater detail.

Farm samples. Detailed examination of the results for the creamery A producers was not warranted at this stage because the individual producers had not been sampled more than once. However, some information could be extracted from the creamery B results. The thirteen positive samples at this creamery were due to seven producers. Results for these seven producers, covering all occasions of sampling, are given in Table 33. It can be seen that 54% of all samples from these farms were positive,

TABLE 34.

Frequency of occurrence of 'S. thermophilus type' organisms at country creameries

Creamery	Total samples		Positive samples		
	No.	Mean Count/ml.*	No.	%	Mean Count/ml.*
ZA	2	125	1	50	250
YB	3	160	3	100	160
XC	4	60	1	25	240
WD	10	180	9	90	200
TG	3	750	2	67	750
QK	3	2,300	3	100	2,300
PL	7	180	7	100	180
OM	1	120	1	100	120
HS	3	60	3	100	60
GS	2	120	1	50	230
DW	2	10	1	50	20
Total	40	320	32	80	400

* 'S. thermophilus type' organisms

TABLE 35.

Monthly variation in counts of '*S. thermophilus* type' organisms in samples from country creameries

Month	No. Samples	'*S. thermophilus* type' + Samples		
		No.	%	Mean count/ml.
April	6	6	100	170
May	14	13	93	250
June	8	5	63	280
July	4	1	25	240
August	8	7	88	1,000
Total	40	32	80	400

TABLE 36.

Association of '*S. thermophilus* type' organisms with different parts of the plant at creamery A

Plant part	Occasion of sampling									Total samples		Positive samples		
	1	2	3	4	5	6	7	8	9	No.	Mean* Count/ml.	No.	%	Mean* Count/ml.
Weigh tank	-	50	<10	<10	<10	<10	-	-	-	5	<10	1	20	50
Before 1st pump	-	110	<10	<10	<10	<10	50	-	<10	7	<10	2	28	80
After 1st pump	-	120	<10	<10	<10	<10	<10	<10	<10	8	<10	1	12	120
Storage tank (1)	60	6,000	300	1,000	80	80	120	120	430	14	1,000	13	93	1,000
(2)	-	6,000	80	100	<10	120	-	-	-					
Before 2nd pump	-	-	90	1,000	<10	40	50	-	290	6	250	5	83	400
After 2nd pump	-	5,000	190	500	20	120	320	250	350	8	800	8	100	800
Balance tank	120	6,000	110	750	30	230	90	220	360	9	900	9	100	900
Cooler exit	-	7,000	250	700	200	200	120	400	400	11	1,000	11	100	1,000

* '*S. thermophilus*' type organisms

showing a tendency for the organism(s) to be associated with particular farms. Ten strains from farm 11B and ten from farm 25D were characterized, and all were found to be varieties of S. bovis. This preliminary finding, though by no means conclusive; was of considerable interest and was in accordance with the work of Abd-el-Malek & Gibson (1948) who did not isolate a single strain of S. thermophilus from the raw milks which they examined. For the moment, the work was left at this point.

Country Creamery samples. Results for the individual country creameries are summarized in Table 34. There is no marked tendency for the organism(s) to be associated with particular creameries, a small contamination at all creameries appearing in the majority of samples.

Abd-el-Malek & Gibson (1948) found that counts of S. thermophilus in pasteurized milks were fairly constant throughout the year. It was thus of interest to examine these results from the point of view of monthly variation in counts. From Table 35, it can be seen that there was no definite seasonal trend. However, in August a small increase occurred which coincided with a spell of very warm weather.

Creamery plant samples. Results for creamery A appear in Table 36. On each occasion, counts were low up to the stage of the storage tanks, when an increase occurred which was maintained until after pasteurization.

Late in August, another series of samples was taken from

TABLE 37.

Occurrence of '*S. thermophilus* type' organisms on creamery plant at the beginning of an outbreak of 'pin-point' colonies

Plant part	'*S. thermophilus* type' (count/ml.)
Weigh tank	<10
Before 1st pump	<10
After 1st pump	<10
Storage tank	27,000
Before 2nd pump	36,000
After 2nd pump	31,000
Balance tank	28,000
Cooler exit	2,500,000

FIG. 20

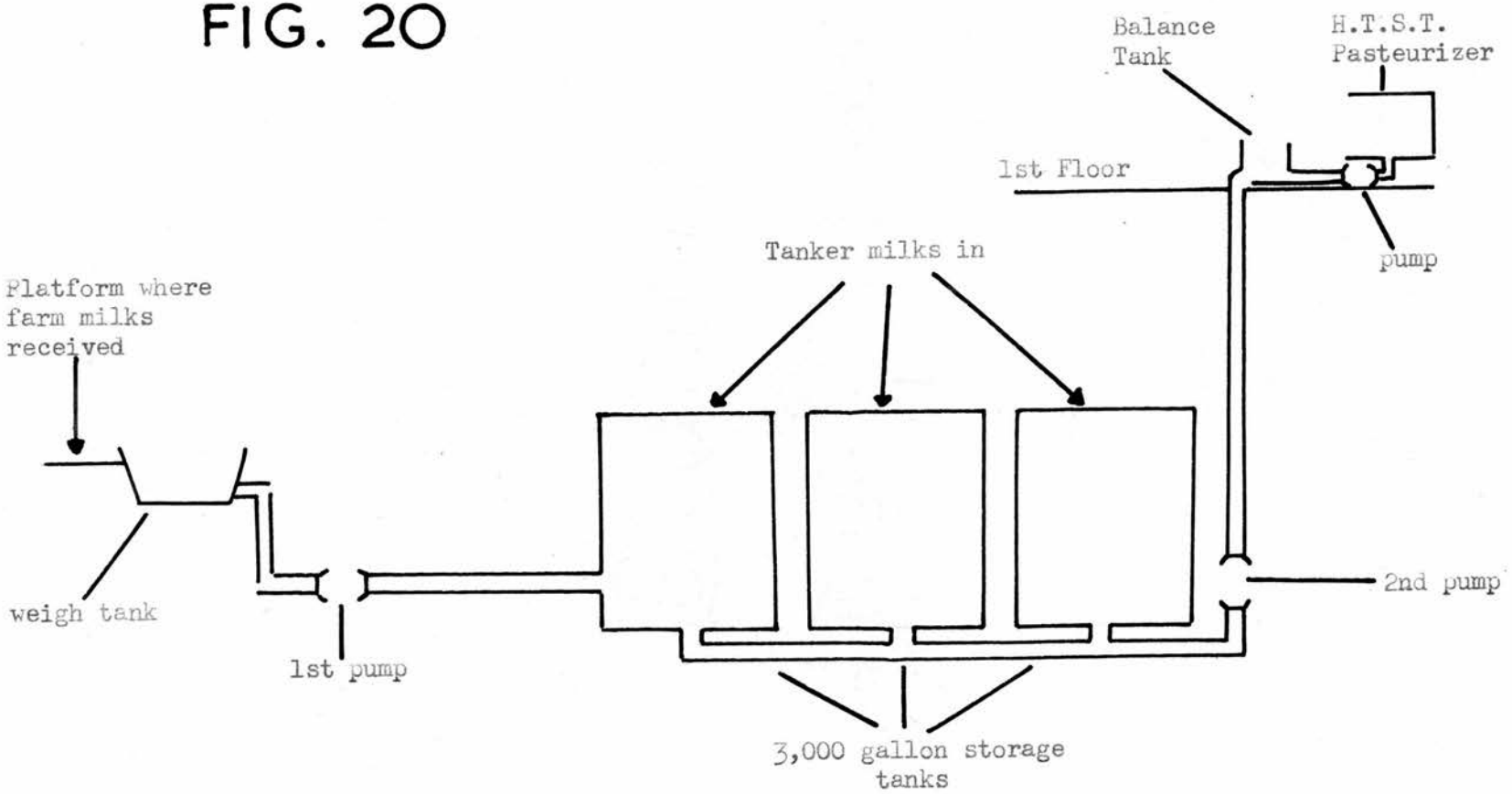


Fig. 20. Diagrammatic representation of lay-out of creamery where outbreak of S. thermophilus occurred.

creamery A plant with the results given in Table 37. Sixty-four colonies were isolated from these plates, and 63 were identified as S. thermophilus while the other one was a variety of S. bovis. There seemed to be sufficient evidence from these results that an outbreak of S. thermophilus had occurred in the creamery plant. The results of the preliminary survey had indicated that S. thermophilus was characteristically associated with creamery plant, the organism being of small importance - if indeed of any importance at all - in farm samples. It therefore seemed logical to proceed to a detailed investigation of the creamery A outbreak with the idea of elucidating the conditions leading to it.

Investigation of an outbreak of S. thermophilus in a creamery plant

The outbreak to be described occurred in late August, 1955, and continued through the month of September.

Before proceeding to the results of this investigation, it is necessary to give a brief description of the creamery plant and the methods in use at the time, as far as they could be ascertained.

Description of the plant. A diagrammatic representation of the lay-out of the creamery is given in Fig. 20. Milk received in cans at the platform was tipped into the weigh tank, flowed by gravity to the first pump and from there was pumped to one of the three storage tanks. These tanks, 3,000 gal. in capacity, were of 'glass-lined' steel and non-insulated. Agitation and aeration were achieved by a revolving propellor

at the bottom of each tank and by compressed air blown in from one side. The tanks were emptied by gravity and the milk passed along pipe-lines to the second pump which lifted it to the balance tank on the floor above. From there, another pump carried it to the pasteurizer itself, which was an APV Paraflow plate type heat exchanger of capacity 2,000 gal/hr..

Handling methods. At the time of the outbreak, about 17,000 gal. of milk were being pasteurized each day. In addition, a further 2-3,000 gal. surplus milk were being pre-heated at the end of the day's run.* After pre-heating, the surplus milk was pumped to one of the storage tanks where it was held overnight and pasteurized first the next day.

Pasteurization commenced each day at about 6 a.m. and the plant was in continuous operation for up to ten hours without cleaning. Whenever a break in supply occurred during the day, water was pumped through the plant to maintain pasteurizing temperature and allow the plant to be brought into operation again without delay whenever the supply of milk became available again.

Sources of milk. At the time, 6-9,000 gal./day were coming

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* During any warm spells of the year, it is the practice at this creamery to hold all surplus raw milk at 150°F for 10 sec. before the overnight storage period. This is achieved by passing the milk through the pasteurizer with the holder adjusted to the temperature of the pre-heater. The milk so treated is thus referred to as pre-heated.

in road tankers from country creameries, mostly in the raw condition. This milk arrived early in the day and followed the pre-heated milk through the plant, being pumped direct into one of the storage tanks. The rest of the milk came from individual farms and passed via weigh tank and storage tanks to the pasteurizer.

Cleaning of plant. After use, the pasteurizer was rinsed with cold water, followed by a paracleanser to emulsify fats and another cleanser to dissolve proteins. It was then rinsed again, and water at a temperature of 185°F was circulated through it for 30 min.. Only then was it dismantled and each plate brushed and scrubbed with a detergent and water. Filter cloths were changed every half hour during pasteurization.

The storage tanks were rinsed with cold water, scrubbed with detergent and rinsed again. Steaming was for 15 min., but this step was often omitted because the cooling process was very slow.

The tanks were used in rotation. Thus a single tank was never used for the same type of milk on successive days.

Consideration of the results of Tables 36 and 37. From these tables, three main points emerge. Firstly, the organism appears to be absent from, or present in very small numbers in, the farm milks. Secondly, contamination appears to be occurring at the storage tanks. Lastly, in the case of the samples taken during the outbreak, there appears to be an increase in count in the pasteurizer. Each of these points was considered in turn.

TABLE 38.

Examination of equipment at a farm showing high counts of S. bovis

Sample source	Type of sample	Count/ml. S. bovis			
		1	2	3	4
Clusters	Rinse	5,000,000	1,000,000	2,000,000	-
Unit cans	Rinse	<10	<10	<10	<10
Cans	Rinse	<10	<10	<10	<10
Brushes	Rinse	<10	<10	<10	-
Cooler (in can)	Swab	<10	-	-	-
Tank water	-	<10	-	-	-
Unit cans	First milk into	20,000	4,000	35,000	-
Cans	First milk into	20,000	-	-	-

1. Apparent absence of the organism from farm milks.

The fact that all samples taken before the storage tanks had negligible counts suggested that farm milks were not responsible for the contamination of the plant. However, in order to establish more certainly that this was so, samples from each of the 177 producers supplying the creamery were examined. Of these, 162 or 91% showed < 10/ml. S. thermophilus type. Of the positive samples, 7 showed less than 100/ml., and a further 5 showed less than 1,000/ml.. The other three samples gave counts of 4,000, 14,000 and 50,000 per ml.. One hundred and sixty two colonies from plates prepared from raw and laboratory pasteurized sub-samples were isolated and examined. Of these, 161 proved to be probable varieties of S. bovis and one w as a strain of S. thermophilus.

A visit was made to one of the farms showing high counts of S. bovis - 4,000, 14,000 and 10,000/ml. on three successive occasions of sampling - and the equipment examined. The results are summarized in Table 38. It can be seen that a build-up of the organism had occurred on the clusters. It was claimed at the farm that the methods of sterilization were satisfactory, but unfortunately it was not possible to see them being carried out. It must be assumed however, from the bacteriological evidence, that steaming was not satisfactory and that at some stage the clusters were being held for long periods at temperatures favourable to multiplication of the organism. However, as far as could be determined, it was not the practice to store the clusters in hot water or in a hot

place. It is of interest to note at this stage that the outbreak of a thermoduric streptococcus at an individual farm reported by Anderson & Meanwell (1933) was, from their subsequent description (Anderson & Meanwell, 1936), very probably S. bovis. This outbreak was traced to the practice of cleaning milk rubbers and teat cups by immersing in hot water for several minutes then plunging them straight into cold water in which they were left to soak between milkings.

Since all milk samples from the farms supplying the creamery were virtually free from S. thermophilus, it was felt that contamination at the farm could be eliminated from consideration as a cause of the outbreak.

2. Contamination at the storage tanks.

The increase in numbers of S. thermophilus at the storage tank stage could have been due to contamination from improperly cleaned tanks or previous contamination of milks entering the plant at this stage.

Cleaning of tanks. Although the cleaning methods in use seemed quite favourable to a build-up of contaminants, it was difficult to see when temperature conditions particularly favourable to S. thermophilus occurred. One possibility was the period of slow cooling following the steaming process. Assuming that steaming was inadequate - at least for some parts of the tank - it is conceivable that some pocket of contamination could have arisen in response to regular steaming. However, the steaming certainly did not occur regularly especially during this warmer period of the year when a larger than usual volume of milk was

TABLE 39.

Occurrence of '*S. thermophilus* type' organisms in creamery tanker milks in relation to outbreaks of 'pin-point' colonies in creamery plant

Month	'Pin-point' Outbreak	Total Samples	'*S. thermophilus* type' + samples		
			No.	%	Mean count/ml.
September, 1955	Present	14	14	100	1,500
March, 1956	Absent	8	6	75	200
April, 1956	Absent	4	1	25	30
May, 1956	Present	5	3	60	6,000
June, 1956	Present	11	8	73	6,500

being handled and economic considerations dictated practice. It could not be determined how often steaming was carried out, but during the course of many visits made by the writer to the creamery she was never once able to witness the process. Consequently all samples taken were from cleaned tanks which had not been steamed. These were uniformly negative for S. thermophilus, although a number showed high counts of S. faecalis. The investigation of tank cleaning had to be left at this rather unsatisfactory point, but it seemed on the whole unlikely that inadequate tank cleaning could account for any appreciable contamination of the enormous volumes of milk passing through the tanks.

The possibility of contamination by milk making its first contact with the plant at the stage of the tanks was now considered. There were two types of milk in this category. These were bulk tanker milk and pre-heated milk.

Creamery bulk tanker milk. Fourteen samples of bulk tanker milks were examined and found to have an average count of 1500/ml. S. thermophilus type. This was a slightly higher mean count than the figure of 450/ml. obtained over the six months preceding the outbreak. It is interesting to note that a similar increase in count occurred during an outbreak in the following year. This is illustrated in Table 39. The outbreak referred to occurred during the months of May and June. Representative strains isolated from these creamery milk samples all proved to be S. thermophilus. This observation was of particular interest because it was the first time that the writer had

succeeded in isolating S. thermophilus from raw milk. Furthermore, here at least was a point where the organism could enter the plant. Most of the tanker milk coming in to the central creamery was from a single country creamery, PL. It was therefore decided to visit this creamery.

It was found that creamery PL was mainly concerned with cheese making. Thus, most of the milk coming in to this creamery was subsequently pasteurized. The raw surplus milk which went to the central creamery was chilled before being loaded into the road tanker. It was found that this chilling was carried out in the cooling section of a pasteurizer normally used for the cheese milk, thus allowing the possibility of contamination from pasteurizing plant. Counts were made of the pasteurized cheese milk and of the chilled raw milk in the storage tanks. Unfortunately, it was not possible to obtain a sample of unchilled milk. The cheese milk showed a count of 20,000/ml., while two samples of raw milk contained 1,500 and 3,000/ml. S. thermophilus type respectively. Five isolates were made from each type of milk, and all were found to be S. thermophilus. No investigation was made of the milks from individual farms supplying this creamery, for it was felt that little was to be gained from such an undertaking, in view of the virtual absence of the organism from a total of 457 farm samples examined previously. Moreover, a relatively large number of producers would need to show high counts to produce significant contamination of the bulk. The most likely explanation seemed to be that the raw milks were being contaminated

TABLE 40.

Contamination of creamery plant with '*S. thermophilus* type' organisms from pre-heated milks

Type of milk	Time of Pumping (min.)		Sample source	'*S. thermophilus* type'
	Start	Finish		count/ml.
Creamery tanker	0	15	Road tanker	600
			Storage tank	3,000
			Balance tank	3,000
			Cooler exit	1,400
Pre-heated	15	60	Storage tank	2,000,000
			After 2nd pump	1,600,000
			Balance tank	2,000,000
			Cooler exit	970,000
Creamery tanker	60	?	After 2nd pump	128,000
			Balance tank	90,000
			Cooler exit	118,000

TABLE 41.

Counts of '*S. thermophilus* type' organisms before and after pasteurization

Occasion	Time of day	Count/ml.	
		Balance tank	Cooler exit
1	6.45 a.m.	2,500	1,400
2	7.00 a.m.	2,000,000	970,000
3	8.00 a.m.	90,000	118,000
4	3.30 p.m.	32,000	213,000

from the pasteurizing plant, which once more focused attention on this stage in production.

Pre-heated milks. Six samples of stored, pre-heated milks gave an average count of 1,000,000/ml.. Fifty isolates were examined and all were found to be S. thermophilus. A small experiment was now arranged, with the co-operation of the creamery, to demonstrate contamination of the plant by pre-heated milks. First, at the beginning of a day's pasteurization, a creamery tanker milk was pumped through the plant for 15 min.. Then, the pump was switched over to the pre-heated milk and this flowed through for 45 min.. This was followed by the rest of the creamery tanker milk. Samples were taken at each stage and the results are given in Table 40. It can be seen from this table that the pre-heated milk can cause a considerable contamination of the milk which follows it through the plant.

It remains, however, to account for the very high counts in the pre-heated milks. This brings us to the third point emerging from Table 37, namely the increase in count of S. thermophilus during pasteurization.

3. Increase in count of S. thermophilus during pasteurization.

In Table 41, counts before and after pasteurization on four different occasions are given. It will be noted that in only one case did an increase in count between balance tank and cooler exit occur, and this was in the sample which had been taken in the afternoon, i.e. after the plant had been in operation for several hours without being cleaned. Thus, it was thought that the increase might be related to the length of run.

TABLE 42.

Counts of 'S. thermophilus type' organisms before, during and after pasteurization - results of a single day's run

Time of day	Count/ml. 'S. thermophilus type' organisms				
	Balance tank	Before filter	Filter	After filter	Cooler exit
6.30 a.m.	800	-	29,000	-	220
7.30 a.m.	23,000	19,000	24,000	15,000	16,000
8.00 a.m.	4,000	-	9,000	-	12,000
8.35 a.m.	5,000	-	5,000	-	4,000
9.10 a.m.	800	-	4,000	-	400
9.30 a.m.	6,000	-	8,000	-	400
10. 10 a.m.	4,000	3,000	2,000	2,000	2,000
10. 35 a.m.	18,000	-	25,000	-	20,000
11. 05 a.m.	-	-	13,000	-	-
11. 30 a.m.	6,000	7,000	7,000	7,000	122,000
12. 00 a.m.	2,000	-	8,000	-	89,000
* 1. 30 p.m.	<10	-	-	-	52,000
* 2. 05 p.m.	<10	-	6,000	-	331,000
* 2. 30 p.m.	<10	-	1,000	-	180,000

* Farm milk only.

To test this point, samples were taken at intervals throughout one day's pasteurization. From Table 42, in which these results are recorded, it can be seen that the suspected increase during the day did, in fact, occur. On this particular day, a sharp increase between balance tank and cooler exit occurred after the plant had been in operation for five hours without a break, and was maintained thereafter.

Having established that an increase did occur during pasteurization, it was necessary now to try to explain it.

One possible cause seemed to be that of contamination from filter cloths. It is the practice in this creamery to leave the hot filter cloths lying on the floor after their removal from the pasteurizer. After varying lengths of time, these cloths are hosed out. Later they are washed and are reused without sterilization. Should multiplication of S. thermophilus have occurred at any stage while the cloth was outside the pasteurizer, the opportunities for re-contamination of milk passing through it when the cloth was used again would be excellent. Results for samples taken from the filter section after each filter change are included in Table 42. It can be seen that the observed increase in count occurred subsequent to the filter section.

The possibility of multiplication within the plant while in operation was now considered. According to the literature, the temperature range over which growth of S. thermophilus could conceivably occur may be stated broadly as 15-50°. (In practice,

the majority of strains isolated in this work were unable to grow at either of these extremes.). Therefore, the H.T.S.T. plant was considered from the point of view of sites where temperatures would permit growth of S. thermophilus. The filter, pre-heater and holder sections, where temperatures are above 60°, were all immediately eliminated from consideration. Similarly at the other end of the temperature scale, the cold water and brine sections were thought to be unlikely sites. Thus, there remained the possibility of the regeneration section. In this section, the raw milk is raised to about 60° and the pasteurized milk cooled from about 73° to about 24°. The theoretical length of time taken by a milk particle to pass through the regeneration section in either direction is about 22 seconds (Ashton, 1950). Thus, if multiplication occurs in this section it must be interpreted as meaning that there is some pocket or groove in the equipment where milk can lodge and remain for sufficiently long periods for the organism to multiply. Davis (1950) states that an important weakness of plate type heat exchangers is generally acknowledged by manufacturers to be a tendency for the rubber gaskets between the plates to work loose. In cases short of the actual blowing out of the gasket, which sometimes occurs, the result is that a groove of the kind visualized is formed between gasket and plate. It therefore seemed feasible that multiplication of S. thermophilus could occur in the regeneration section. In order to test the point, samples were taken three times daily for a week at the balance

TABLE 43.

Variation in count of '*S. thermophilus* type' organisms before, during and after pasteurization with increasing lengths of run of an H.T.S.T. pasteurizer

Occasion	Time	Length of run (hr.)	Count/ml. '*S. thermophilus* type' organisms			C.E.:B.T.*	Swab of regeneration section
			Balance tank (B.T.)	End of holder	Cooler exit (C.E.)		
Day 1	6.00 a.m.	0	10	-	10		Negative
	10.30 a.m.	4.5	32,000	34,000	196,000	6	
	3. 00 p.m.	9	7,000	7,000	131,000	19	
	4.30 p.m.	10.5	2,000	9,000	95,000	11	
Day 2	9.45 a.m.	3.5	235,000	255,000	344,000	1	Negative
	2.30 p.m.	8.5	14,000	17,000	56,000	3	
	5.20 p.m.	11.5	4,000	16,000	79,000	5	
Day 3	10.20 a.m.	4.5	70,000	66,000	138,000	2	Negative
	3.00 p.m.	9.0	5,000	6,000	35,000	6	
	5.30 p.m.	11.5	3,000	5,000	77,000	15	
Day 4	10.30 a.m.	4.5	106,000	86,000	217,000	2	Negative
	2.30 p.m.	8.5	2,000	2,000	9,000	4	
	4.00 p.m.	10.0	500	1,000	29,000	29	
Day 5	11.50 a.m.	5.0	15,000	19,000	10,000	1	Negative
	2.00 p.m.	8.0	2,000	3,000	5,000	1	
	4.20 p.m.	10.5	500	1,600	56,000	30	

* Ratio of counts

tank, after the holder and at the cooler exit. In addition swabs were taken each day after the plant was dismantled to detect any pockets of contamination. Unfortunately, these swabs could not be obtained before a rinse, cleanser and paracleanser had been passed through the plant, but they were taken in the hope that the preliminary cleaning had missed some pockets. Results are given in Table 43. Although the results of the swabs were negative, there is good evidence that the observed build-up occurs between the end of the holder and the cooler exit, i.e. in the regeneration section on the cooling side.

DISCUSSION

The results given in this section of the work have emphasized the elusiveness of S. thermophilus in raw milks. Of a large number of isolates made from farm milks, only one strain of S. thermophilus has been found. This finding is in agreement with those of other authors who have either not detected S. thermophilus in raw milks (e.g. Abd-el-Malek & Gibson, 1948) or have found it to be present in very small numbers (e.g. Galesloot, 1951a).

On the other hand, high counts of S. thermophilus are often obtained in freshly pasteurized milks. This can be associated in part, as the foregoing results have shown, with the ability of the organism to multiply during H.T.S.T. pasteurization under certain conditions. The conditions necessary seem to be firstly that there be cracks or grooves in the regeneration section where milk can lodge, away from the general milk flow; and secondly that there be 5 or 6 hours' continuous pasteurization.

In the case of the creamery described in the present work, although a small inoculum of S. thermophilus was entering the plant throughout the year through the creamery tanker milks, the actual outbreaks were confined to the warmer months. This was found to be associated with the practice of pre-heating. In the year subsequent to the outbreak investigated, it was observed that spasmodic outbreaks followed spasmodic pre-heating in the spring. Finally, in mid-summer, the adoption of pre-heating as a daily routine resulted in a prolonged

outbreak, which ceased a few days after the process was discontinued. The effect of pre-heating is probably twofold. Firstly, once the outbreak has occurred, it maintains and intensifies it by contaminating the plant to a considerable extent early in the day. However, its most important effect is that, while it is being carried out, the plant is run for several hours longer than usual without cleaning. The importance of this was shown when, at one stage, the manager of the creamery was persuaded to change to a second plant for a few hours each day allowing the main plant to be cleaned. The result was the virtual elimination of S. thermophilus from the plant even though pre-heating was continuing. When the normal procedure was recommenced, the outbreak occurred again. Murdock, Brokaw & Allen (1955) have noted in the case of pasteurized orange juice that large numbers of Lactobacillus buchneri appeared in the regeneration section of a plate type heat exchanger after six hours' continuous operation. Similarly, Yale & Kelly (1933) and Rowlands (1945) reported multiplication of thermophilic organisms in an H.T.S.T. pasteurizer after a prolonged plant run.

Thus, while there seems to be good evidence for multiplication of S. thermophilus during pasteurization, as pointed out previously, this only occurs when certain conditions are fulfilled. It remains then to explain why S. thermophilus is so common a contaminant of freshly pasteurized milk and yet so rarely isolated from raw milk. The sudden appearance of the organism in heat treated milk leads one to suspect that it might

in fact, be a heat-induced variant of some other species. The obvious possibility is S. bovis since this organism occurs commonly in raw milk and appears to be closely related biochemically to S. thermophilus (Abd-el-Malek & Gibson, 1948; Galesloot, 1951a,b, 1952).

Burkey & Rogosa (1940) grew cultures of S. thermophilus and Lactobacillus helveticus for several months at maximum growth temperatures in unheated milk. In this way they induced the organisms to adapt themselves to survive higher temperatures, to grow at a temperature range above that of the parent culture and to grow actively in unheated milk.

A note on some initial attempts along these lines to induce such variants in S. bovis follows. Unfortunately, very little time remained for this work so that they were of a very preliminary nature.

Note on initial attempts to induce S. thermophilus-like variants in strains of S. bovis under the influence of heat and incubation at high temperatures

Fourteen isolates representative of the group II strains given in Section III (p.118) were used. Lactose broth cultures, incubated at 37° for 18 hr., provided the inocula for two series of tubes.

Series I. Litmus milk with 0.25% yeastrel and 0.25% glucose was inoculated with 2 drops from a Pasteur pipette and placed in an incubator at 46°. When acid production was observed (1-2 days), transfers were made into fresh medium. This was continued for 24 days. After 2 transfers, 11 strains were no longer viable. The 3 remaining strains were carried on. By the end of 3 weeks, daily transfers were essential because of the vigorous acid production. In fact, one culture was incubated for 2 days accidentally and was found to have lost its viability as a result. The 2 surviving strains were tested and it was found that in fermentation reactions, ability to hydrolyse starch and failure to survive 65° for 30 min., their properties remained unchanged. However, there had been a marked change in the vigour of growth as indicated by rate of acid production. It is probable that transfers would need to be carried on for several months before adaptation in other properties could be observed.

Series II. Lactose broth was inoculated as above and incubated at 37° for 18 hr. The tube was removed from the incubator, and the entire contents held at 63° for 20 min.. Six drops of the

heated culture were transferred to a fresh broth and re-incubated. The next day, the whole process was repeated. After 2 transfers, all strains had lost their viability. The weakness of this second series was that heating was carried out in acid conditions and that cultures in their stationary phase were used for heating. As Anderson & Meanwell (1936) and White (1954) have shown with S. bovis and S. faecalis, maximum heat resistance is obtained in the lag and early logarithmic phases of growth.

It is felt that further investigations along these lines are warranted. The negative results obtained here are not surprising since the methods were not worked out properly and the time available was too short.

SECTION III

Characterization of strains of the *S. thermophilus* - *S. bovis* group.

Representative isolates from the milks examined in Section II were studied for the purpose of characterization of strains. The results seemed of sufficient interest to warrant considering them in some detail.

REVIEW OF LITERATURE

It was pointed out in the introduction to this part of the thesis that S. thermophilus has proved difficult to place within the genus. Sherman (1937a), who recognized the value of the temperature limits of growth in a primary grouping of the streptococci, placed S. thermophilus in the so-called 'viridans' group. Most members of this group shared with the enterococcus group the ability to grow at 45° and relative heat resistance, and were distinguishable from it on the basis of inability to grow at 10°, inhibition by 6.5% salt and 0.1% methylene blue and failure to give strong reduction in litmus milk. Thus, since its claim to recognition as a distinct group rested solely on negative characteristics, it was probably inevitable that it should comprise a heterogeneous collection of species whose chief feature in common was that they could not be fitted into any other group. Sherman himself regarded the group as an interim one, pending the recognition of more satisfactory criteria.

Of the species other than S. thermophilus included within the viridans group, only Streptococcus bovis seems to show any possibility of relationship to it. Abd-el-Malek & Gibson (1948) from a study of a large number of strains isolated mainly from pasteurized milk, found a number which

on biochemical grounds, they regarded as intermediate between the two species. Galesloot (1951a,b, 1952) reported a similar situation with his strains from milk. On the other hand, serological evidence does not support this view. Early reports that a proportion of strains of S. bovis reacted with group D anti-sera (Sherman, 1938; Shattock, 1944) culminated in the work of Shattock (1948, 1949), which satisfactorily established the serological identity of the species as group D and simultaneously demonstrated its relationship with the enterococci. On the other hand, attempts to group S. thermophilus have consistently failed. Shattock (1949) could find no serological relationship with group D or with any of the groups A to N, and no further progress has been reported up to the present time.

It seemed of particular interest to follow the biochemical approach further. The criteria used by Abd-el-Malek & Gibson (1948) formed the basis for the work described here.

METHODS

Every isolate was picked from an individual colony into litmus milk and incubated at 37°. After the preliminary grouping of strains, the purity of the culture was checked by streak plating twice on the basal medium (p. 86) and picking isolated colonies into lactose broth (p. i).

Stock cultures were carried in litmus milk incubated overnight at 37° or until the acid reaction was just visible, and stored in a refrigerator. Transfers were made every 6-8 weeks.

Unless otherwise stated the inoculum for all tests was from an 18 hr. culture in lactose broth, obtained by transfer from the stock litmus culture and incubated at 37°.

Tests used in initial selection of strains.

1. Growth in litmus milk: Litmus milk was prepared in the normal way. The changes looked for were acid production, clot production and the time and degree of reduction.

2. Production of catalase: The strain was grown for 2 days at 37° on slopes of basal medium. At the end of this time, an aliquot of hydrogen peroxide (5 vol.) was poured over the slope, which was then examined for the formation of bubbles of oxygen.

3. Morphology: This was determined in lactose broth incubated for 18 hr. at 37°. A drop of the culture was placed on a glass slide and rapidly dried out at about 45°. This was then stained by the Gram method or with dilute methylene blue.

4. Hydrolysis of arginine: The method of Niven, Smiley &

Sherman (1942b) was used, with incubation for 2 days at 37° before testing.

5. Growth at 45°: This was determined using litmus milk fortified with 0.25% yeastrel and 0.25% glucose. Incubation was in a circulated water bath, the tubes being raised to the temperature of the bath before inoculation. One drop from a Pasteur pipette was the inoculum used. Readings were made after 24 and 48 hr..

6. Inhibition by 0.1% methylene blue: Skim milk, sterilized in bulk by steaming on three successive days, was mixed with 1% medicinal methylene blue, sterilized separately, to give the required concentration. The methylene blue milk was then distributed in approximately 2 ml. quantities in sterile ¼ oz. vials. Incubation was at 37° for 2 weeks.

Tests used in preliminary grouping of strains.

7. Inhibition by 40% bile: Ox-bile taken from the gall bladder as aseptically as possible was mixed with a broth containing 0.5% lactose, 0.5% tryptone, 0.5% beef extract and 0.5% yeastrel in the ratio of 2 parts to 3. The mixture was then tubed in 5 ml. amounts and used for the test.

8. Survival of 65° for 30 min.: This was determined by the method of Abd-el-Malek & Gibson (1948) except that incubation was for two days at 37°.

9. Hydrolysis of starch: Poured plates were used with an inoculum of about 0.1 ml. and a medium containing 0.1% soluble starch, 0.5% peptone, 0.5% beef extract, 1.5% agar. After two days' incubation at 37°, the non-hydrolysing strains had

produced no growth in the medium and thus were readily detectable from the hydrolysers. However, in addition, the plate was flooded with iodine which stained the unhydrolysed starch deep blue.

10. Preferential utilization of disaccharides: The test substances used were glucose and lactose. The tubes were inoculated in pairs, using a multiple loop capable of delivering about 3-4 loopsful. Incubation was at 37° and readings were made at 8 and 24 hr.. Results for the majority of strains could be determined after 8 hr., but where no growth was visible at this time, the differentiation could be clearly seen at 24 hr.. The method was preferred to the use of a smaller inoculum because it usually gave results within 8 hr..

11. Fermentation of maltose: As in the case of all the fermentation tests, a 5% solution of maltose was prepared separately and sterilized by filtration. The appropriate amount was then pipetted into broths containing 0.5% peptone, 0.5% tryptone, 0.5% beef extract, 0.5% yeastrel to give a final concentration of 0.5%. Brom cresol purple was the indicator used. Incubation was at 37°, and readings were made at 24, 48, 72 hr. and after one week.

Additional tests:

12. Fermentation of sugars and alcohols: The method used was the same as in the case of maltose. The substances tested in addition to maltose, glucose and lactose, were sucrose, raffinose, arabinose, cellobiose, galactose, salicin, inulin, sorbitol, mannitol and glycerol.

13. Inhibition by sodium chloride: A solution of sodium chloride (20%) was prepared and sterilized separately. Following the method of Abd-el-Malek & Gibson (1948), the salt was incorporated into lactose broth to give final concentrations of 2, 3 and 4%. Readings were made after 24 hr. at 37°. Longer incubation periods reduced the sensitivity of the test.

14. Hydrolysis of aesculin and hippurate: These properties were determined simultaneously using the method of Davis & Rogers (1939), which was developed for the identification of mastitis organisms and had been used successfully by Abd-el-Malek & Gibson (1948) in their general classification of milk streptococci.

15. Growth on blood agar: Plates were prepared incorporating 5% ox-blood into a medium containing 0.5% peptone, 0.5% beef extract and 0.5% sodium chloride. These were streaked with the test strain and examined after 40 hr. at 37°.

16. Growth at 50°: This was carried out in the same way as growth at 45°. (p.112).

17. Survival of 60° for 30 min.: This was carried out in the same way as survival of 65°. (p.112).

18. Growth on 5% sucrose agar: Several workers have reported that a few strains of S. bovis are capable of polysaccharide production in the presence of high concentrations of sucrose (e.g. Sugg, Hehre & Neill, 1942), resulting in the development of mucoid colonies on agar containing 5% sucrose. It was possible that this might be of some value in differentiating

strains. Streakings were made on to the surface of a medium containing 0.5% tryptone, 0.5% beef extract, 0.5% yeastrel and 5% sucrose. Incubation was for two days at 37°.

Additional tests with inhibitory substances

The work of Richards, Soulides & Soulides (1945) suggested that crystal violet (1/200,000) and thallium acetate might be of value in differentiating the strains. Potassium tellurite was also tested further (for reasons, see p.84).

19. Inhibition by 0.2% thallium acetate: A solution of 1% thallium acetate was prepared and sterilized separately. It was then incorporated at the required concentration into lactose broth with brom cresol purple as indicator. Preliminary trials with about 30 strains indicated that 0.2% was the most useful concentration for grouping strains. Where acid production failed to occur after 24 hr. at 37°, this was regarded as representing inhibition.

20. Inhibition by 1/200,000 crystal violet: A solution of 1/5,000 crystal violet was prepared and sterilized separately. It was then incorporated into lactose broth with brom cresol purple at the concentration recommended by Richards et al. (1945). Where acid production failed to occur after 48 hr. at 37°, this was regarded as representing inhibition.

21. Inhibition by potassium tellurite: A solution of 1/10 potassium tellurite was prepared and sterilized by filtration. A fresh solution was made up on each occasion of testing. Preliminary trials with a number of the strains indicated that 1/3,200 and 1/5,000 were the most useful concentrations for

differential purposes. The solution was incorporated into lactose broth at these concentrations. The formation of a black precipitate in the bottom of each tube representing growth in the presence of the inhibitor was looked for after 24 and 48 hr. at 37°.

TABLE 44.

Criteria used for the primary division of the *S. bovis*-*S. thermophilus* group

Criterion	Group		
	I	II	III
No. strains	162	140	36
Survival of 65° for 30 min.	+	-	+
Growth in 40% bile	-	+	+
Preferential utilization of disaccharides	+	-	±
Fermentation of maltose	-	+	±
Hydrolysis of starch	-	+	±

RESULTS

A total of 324 isolates from raw and pasteurized milk were examined. All of these were catalase-negative, Gram-positive cocci, occurring in medium to long chains in lactose broth incubated 18 hr. at 37°. On these bases, they were regarded as members of the genus Streptococcus.

In addition, they were able to grow at 45°, did not hydrolyse arginine, were inhibited by 0.1% methylene blue and produced acid and usually a clot in litmus milk with little or no reduction. These properties were considered sufficient justification for placing them in the S. bovis-S. thermophilus group of Abd-el-Malek & Gibson (1948).

These isolates were examined in greater detail and a primary division made into three groups according to the criteria given in Table 44. (The criteria used were those which in practice were found to give the clearest division.)

Group 1

This group included 162 strains. In addition to the characteristics given in Table 44, all strains produced acid in sucrose and galactose (slowly), but had no action on raffinose, inulin, salicin, sorbitol, glycerol and mannitol. One strain fermented arabinose, while eleven were able to ferment cellobiose. Occasionally, there was no growth on blood agar, but usually there was slight growth with faint greening. There was no action on aesculin or hippurate. Very long chains tended to form in lactose broth after 18 hr. at 37°. Most strains showed

TABLE 45.

Variations in biochemical activities of strains of Group I (*S. bovis* - *S. thermophilus*)

Criterion	Type			
	A	B	C	D
No. strains	4	146	11	1
Growth in 2% NaCl	+	-	-	+
Growth in 3% NaCl	-	-	-	+
Fermentation of cellobiose	-	-	+	-

marked salt sensitivity. Fifty representative strains were examined for growth at 50°. Of these, six were able to grow.

This group was, in general, remarkable for its uniformity. The slight variations in biochemical activity are recorded in Table 45. The strains examined were isolated from laboratory pasteurized farm milk (1 strain), creamery raw tanker milk (12 strains) and creamery plant (139 strains). Thus, they could almost all be associated either directly or indirectly with creamery plant.

The description of the group agrees well with the classical Streptococcus thermophilus of Orla-Jensen (1919), and with most subsequent descriptions of this organism. The findings with respect to growth at 50° - in common with those of most other authors - are in disagreement with those of Sherman & Stark (1931), all of whose strains were able to grow at this temperature. In fact, Sherman (1937a) used this property as a means of distinguishing S. thermophilus from all other streptococci, including S. bovis, a procedure which has been followed in the classification in Bergey's Manual (1948). The majority of strains examined by Orla-Jensen (1919) were also able to grow at this temperature. It is perhaps significant that both these authors used an isolation method involving a period of enrichment at relatively high temperatures.

Group II

This group included 140 strains. Unlike group I, there was considerable variation between strains, especially with

TABLE 46.

Variations in biochemical activities of strains of Group II (*S. bovis* - *S. thermophilus*)

	Type											
	A	B	C	D	E	F	H	I	J	K	L	M
No strains	36	20	15	11	6	6	7	5	3	7	11	3
Growth in 3% NaCl	+	+	+	+	+	+	+	+	+	+	-	+
Hydrolysis of aesculin	-	-	-	-	-	-	+	+	+	-	-	+
Fermentation of												
Raffinose	+	-	-	+	-	+	-	-	-	±	+	+
Inulin	+	-	+	+	-	-	-	+	+	+	+	+
Salicin	-	+	+	+	+	-	+	+	+	±	-	+
Arabinose	-	-	-	-	+	-	-	-	+	+	-	-
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-	-	-	±	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	±	-	-

respect to sugar fermentations.

All strains, in addition to the features mentioned in Table 44, produced acid in sucrose, cellobiose and galactose. No strain showed any action on glycerol or hippurate. In general, members of this group produced shorter chains in lactose broth than did those of group I. On blood agar, growth was fair with some greening and occasional slight clearing. Most strains showed a high degree of salt tolerance, but this was variable. The action on aesculin, raffinose, inulin, salicin and arabinose was variable. A few strains could ferment mannitol and a few sorbitol. No strain was able to grow at 50° or to produce mucoid growth on 5% sucrose agar. Thirty four representative strains were tested for survival of 60° for 30 min. and all showed less than 1% survival.

Some of the variations which were encountered are summarized in Table 46.

The 11 strains of type D were the closest of the collection to typical S. bovis, deviating from it only in their inactivity towards aesculin. All of these were isolated from milk from individual farms. Four survived laboratory pasteurization followed by incubation at 45°. The rest were isolated from raw samples at 45°. The 36 strains of type A were atypical in their inactivity towards both aesculin and salicin. Four isolates were from creamery tanker milk, 4 from creamery A balance tank and the rest from samples from individual farms. Of these, 4 were from laboratory pasteurized samples. All 20 strains of type B, which fermented salicin but not raffinose

were isolated from samples from individual farms - 4 laboratory pasteurized and 16 raw. The strains of type C, fermenting inulin and salicin but not raffinose, were isolated from creamery A balance tank (1 strain) and individual farms (14 strains). Of the latter, 7 were from laboratory pasteurized samples and 7 from raw samples. The 11 strains of type L, which were similar to type A but showed an unusual susceptibility to salt, were all isolated from individual farm samples. Of these, 10 were from laboratory pasteurized samples suggesting an association between relative heat resistance and salt susceptibility, i.e. a deviation in the direction of S. thermophilus. The details of the sources of the other organisms will not be given because the different types were represented by few strains. Moreover, all were isolated from samples from individual farms.

While the members of this group resemble Streptococcus bovis (Orla-Jensen) in their bile tolerance, active hydrolysis of starch, relative salt tolerance (except type L), inactivity towards arginine, hippurate and in general, towards alcohols, they show considerable deviation from the type species in their fermentative patterns.

Raffinose fermentation has come to be regarded as an almost invariable property of the species (Ayers & Mudge, 1923; Sherman & Stark, 1931; Abd-el-Malek & Gibson, 1948). Of the strains examined here, only 85 or 61% fermented this sugar. Again, S. bovis strains from cow faeces can generally ferment

salicin (Ayers & Mudge, 1923; Sherman, 1937b, etc.). However, of the strains examined here, only 80 or 57% were able to do so, and there was no evidence to support the suggestion of Sherman (1937b) that failure to ferment salicin is associated with failure to hydrolyse starch. Of 513 strains from milk studied by Abd-el-Malek & Gibson (1948), only 3 were unable to ferment salicin.

Orla-Jensen (1919) and Sherman & Stark (1931) found that typical S. bovis strains from cow faeces fermented arabinose. However, Sherman (1937b) later recorded this property as variable. Only 10 strains of those examined here fermented this sugar.

Fermentation of inulin was variable, a finding which is in agreement with other authors.

Sherman (1937b) describes S. bovis as capable of hydrolysing aesculin. Only 18 or 13% of strains examined here possessed this property. This is in agreement with the findings of Abd-el-Malek & Gibosn (1948) for milk strains.

In the fermentation reactions, there is some resemblance to S. salivarius (Andrews & Horder) as described by Safford, Sherman & Hodge (1936), but this organism does not hydrolyse starch and generally produces mucoid colonies on 5% sucrose agar. While some strains can withstand high concentrations of bile (Niven, Smiley & Sherman, 1942a), this is not usual.

In their inability to ferment arabinose, the strains resemble Streptococcus inulinaceus (Orla-Jensen), but this organism does not hydrolyse starch.

The finding that representative strains were unable to withstand 60° for 30 min. is contrary to the results of most workers for S. bovis but is in agreement with Shattock & Mattick (1943). It seemed rather surprising that many of these strains had been isolated originally from pasteurized milk. However, Galesloot (1952) reported a similar phenomenon with strains of S. bovis-S. thermophilus isolated by him from pasteurized milk, namely, that a number were unable to survive 63° for 35 min.. Very probably, as suggested by him, the explanation lies in the relationship between heat resistance and phase of growth. Anderson & Meanwell (1936) showed that the maximum heat resistance of a thermoduric streptococcus, which was probably S. bovis, was obtained in the lag and early logarithmic phases of growth. White (1954) reported a similar relationship in the case of S. faecalis. Galesloot (1952) attributed an apparent increase in count of strains of S. bovis-S. thermophilus which had been stored at relatively high temperatures before pasteurization to an increased survival of the heat treatment associated with the stage of growth reached during the storage period. With pure cultures in the stationary phase of growth, Galesloot (1951a) found an average survival of pasteurization by strains of S. thermophilus, S. bovis and S. faecalis of approximately 86%, 8% and 0.1% respectively.

The inoculum used by Abd-el-Malek & Gibson (1948) in their heat resistance tests was described as "0.2 ml. of a fresh, moderately turbid lactose broth culture". No details of the incubation conditions were given, but it was assumed at the

TABLE 47.

Variations in biochemical activities of strains of Group III (S. bovis - S. thermophilus)

Criterion	Type A				Type B			
	E209	E213	E221	B239	E204	B13	B2	D4
Survival of 65° for 30 min.	+	+	+	+	+	+	+	+
Growth at 50°	+	+	+	-	+	+	-	-
Growth in 40% bile	+	+	+	+	+	+	+	+
Growth in 3% salt	-	-	-	?	+	+	?	+
2% salt	+	+	+	?	+	+	?	+
Fermentation of								
Maltose	-	-	-	-	+	+	+	+
Raffinose	-	-	-	-	+	+	+	-
Inulin	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	+	-	+	+
Cellobiose	-	-	-	?	+	+	-	?
Preferential utilization of disaccharides	+	+	+	+	-	-	-	-
Hydrolysis of starch	-	-	-	?	+	+	-	?

beginning of the present work that a culture grown at 37° for 18 hr. would meet the above requirements. However, the lack of agreement with their results leads one to suspect that the cultures used by them may have been considerably younger than those used in the present studies. The latter were almost certainly in the stationary phase of growth at the time of testing.

The need for strictly standardized conditions in the carrying out of heat resistance tests is thus emphasized.

From these results, it seems that at least in terms of its heat resistance, S. thermophilus is clearly distinguishable from S. bovis. Probably, in practice, a relatively small proportion of strains of S. bovis survive pasteurization - the proportion being determined to some extent by pre-pasteurization environment.

The bulk of evidence favours the point of view that the strains in this group should be regarded as varieties of S. bovis. The atypical fermentation patterns may possibly reflect the previous history of the strains.

Group III

An intermediate group comprising some 36 strains were found to combine the bile tolerance of S. bovis with the heat resistance of S. thermophilus. Results for 8 representative strains are given in Table 47. All strains except B13 gave the very faint growth with slight greening on blood agar typical of group I strains. In addition, all eight strains survived 65° for 30 min., and five grew at 50°, features characteristic of S. thermophilus. Strains E209, E213, E221 and B239 can be

TABLE 48.

The growth of representative strains of Groups I, II & III (*S. bovis* - *S. thermophilus*) in the presence of inhibitors

Criterion	Group I						Group II								Group III			
No strains	1	7	1	7	1	1	3	7	7	6	2	1	7	1	4	1	2	1
Growth in the presence of																		
Thallium acetate (0.2%)	+	+	+	+	+	-	-	-	-	+	+	-	+	+	+	+	+	+
Crystal violet (1/200,000)	-	-	-	+	+	-	-	-	-	-	-	+	-	+	+	+	+	+
Potassium tellurite (1/5,000)	-	+	+	+	+	+	+	+	-	+	+	-	-	-	+	-	+	+
(1/3,200)	-	+	+	+	+	+	+	-	-	-	+	-	-	-	+	-	+	+
Growth at 50°	+	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-

placed with little hesitation as atypical, bile tolerant strains of S. thermophilus. Strains E204, B13 and probably D4 are perhaps best regarded as atypical strains of S. bovis, showing a remarkable degree of heat resistance. Strain B2, although not hydrolysing starch, is probably also best placed in this group.

It is of interest that strains E204, E209, E213, E221 and B239 were all isolated from creamery PL raw tanker milk (see p.99). The group III B strains, B13 and D4, were both isolated from laboratory pasteurized samples from individual farms, while B2 was isolated from bulk raw milk in creamery A balance tank.

The strains of this group seem to represent an intermediate group between the two species, comprising group IIIA - atypical S. thermophilus strains - and group IIIB - atypical S. bovis strains.

The effect of inhibitory substances on members of the three groups

Each strain having been placed in one of the three major groups, the effect of certain inhibitors on representative members of each group was tried.

It can be seen from Table 48 that, in general, organisms of groups I and III showed a greater resistance to inhibitory substances than did members of group II. It may be noted that all 8 group III strains and 17 out of 18 group I strains, compared with 16 out of 34 group II strains were able to grow in the

presence of of 0.2% thallium acetate. Again, all group III and 8 group I strains grew in the presence of 1/200,000 crystal violet while only 2 group II strains were able to do so. Seven group III, 17 group I and 5 group II strains grew in the presence of 1/3,200 potassium tellurite. It is interesting to note also that 6 of the 8 group III strains were able to grow at 50° compared with 3 out of 18 group I strains. Thus it seems that the strains of group III combine a high degree of heat resistance with an ability to resist inhibitory substances including 40% bile.

DISCUSSION

A large proportion of the strains examined in this part of the work could be regarded with reasonable certainty as typical Streptococcus thermophilus (Orla-Jensen). These strains exhibited a remarkable degree of uniformity in their reactions to the tests used here, especially in their heat resistance, preferential utilization of disaccharides and inability to ferment maltose. Although maltose fermenting varieties have been reported by some authors (Orla-Jensen, 1919; Wright, 1936a; Burri, 1941; Burri & Elser, 1941), none was encountered in this work. Several strains did, however, appear, which were atypical in that they were able to grow in the presence of 40% bile. This bile tolerance was associated with a general increase in resistance to inhibitory agents including high temperatures. For this reason, there is some doubt as to whether the bile tolerance of these strains can be regarded as a deviation from the type of species in the direction of S. bovis or indeed as evidence in support of an intestinal origin. More probably it should be interpreted as an indication of the doubtful validity of the use of 40% bile tolerance as a means of differentiating the two species.

The position with respect to the strains designated as S. bovis was less satisfactory. Not only was there not a single strain which showed all the characteristics of typical S. bovis as reported by other authors, but some surprising variations in sugar fermentation reactions were obtained which had not been reported previously. Galesloot (1951a) also

reported that strains of S. bovis-S. thermophilus from milk showed considerable differences in biochemical reactions from those described in the literature. Unfortunately, no detail of the precise nature of the differences was given. The strains from milk examined by Nichols (1939) and described by her as varieties of S. bovis also showed many deviations from the type species.

In considering this situation and its possible causes, one is struck by the fact that with the exception of the work mentioned and that of Abd-el-Malek & Gibson (1948) most of the earlier findings were made using strains isolated directly from cow faeces. Differences in fermentation patterns are often associated with differences in habitat. Thus, atypical fermentation patterns shown by strains of S. bovis isolated from milk may not be of very great significance and probably do not constitute sufficient grounds for excluding these strains from the species. In support of this, Sherman & Stark (1931) noted differences in the ability of strains to ferment inulin according to whether they were isolated from milk or from faeces. Those from milk were unable to ferment inulin while those from cow faeces could do so.

Further, it is questionable whether a loss in certain fermentative powers by strains of S. bovis can be regarded as sufficient grounds for claiming a relationship with S. thermophilus which happens to be characteristically inactive towards the majority of the conventional test substances. In the case of group II strains whose bile tolerance, active starch

hydrolysis and inability to survive 60° for 30 min. are quite unlike S. thermophilus, it is felt that no such claim could justifiably be made. However, in the case of the strains examined under group IIIB, there does seem to be sufficient evidence for claiming the existence of an intermediate group. These strains, while resembling S. bovis in many respects, show a degree of heat resistance possessed by S. thermophilus alone among the streptococci.

To summarize, it may be said that typical S. bovis and S. thermophilus may be distinguished without difficulty according to the criteria given in Table 44. There is some evidence for the existence of an intermediate group of strains.

Conclusions from Part II of this thesis

CONCLUSIONS

The aim of this part of the thesis was to trace the source of *S. thermophilus*. The approach used failed to give the required information, but it at least seems clear that either the organism does not have a source outside milk, or that it normally gains entry into raw milks in numbers so small that to determine its source would be very difficult.

If *S. thermophilus* does not have a habitat outside milk but arises through adaptation from some other species such as *S. bovis*, one would expect to find a large number of intermediate strains such as those reported by Abd-el-Malek & Gibson (1948). Yet, after handling a fairly large number of isolates, this author was left with the impression that the gulf separating *S. thermophilus* from *S. bovis* is very wide. Indeed, even allowing for its large proportion of negative reactions, *S. thermophilus* appeared to be a well-defined and remarkably uniform species. Although many atypical fermentation patterns were exhibited by the *S. bovis* strains, few appeared to represent deviations in the direction of *S. thermophilus*. On the other hand, a small number of strains of *S. bovis* possessing the heat resistance of *S. thermophilus* were isolated, and, although these were few, they provide a certain incentive for further investigation along these lines.

SUMMARY

The thesis is divided into two parts.

PART I. The aim of this part of the thesis was to study the occurrence and behaviour of Bacillus cereus in milk in so far as these properties are concerned in the appearance of bitty cream. As a preliminary step, a method of quantitative estimation of the organism in raw milk was developed. This consisted in the use of a selective medium (yolk inhibitor agar) by means of which as few as 1 or 2 cells/ml. could be detected. The properties of B. cereus utilized in this medium were its ability to produce a lecithinase, as shown by its action on egg yolk, and its resistance, relative to that of other milk organisms, to certain inhibitors (namely polymyxin and lithium chloride). Yolk inhibitor agar was used to detect B. cereus in 1 ml. sub-samples in a survey of 426 samples of milk from farms, bulk road tankers and creamery plant. At the same time, raw and laboratory pasteurized sub-samples were examined for the development of bitty cream after storage at 22° for 48 hr.. Thirteen percent of these samples were recorded as positive on yolk inhibitor agar, the average count being 4/ml.. Fifteen percent of laboratory pasteurized sub-samples developed bitty cream, compared with considerably less than 1% in the case of the raw sub-samples. However, there was no relationship between the presence of B. cereus in 1 ml. and the development of bitty cream. This could not be related to the differences shown by the samples in total count, laboratory pasteurized count, numbers of aerobic spore-formers,

other than B. cereus, or numbers of thermoduric streptococci. Attempts to isolate, from raw milk, organisms inhibitory to B. cereus showed that these were few but that certain Pseudomonas species and one strain of S. lactis, which was probably a nisin producer, were able to do so. The presence of B. cereus in 1 ml. sub-samples was found to be associated with particular farms and creameries. This was traced to contamination of milk from inadequately sterilized cans, which was in turn traced to the practice of allowing cans to stand for long periods, with watery residues lying in them before cleaning. The survival of heating suggested that the organism was in the spore stage at the time of 'sterilization'. The importance of this stage had previously been emphasized by the common appearance of the fault in pasteurized milks. It was found that B. cereus sporulates prolifically in diluted milk under conditions of good aeration (i.e. the conditions obtained in watery residues in cans). Thirteen strains of B. cereus from milk formed 100% spores within 48 hr. at 30° in milk diluted 1/50. The addition of K^{+} and Mn^{++} to undiluted milk had no effect on sporulation. The germination of unheated spores of B. cereus in raw milk was found to occur relatively slowly, while laboratory pasteurization caused stimulation in rate. Laboratory pasteurization of previously heated spores caused a further marked stimulation. The activating effect of the first heating persisted at least for several weeks. Germination rate following pasteurization declined with

temperature, between 30° and 15°, and with dilution, up to 1/50. It was concluded that delayed germination of spores in raw milk and the consequent advantage possessed by competing organisms, may be at least part of the reason for the relative absence of bitty cream from raw milk.

PART II. The aim of this part of the thesis was to determine the source of Streptococcus thermophilus. A preliminary survey of samples of milk from farms, bulk tankers and creamery plant was carried out, using a method of rough screening for S. thermophilus. This method consisted in subjecting a sub-sample to laboratory pasteurization, pour plating with a fairly rich medium and incubating at 45°. Apart from spore-formers, only S. thermophilus and closely related organisms grew under these conditions. A corresponding raw series was set up at the same time and representative isolates were characterized and identified from each series. Samples from farms were virtually free from S. thermophilus, while the organism appeared to be characteristically associated in small numbers with creamery plant. Samples were taken regularly from one creamery and there was little monthly variation between samples until mid-summer when very high counts of S. thermophilus were suddenly obtained. All sources of milk entering the creamery at the time were examined and the absence of S. thermophilus in milk direct from farms was confirmed. A certain relatively small contamination was entering the plant from the bulk tanker milks from country creameries, but this could not account for counts of the order obtained in the

bottled milk. It was found that multiplication of S. thermophilus occurred in the H.T.S.T. pasteurizer, probably in the regeneration section. This was only obtained after the plant had run for greater than 5 or 6 hours without cleaning. The appearance of the outbreak in the summer months could thus be traced to the increased length of plant run consequent upon the practice at this time of the year, of pre-heating surplus milk before storage to avoid deterioration overnight. The common occurrence of S. thermophilus in pasteurized milk and its virtual absence from raw milk was thus still unexplained. It was suggested that S. thermophilus ~~varieties~~s might arise as a heat-induced variant of S. bovis. Some very preliminary experiments were unsuccessful, but further investigations are warranted. Over 300 isolates obtained in the above survey were characterized. On the basis of heat resistance, action on starch and maltose, rate of utilization of disaccharides relative to monosaccharides and bile tolerance, they were placed in 3 groups. The strains of group I conformed well with previous descriptions of S. thermophilus. Those of group II were regarded as varieties of S. bovis but showed considerable variation from the type species in their fermentation reactions and action on aesculin. Group III combined relative heat resistance with tolerance to bile and other inhibitions. Some were probably intermediate between S. bovis and S. thermophilus.

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APPENDIX

P. 7. Yolk saline (1:1)

A whole egg was soaked for 10 min. in industrial methylated spirits. It was then carefully withdrawn and the shell was broken. The yolk was separated from the white, by rapid transfers in the shell halves, and placed in a sterile petri dish. The fluid portion of the yolk was pipetted into a sterile 1 oz. vial and shaken with an equal volume of sterile saline. The resulting suspension was held in a refrigerator and its sterility checked from time to time by plating. Contamination was rare in storage periods of up to several months.

P. 7, P. 64. Nutrient agar (weak nutrient agar)

Peptone, 5g.; beef extract (lab lemco), 5 g.; agar, 15g.; distilled water, 1,000 ml.; pH 7.0.

P. 29. Yeastrel milk agar

Yeastrel, 3g.; peptone, 5g.; skim milk, 10 ml.; agar, 15g.; distilled water, 1,000 ml.; pH 6.8.

P. 30. Lactose-tryptone beef extract agar.

Lactose, 5g.; tryptone, 10g.; beef extract (lab. lemco) 10g.; yeastrel, 5g.; agar, 15g.; distilled water, 1,000 ml.; ph 7.0.

P. 50. Glucose peptone water

Glucose, 5g.; peptone, 5g.; distilled water, 1,000 ml.; brom cresol purple; pH 7.0. The medium was tubed in 5 ml. quantities in $\frac{5}{8}$" tubes with Durham tubes.

P. 111. Lactose broth

Lactose, 5g.; peptone, 5g.; beef extract (lab. lemco), 5g.; distilled water, 1,000 ml.; brom cresol purple; pH 7.0.

P. 69. Statistical data relating to Fig.13, 14, 15 and 16

All calculations were made according to the methods given in Snedecor (1946), Chapters 10, 13 and 14.

Fig. 13 Strain C.12. Once-heated spores.

x = min.; y = $\log_{10}$ residual spores.

Regression line: $\bar{y} = 2.30 - 0.00026x$.

Correlation coefficient: $r_{xy} = -0.7679$.

Significance of linear regression

Source of variation	d.f.	S.S.	M.S.
Regression (b Sxy)	1	0.0147	0.015
Error of estimate	17	0.01	0.006
Total	18	0.025	

$F = 25.00^{***}$ $(P < 0.01)$

Fig.14 Strain C.15. Once-heated spores.

x = min.; y = $\log_{10}$ residual spores.

Regression line: $y = 2.39 - 0.0004x$

Correlation coefficient: $r_{xy} = -0.9305$

Significance of linear regression

Source of variation	d.f.	S.S.	M.S.
Regression (b Sxy)	1	0.037	0.037
Error of estimate	17	0.006	0.00035
Total	18	0.043	

$F = 104.8^{***}$ $(P < 0.01)$

Fig.15 Strain C.12. Twice-heated spores.

1. Linear regression. x = min.;
$y = \log_{10}$ residual spores.

Regression line: $y = 3.13 - 0.001x$

Correlation coefficient: $r_{xy} = -0.8716$

Significance of linear regression

Source of variation	d.f.	S.S.	M.S.
Regression (b Sxy)	1	0.20	0.20
Error of estimate	17	0.06	0.0035
Total	18	0.26	

$F = 56.66^{***}$ $(P < 0.01)$

2. Curvilinear regression. x_1 = min.; $x_2 = (\text{min.})^2$;
$y = \log_{10}$ residual spores

Regression line: $\hat{y} = 3.22 - 0.0027x_1 + 0.000005x_2$.

Multiple correlation coefficient: $R = 0.9783$.

Significance of curvilinear regression

Source of variation	d.f.	S.S.	M.S.
Regression (R^2Syy)	2	0.2486	0.1243
Error of estimate	16	0.0118	0.0007
Total	18	0.2604	

$F = 177^{***}$ $(P < 0.01)$

Significance of departure from linear regression

Error	d.f.	S.S.	M.S.
Linear regression	17	0.1362	
Curved regression	16	0.00081	0.00005
Difference	1	0.1292	0.1292

$F = 5,584^{***}$ $(P < 0.01)$

Fig.16 Strain C.15. Twice-heated spores.

1. Linear regression. x = min.;
   $y = \log_{10}$ residual spores.

   Regression line: $y = 3.25 - 0.0008x$

   Correlation coefficient: $rxy = -0.7300$

Significance of linear regression

Source of variation	d.f.	S.S.	M.S.
Regression (b Sxy)	1	0.14	0.14
Error of estimate	17	0.13	0.0076
Total	18	0.27	

$F = 0.14/0.0076 = 18.30^{***}$ $(P < 0.01)$

2. Curvilinear regression. x_1 = min.; $x_2 = (\text{min.})^2$;
   $y = \log_{10}$ residual spores

   Regression line: $\hat{y} = 3.35 - 0.0028x_1 = 0.000006x_2$

   Multiple correlation coefficient: $R = 0.9986$

Significance of curvilinear regression

Source of variation	d.f.	S.S.	M.S.
Regression (R^2Syy)	2	0.2692	0.1346
Error of estimate	16	0.0008	0.00005
Total	18	0.27	

$F = 0.1346/0.00005 = 2692^{***}$ $(P < 0.01)$

Significance of departure from linear regression

Error	d.f.	S.S.	M.S.
Remainder after linear regression	17	0.1362	
Remainder after curved regression	16	0.00081	0.00005
Remainder	1	0.1292	0.1292

$F = 5,584^{***}$ $(P < 0.01)$

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THE UNIVERSITY OF EDINBURGH
DEPARTMENT OF AGRICULTURE
10 GEORGE SQUARE
EDINBURGH 8.