THE GROWTH OF MICRO-ORGANISMS IN PASTEURIZED AND STERILIZED MILK

A Thesis submitted to the University of Glasgow for the Degree of Doctor of Philosophy in the Faculty of Science

by

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Synopsis

This work describes investigations into the growth of mesophilic Bacillus spores from very small inocula in pasteurized and sterilized milk. A comparison of growth in sterilized milk with that in pasteurized milk, showed that the process of sterilization could set up inhibitory conditions. Over the range of heat treatment, 107.5°-117.5°C for 15 min., inhibition was related to the method of processing, in particular to the presence of a partial vacuum in the headspace of the bottles during heating and not to the degree of heating used for sterilization. The inhibition was manifested by a prolongation of the lag phase. The logarithmic phase was not affected, but under inhibitory conditions there was a depression of the maximum population. The degree of inhibition became more pronounced as the level of inoculum was decreased; but at any one level of inoculum, inhibition was least when incubation was carried out at the optimum growth temperature for the organism. There was some variation between milks in their ability to inhibit the growth of small inocula.

The changes in acidity, reducing powers and browning of the milk caused by heat were examined over the range of heat treatments from 104.5°-117.5°C for 15 min. Although most of the changes varied with the degree of heating, it was found that the oxidation-

reduction potential was not affected by the temperature of heating within the above range but was affected by the treatment of the milk before and after heating. The capacity of milks to resist atmospheric oxidation varied, and the results suggested that a good poising capacity against oxidation was associated with a high lactoglobulin content.

It was shown that there was an association between the oxidation-reduction potential and the inhibition of bacterial growth. This association was distinct from any effect due to the presence or absence of air during the incubation of the inoculated milk. Milks with good powers to resist oxidation were more inhibitory towards bacterial growth than milks with a poor poising capacity.

The absence of bacteriological standards for sterilized milk gives a practical importance to studies of the growth rates of micro-organisms in heated milk. The results obtained experimentally gave a pattern of inhibition which appears to offer an adequate explanation of the various observations on commercially sterilized milk which have been published. Samples of milk sterilized by three commercial processes were shown to have oxidation-reduction potentials compatible with the theory that inhibition was associated with Eh.

The production of instability of the milk towards 80% alcohol was found not to be suitable as a means for detecting the growth of the seven strains of Bacillus used in this work when the incubation period was less than 2-3 days at 37°C.

SECTION I

INTRODUCTION

1. Methods for the heat treatment of milk

Since pasteurization only reduces the microflora of milk, other methods have been sought whereby a sterile product might be obtained. In 1894 a process known as sterilization was introduced in which milk was heated at or above 100°C. The product was not very satisfactory until in 1904 the introduction of homogenization gave a uniform product in which the cream did not separate during the heat treatment.

Thomsen (1) and Gammack (2) have reviewed the types of plant which are used for the sterilization process. Of these only the Webster continuous process and a static horizontal batch heater are in common use in Great Britain. A third method employing a temperature of 138°C for 2 sec. is being developed (Cuttell (3)) but this has not yet been used commercially to produce "Sterilized" milk without a further in-bottle heat treatment.

The production of milk designated as "Sterilized" is now subject to Statutory Instruments (1949) No. 1589 and (1951) No. 646 (44, 45). Under the Order for Scotland (1951 No. 646) the milk must first be filtered or clarified, and after homogenization it must be heated to a temperature of not less than 220°F and not more than 235°F (104.6-

112.8°C) and be maintained at that temperature for a period not exceeding 30 min. The use of the word "sterilized" as a designation is unfortunate since milk heated within the required limits is not necessarily bacteriologically sterile. However, the term sterilized in this connexion is recognised in the industry and has been used in this thesis to denote homogenized milk which has been heated at temperatures above 100°C either in commercial plants or in the laboratory.

In the commercial sterilization process, homogenized milk at a temperature of 65-70°C is filled under partial vacuum into heated bottles. The temperature of the milk and the use of vacuum fillers result in a tendency for the gases dissolved in the milk to be driven off. Steam from the milk displaces air in the headspace so that when the Crown caps are applied, a vacuum of 12-13 in. Hg is formed. The degree of vacuum set up will depend on the temperature of the milk and the bottles at the time of capping (4). The milk is sterilized in the bottles after capping. It is a Statutory regulation that the seal must be airtight, thus the vacuum in the bottle should be maintained until the bottle is opened.

The only control to which the finished product is subject is that it shall pass the Aschaffenburg turbidity test (5). This test is used to detect the presence or absence of undenatured serum proteins in milk. Sterilized milk is considered to have received

a satisfactory heat treatment when the absence of a turbidity in the test indicates that all the serum proteins have been denatured. There is no bacteriological standard for Sterilized milk in Great Britain

2. Heat-induced changes in milk

Extensive research has been done on the heat-induced chemical changes in milk, and before discussing the growth of organisms in heated milk it is pertinent to mention some of the changes which occur when milk is heated.

The denaturation of proteins begins at temperatures below 100°C (6). Although casein is relatively heat-stable, the precipitation of serum proteins is essentially complete in 10 min. on heating at 95°C. Denaturation is accompanied by the release of ammonia and volatile sulphides. The liberation of sulphides rises to a maximum and then falls off as heating proceeds (7, 8, 9, 10). Lactose reacts with basic $-NH_2$ groups to form a stable complex which is dissociable in the early stages of heating (11, 12). The formation of this complex is associated with the browning of the milk, a subject that has been reviewed by Patton (13). It has been suggested also that lactose is broken down by way of a ketose to give an acid (14, 15). A wide variety of dialysable compounds have been isolated from heated milk. Many of the lower fatty acids (especially formic acid),

furfuryl alcohol, maltol, acetol, methyl glyoxal and acetaldehyde have all be identified (16, 17, 18, 19, 20, 21, 22, 23, 24). The formation of acids is associated with an increase in titratable acidity and a fall in pH (25). Heat treatment increases also the reducing powers of milk especially if heating is preceded by deaeration or is carried out in the absence of air (26, 27, 28, 29). The availability of the reducing groups depends on the Eh of the oxidant and the conditions used (10). The oxidation-reduction potential of heated milk is affected by deaeration or by heating in the absence of oxygen (27, 28). There is a decrease in soluble calcium and inorganic phosphate during heat treatment (30, 31) which increases the stability of the milk towards alcohol. Differences between various reports on the effects of heat on the vitamin content, biological value and nutritive index of milk are probably due to the use of different degrees of heating rather than to variation between experimental techniques (32, 33, 34, 35, 36, 37).

3. The microflora of sterilized milk

Both mesophilic and thermophilic species of the genus Bacillus have been reported as causing a poor keeping quality in sterilized milk (38, 39). Recently three surveys of the organisms which survive in the commercial product have been published (4, 40, 41). The Bacillus subtilis group formed 70-90% of

all isolates. Of the other species, B. circulans was most frequently isolated. These three surveys give information on the frequency with which different species normally survive the sterilization process and do not refer to sterilized milk which has a poor keeping quality. Any reference to the growth of these species in heated milk is very limited. Grinsted & Clegg (42) concluded that because these bacilli are capable of growth on milk-starch agar slopes at 15°C any organisms surviving the sterilization process will cause spoilage. Mossel & Drion (43) deduced that because of the ability of many of these species to grow at reduced oxygen tensions, the Eh of sterilized milk is insufficient to limit bacterial growth. The effect of sealing bottles at various temperatures to give 10-70% absolute vacuum on the subsequent growth of spores in the sterilized milk was investigated by Ridgway (4). He found that "there was a slight indication that at a higher vacuum germination and reproduction were retarded, since faults took a little longer to become apparent". Unfortunately no mention is made of the size of inoculum or the species of bacteria used.

4. Present investigation

Since the designation of sterilized milk in 1949 in England and Wales and 1951 in Scotland (44, 45) no provisions have been made for bacteriological standards for sterilized milk. Such a standard is

desirable and it is therefore of practical importance that as much as possible should be known about the growth of micro-organisms in sterilized milk.

There is no published information on the levels of bacterial contamination in sterilized milk that are associated with a poor keeping quality. Burton, Akam, Thiel, Grinsted & Clegg (46) have shown that the number of bacteria normally surviving commercial sterilization is about 1 spore/100 ml. milk. The spoilage of bottled sterilized milk caused by these survivors usually tends to occur erratically within the same batch of milk. This suggests that either the distribution of surviving spores varies from bottle to bottle or that there are some variable factors which affect the germination or growth of the spores. Hence it is desirable to know more of the conditions affecting the growth of small numbers of spores in sterilized milk. To this end, investigations have been made into the growth rates of Bacillus species from small inocula in milk sterilized under varying conditions.

In order to determine the effect of the heat treatment used in "sterilization" on milk as a growth medium for Bacillus species, comparisons were made initially with pasteurized milk prepared from raw milk obtained with aseptic precautions. Even under such conditions, the milk was rarely sterile. Its use was therefore discontinued but not before it had been shown that the growth rate of Bacillus species in milk

sterilized under certain conditions was similar to that in pasteurized milk. Preliminary chemical examination of sterilized milk showed that the changes caused by the sterilization process could be varied by the manner in which the bottles were closed. Different levels of vacuum in the headspace of the bottles were associated with variations in the properties of the milk. It was decided to concentrate on examining bacterial growth rates in milk sterilized under different conditions. The effects of vacuum in the headspace of the bottle during sterilization and of permitting air to enter the bottle after heat treatment were studied in relation to the chemical changes produced and to the effect of these changes on the growth of Bacillus species. The changes in acidity, reducing powers and colour of the milk associated with the different treatments are reported in Section III. The influence of heat-induced changes in the milk and of other factors on the growth of Bacillus species is considered in Section IV.

SECTION II

METHODS

1. Treatment of milk

(a) Source. Raw milk of low bacterial count was obtained under controlled conditions as single cow samples. The cows were all free from mastitis, gave not less than 15 lb. milk at one milking and received no marked change in feed during the experimental period.

The milker washed his hands and arms well, drying them on a sterile towel before washing down the hind quarters of the cow with a fresh solution of a quaternary ammonium compound using autoclaved udder cloths. The milk was collected in an autoclaved milking machine. The teat cups were covered with paper before sterilization and the paper was not removed until a cup was ready to be placed over a teat. Care was taken not to apply the vacuum to any teat cup before it was placed over the teat. After weighing the milk in the sterile can, about 1½-2 litres were transferred aseptically to a sterile conical flask for pasteurization. If necessary the remainder of the milk was held at 4-5°C until the following day (not longer than 18 hr.) before it was homogenized and sterilized.

Later it was found to be equally satisfactory to use bulk farm supplies and thus avoid the variation in chemical composition of single cow samples.

(b) Pasteurization. The temperature of the milk in the conical flask was recorded by a sterilized thermometer held in place by the bung. A lead collar was placed round the flask so that it could be submerged as far as possible in a water bath at 63°C. At intervals the milk was swirled gently to mix it. The temperature of the milk rose to 63°C in about 30 min., and after being maintained at that temperature for a further 30 min. it was rapidly cooled to below 20°C in iced water. The milk was kept at less than 20°C until the following day when the heating and cooling was repeated. The pasteurized milk was filled into sterile bottles (440 ml. per bottle) which were held at 4-5°C until required.

(c) Homogenization. Tap water was run through the homogenizer to check that the machine was working correctly. This was followed by hypochlorite solution (200 p.p.m. available chlorine) and then the machine was flushed with tap water, each process lasting for not less than 5 min. Milk which had been preheated to 80°C was homogenized at 2,500 lb./sq. in., the milk from the first minute of the run being discarded to ensure that all the water had been flushed out. After use, the homogenizer was flushed with cold water, stripped down, all parts washed in hot detergent, rinsed and re-assembled. Since the homogenizer was not used solely for this work, it was sometimes necessary to autoclave the block of the

homogenizer (10 lb./sq. in. for 30 min.) before use. Because of its size, the holding tank was sterilized in hot air at 120°C for 4 hr.

Oil-impregnated piston packings in the homogenizer were not found to be suitable because oil got into the milk and the milk leaked into the packings. They were therefore replaced by dry fibre packings, and a water cooling system was added to prevent seizing of the pistons.

(d) Sterilization. Suitable bottles were filled with quantities of homogenized milk to give approximately the same headspace in relation to volume as was used in commercial practice (9% of the total). The bottles which were 8 or 16 oz. medical flats or 40 oz. bottles were provided with a perforated metal screw cap and rubber diaphragm. Glass hoods were used to prevent contamination of the diaphragm after sterilization.

The bottles were sterilized either open or closed. The caps of bottles to be sterilized open were placed at an angle across the neck of the bottle. If necessary the glass hood was prevented from lying flat on the shoulders of the bottle by two curtain hooks placed at an angle to each other (Plate 1). The caps of bottles which were to be sterilized closed were screwed down and the bottles evacuated for 30 min. by means of a hypodermic needle inserted through the rubber diaphragm and attached to a water pump. The

i ii

The bottles used in the laboratory sterilization of milk.
(i) "evacuated" bottle. (ii) "open" bottle.

Plate 1.

hypodermic needles used throughout this work were 0.55 mm. diam. $\frac{7}{8}$ in. length Record fitting. Frothing was reduced to a minimum by cooling the milk and deaeration was carried out at a vacuum equivalent to 25 in. Hg (635 mm. Hg). For simplicity, bottles will be referred to as being either "open" or "evacuated".

"Open" and "evacuated" bottles were sterilized together in one batch in a small autoclave usually at 115.5°C (10 lb./sq. in. steam pressure) for 15 min. The temperature of the milk during autoclaving was controlled using a thermistor placed in a bottle of the same size containing an equal volume of water. This control bottle was placed in the centre of the autoclave and the other bottles placed round it, not touching one another. The reading obtained by balancing the current produced by the thermistor could be used to obtain the value for the temperature from a calibration curve. Later a second thermistor was included for use in an evacuated bottle. The apparatus for recording temperature was made by J.A. McLean, B.Sc. of the Biophysics laboratory of this Institute.

The total time that the bottles of milk remained in the autoclave ranged from 50 to 90 min. depending on the size of the bottle and the temperature used. The bottles were placed in a hot autoclave and held in an atmosphere of free steam until the temperature in the control bottle was 100°C. The valve of the autoclave was then closed and the

pressure allowed to rise to the required value. When the control bottle showed the correct temperature, the bottles were held for a further 15 min. and then the heating was turned off. After 15-25 min. the autoclave was opened and the bottles placed on a bench to cool to room temperature. The open bottles were closed when cool.

(e) Adjustment of the composition of the headspace. Before the experiment began, the vacuum in the "evacuated" bottles was filled with sterile air or nitrogen. This was necessary to allow the bottles to be inoculated and sampled. The gas was passed through a sterile cotton wool filter and introduced into the headspace of the bottle through a hypodermic needle inserted through the rubber diaphragm of the cap. When it was required to incubate "open" bottles under partially anaerobic conditions, the headspace was evacuated rapidly for about 10 min. using a hypodermic needle attached to a water pump. The vacuum so formed was filled with nitrogen. Commercial nitrogen stated to contain not more than 0.5% oxygen was used.

The treatments given to the milk may be summarized as follows:-

Treatment	Type of milk	Gas in headspace
A	'Evacuated', i.e. deaerated at 25 in. Hg for 30 min. and sterilized with a vacuum in the headspace of the bottle	N_2
B		Air
C	'Open', i.e. sterilized in open bottles	N_2
D		Air
E	Pasteurized	Air

For ease of reference this summary of the treatments has been included as a draw-out sheet at the end of the thesis (p.103).

2. Chemical examination

(a) Reducing substances. The reducing substances in milk were measured by the acid ferricyanide method of Chapman & McFarlane (47) as modified by Crowe, Jenness & Coulter (48). That is, the milk was buffered to pH 6.6 and the reaction carried out at 50°C. The milk sample was diluted 1:9 with distilled water before testing. Results are reported as extinctions (i.e. as drum readings on a Spekker absorptiometer).

Approximate Eh measurements were made by adding to milk suitable oxidation-reduction dyes such as potassium indigo tri-sulphonate to give a final concentration of 0.002%. After heating the milk, the colour was compared with standards. A bottle of milk containing no dye was heated under the same conditions. This milk was used to prepare standards containing various proportions of the dye. The relation of the

percentage colour reduction to the value of Eh'_o was obtained from Hewitt's Oxidation-Reduction Potentials in Bacteriology and Biochemistry (49) and from Knight (50).

The Eh of milk samples was measured electrometrically using a direct reading Pye meter, and the method based on that of Tobler (51). The apparatus used is shown in Fig. 1. The milk was transferred from the bottle in which it was heated to the measuring cell under an atmosphere of air or commercial nitrogen. Where required, anaerobic conditions were maintained by allowing a flow of nitrogen to pass through the headspace of the cell. The milk was not agitated during measurements. The cells were held in a water bath at 50°C and readings taken at intervals until a stationary value was obtained.

Normally, milk sampled from "evacuated" bottles was examined under N_2 whereas the milk from "open" bottles was examined in air. Measurements were also made on both types of milk in which the stationary value was first obtained under N_2 and then in air and vice versa.

Saturated calomel electrodes and platinum electrodes were used. The platinum electrode consisted of a bright Pt disc (5 mm. diameter) fused on to the end of a glass tube. The results given by the platinum electrodes agreed within ± 1 mV in quinhydrone standards and within ± 5 mV in milk. After use both

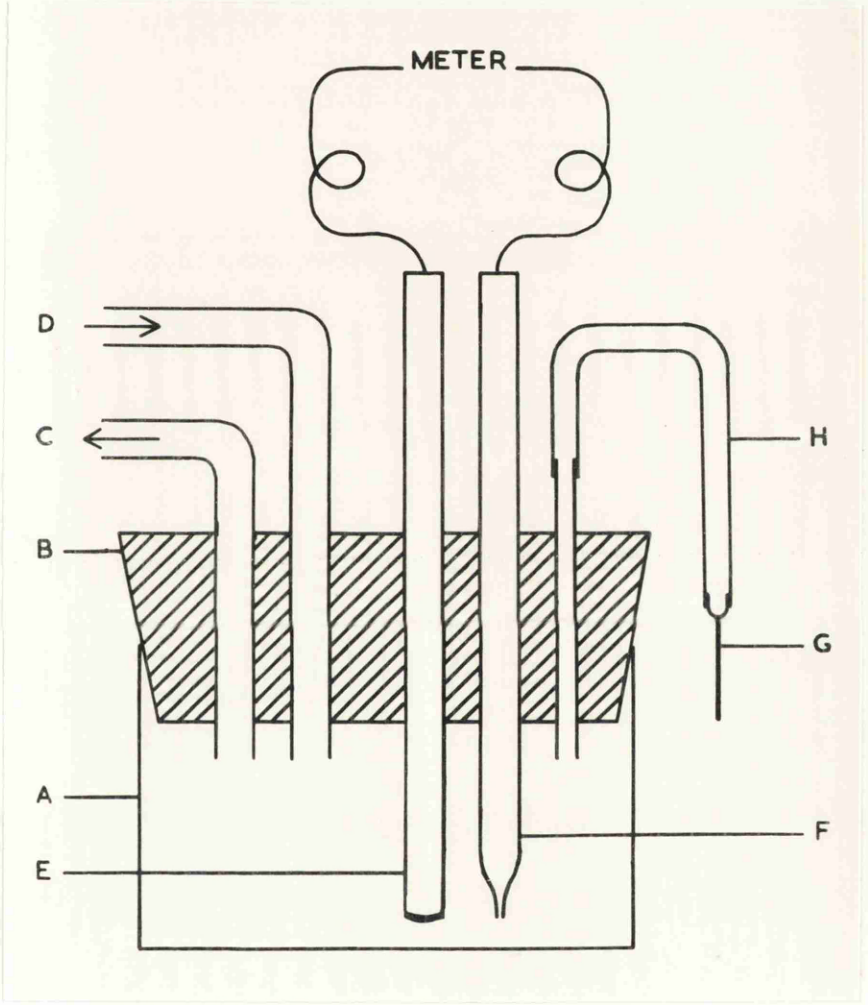


Fig. 1. Cell for the measurement of Eh. A 1 oz. ointment jar, B. rubber bung, C & D gas outlet and inlet tubes, E platinum electrode, F saturated calomel electrode, G hypodermic needle, H rubber tubing.

electrodes were rinsed in distilled water and the glass wiped with lens tissue paper. The electrodes were rinsed again and then stored in a cell containing distilled water. Further cleaning was not normally found to be necessary.

(b) Acidity. The titration of sterilized milk by the usual method of matching the change in colour of phenolphthalein with a rosaniline standard was found to be satisfactory only if the browning of the milk was not extensive. To overcome visual artifacts 25 ml. quantities of the milk were titrated with N/9 NaOH to pH 8.60. The results are expressed as ml. N/9 NaOH/10 ml. milk. The end-point given by the rosaniline method was about pH 8.55. All pH measurements were made using a direct reading Pye pH meter.

(c) Estimation of the degree of heat treatment. The Aschaffenburg turbidity test was used (5). The filtrate was held at 100°C for exactly 5 min. and cooled immediately in ice-cold water. The yellowing of the heated filtrate from the Aschaffenburg turbidity test was read against water in a Spekker absorptiometer using an Ilford 101 filter. The results are reported as extinctions (i.e. drum readings).

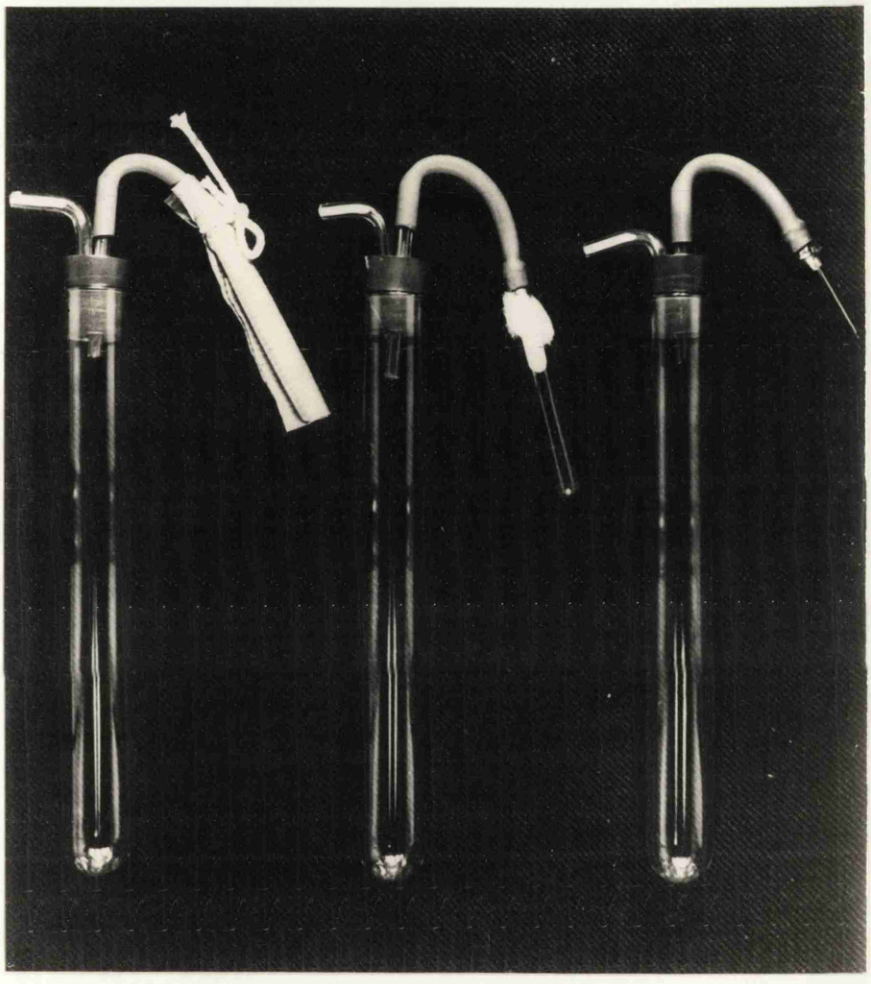
The colour of the milk was matched in a white light cabinet using a Lovibond tintometer, the colour being given in red and yellow units. There was no blue component.

(d) Measurement of vacuum. A Bourdon vacuum gauge fitted with a stainless steel puncturing needle and rubber ferrule was used to measure the vacuum in crown capped bottles. The gauge was also adapted to measure vacuum through a rubber diaphragm by unscrewing the puncturing needle and attaching a hypodermic needle to the gauge by a short length of rubber pressure tubing.

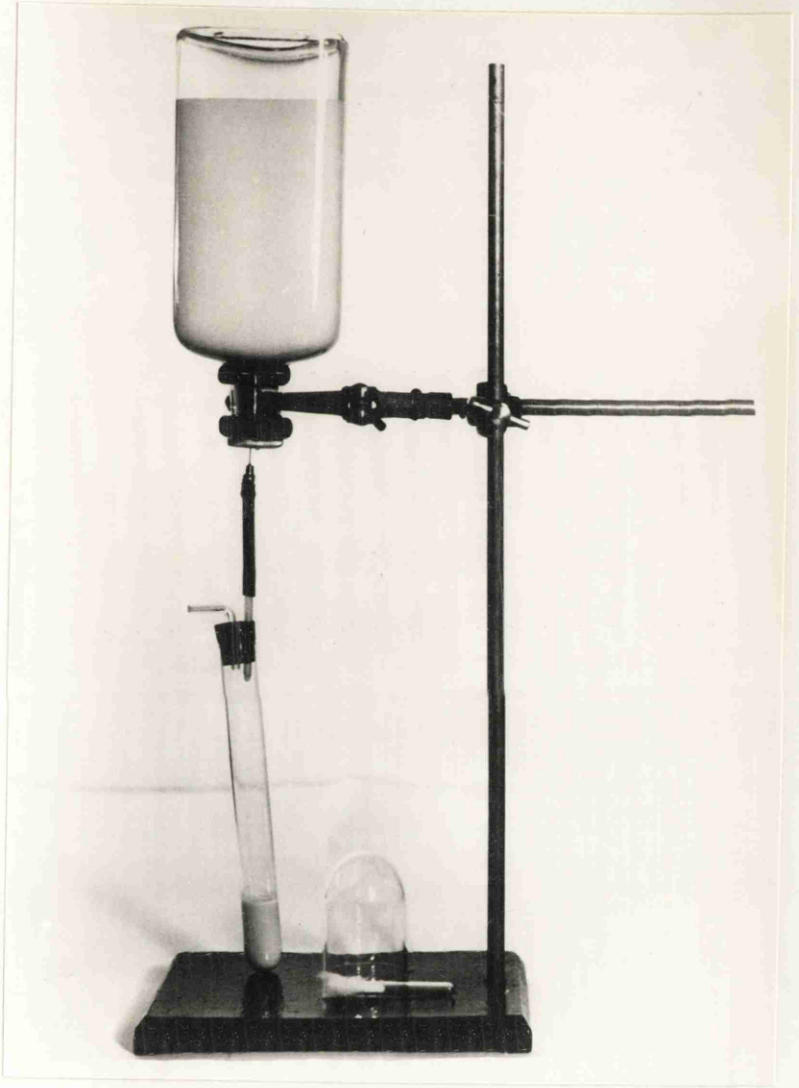
3. Bacteriological examination

(a) Sampling. The method of sampling is illustrated in Plates 2 and 3. The sampling tube consisted of a 6 x $\frac{3}{4}$ in. test tube fitted with a rubber bung carrying inlet and outlet tubes. A hypodermic needle was connected to the inlet tube by a short piece of rubber tubing. For sterilization the outlet tube was plugged with cotton wool. The hypodermic needle was inserted in a plugged Durham's tube and this was protected by a paper cover (Plate 2).

Before sampling, the bottles were well shaken, the glass covers removed and the metal tops cleaned with alcohol using a separate sterile cotton wool swab for each bottle. The bottles were inverted and held at the neck by a clamp. The needle attached to the sampling tube was pushed vertically through the diaphragm of the bottle and the milk sample collected in the tube (Plate 3). Blockages in the needle were usually removed by squeszing the rubber tubing. If this was not successful the flow could be restored by



Sampling outfit. Plate 2.



Sampling outfit in operation. Plate 3.

inserting a small amount of the appropriate gas into the bottle in order to create a small positive pressure. Air was inserted through the plugged side-arm using hand-bellows. Nitrogen was introduced either directly into the bottle using a separate sterile needle for each bottle or by passing it through the side-arm after the sampling tube had first been flushed with nitrogen.

(b) Micro-organisms used. Mesophilic bacilli were identified using the classification of Smith, Gordon & Clark (52) except that optimum growth temperatures were tested in liquid and not on solid media. The organisms used were:-

Bacillus subtilis 6 and D1/2M

B.licheniformis 8

B. cereus 201

B. circulans 152 and 154

B. brevis 58

All these organisms had been isolated from sterilized milk with the exception of B. cereus 201 which was isolated from raw milk. B. subtilis D1/2M was obtained from Dr. A.A. Nichols (National Agricultural Advisory Service, Wolverhampton), the remaining organisms being isolated in this laboratory.

The stock spore suspension used to inoculate the sterilized milk was prepared from the growth on nutrient agar slopes which had been incubated for 48 hr. at the optimum growth temperature for the

particular strain. This growth was suspended in quarter-strength Ringer's solution and filtered through Whatman No. 1 filter paper into a sterile tube. The suspension was heated at 80°C for 10 min., cooled at once to below 20°C and a plate count made in duplicate from suitable dilutions. The suspension was stored at 4-5°C until required.

The suspensions were used for up to 66 days with B. licheniformis 8, up to 94 days with B. cereus 201 and 25 days with B. subtilis D1/2M without showing any changes in viable spore count.

(c) Inoculation. Before the composition of the headspace of the required bottles had been adjusted, a suitable dilution of a stock spore suspension was prepared in sterile quarter-strength Ringer's solution. This was transferred to a sterile 1 oz. Universal container fitted with a perforated cap and rubber diaphragm and paper cover. When the headspace of the bottles was adjusted, the Universal container was well shaken and the spore suspension withdrawn through the diaphragm into a sterile glass/metal Record hypodermic syringe. From this 1 ml. quantities were inoculated into the bottles of sterilized milk. At the same time plates were inoculated with 1 ml. quantities of the suspension from the syringe to determine the number of spores present. The bottles were well shaken before transfer to the incubator.

(d) Counting methods. Dilutions were prepared in

sterile quarter-strength Ringer's solution (2.25 g. NaCl, 0.105 g. KCl, 0.12 g. $CaCl_2.6H_2O$, 0.05 g. $NaHCO_3$, 1 litre distilled water). A fresh pipette was used for each dilution. Poured plates were prepared using 1% starch agar, made from dehydrated nutrient agar (Oxoid) with the addition of 1% soluble starch. The colonies were counted after 24 hr. at 37°C except with B. brevis 58 and B. subtilis D1/2M which were incubated for 48 hr. before being counted.

Spore counts were made in the same manner but the sample was held at 80°C for 10 min. and cooled at once to below 20°C before dilution and plating.

(e) Alcohol test. Equal volumes of milk and 80% alcohol were mixed and examined at once for precipitation.

(f) Direct microscopic examination. Smears were dried quickly at 55°C and flooded with alcohol which was ignited and allowed to burn off. Loeffler's Methylene Blue stain diluted 1:15 with distilled water was poured on the smear and was then washed off immediately in running water. The smear could be dried by blotting and by warming it in a flame but it was preferred to dry the preparation in air after removal of the surplus water.

SECTION III

INVESTIGATIONS INTO THE CHEMICAL CHANGES PRODUCED DURING THE STERILIZATION OF MILK

1. Introduction

Other workers have shown that heat treatment causes progressive chemical changes in milk. For example, Burton (53) examined the changes in colour, Whittier & Benton, acid formation (25) and Harland, Coulter & Jenness examined the reducing powers of heated milk (27). However, these workers tended to study only one type of change. When the present investigations were started it was considered desirable to know more of the general interrelationships of these changes in order to correlate bacterial growth with the environmental conditions established in the milk by the sterilization process. Since it was not known in what manner bacterial growth might be affected only non-specific tests were studied. For this purpose measurements were made of the titratable acidity, pH, Eh, acid ferricyanide reduction, browning of the milk and yellowing of the Aschaffenburg turbidity test filtrate. The Eh was studied in more detail after it had been shown that the ability of bacilli to grow in sterilized milk appeared to be associated with the oxidation-reduction potential of the milk.

2. Experimental results

(a) Preliminary experiments. Before the methods used in the chemical examination of sterilized milk were finally adopted preliminary experiments were made to determine the most satisfactory conditions for testing. These experiments are reported briefly here.

The reactions of sterilized milk with sodium nitroprusside, Grote's reagent for sulphur compounds (54) and alkaline lead acetate proved unsatisfactory as means of estimating reducing substances. With sodium nitroprusside and with Grote's reagent the colour produced was weak and the brown discolouration of the milk made it difficult to read the result. Alkaline lead acetate gave a very strong reaction with raw milk and a brownish-yellow colour with sterilized milk. After aeration the brownish-yellow colour was intensified whereas the reaction with acid ferricyanide was reduced by aeration. Hopkins (55) has shown that aeration of glutathione produced hydrogen sulphide. Therefore it seems possible that while there is a reduction in reducing groups during aeration some H_2S is formed at the same time.

Chapman & McFarlane (47) noted that the reaction between milk and potassium ferricyanide was dependent on the time, temperature and pH of the reaction. They used freshly boiled water saturated with CO_2 to prevent autoxidation in the preparation of standard solutions. They took no precautions to prevent autoxidation in

measuring the reducing powers of milk powders nor did they note the effect of time on the reaction other than for colour development in the filtrate.

In the present investigations it was found possible, taking suitable precautions, to obtain good agreement between duplicate samples of the same filtrate, e.g.:-

Type of milk	Reducing substances (Extinction) Readings for duplicate samples of the filtrate	
Pasteurized milk	0.089	0.090
Homogenized milk	0.093	0.094
Sterilized milk A	0.414	0.416
Sterilized milk B	0.470	0.476

To obtain this degree of accuracy it was necessary to use chemically clean glassware and to ensure that there was no delay during testing.

There was a rapid change in reducing properties when a bottle of sterilized milk was opened. This oxidative process continued at an appreciable rate at room temperature when the milk was diluted 1:9 with distilled water. For example, two different samples diluted immediately before testing gave extinctions of 0.327 and 0.415. After holding the diluted milk for 30 min. the readings were 0.297 and 0.384 respectively.

Chapman & McFarlane considered protein to be the sole source of acid-ferricyanide-reducing groups in dried milk powder. However, Lea (56) noted the reducing activity of dialysable non-nitrogenous

material which he presumed to be a degradation product of lactose. The following results show that the protein-free filtrate was capable of reducing ferricyanide even at 5°C (Table 1).

Table 1. The effect of the time at which colour is developed on the result of the acid ferricyanide reduction test

Type of milk	Immediately	Filtrate stored at 5°C for 90 min. before colour development
Pasteurized	0.105	0.142
Homogenized	0.114	0.144
Sterilized A	0.417	0.442
" B	0.550	0.579

Since the rate of increase was similar for all samples the continuing reaction cannot be due to heat-formed reducing substances. An equivalent change occurred if the filtrate was allowed to stand at room temperature for 30 min. before colour development. It follows that the time taken to perform the test is of importance.

The results of this test are given as extinctions without reference to a calibration curve. Chapman & McFarlane used a glutathione standard but owing to lack of knowledge of the reducing groups taking part in the reaction Lea (56) preferred a ferricyanide standard. Since neither of these standards is affected by variations in the time taken

to perform the test, it has been preferred to report results here directly as the reading on the drum of the Spekker absorptiometer.

As a preliminary to the present investigation oxidation-reduction dyes were used to confirm the value of -50 mV obtained by Wilson (57) for the potential of sterilized milk. Potassium indigo trisulphonate (Eh'_o = -81 mV pH 7.0 at 30°C) was found to be the most suitable, and the results quoted were obtained with this dye.

Experiments to determine the effect of deaeration gave results for which typical values are shown in Table 2. The most negative potential was always obtained in bottles which were deaerated before heating and which remained closed after treatment (treatment A). The calculated value for the potential of milk which had received this treatment was usually more negative than -120 mV and was always more negative than the value reported by Wilson (57). The most positive results were shown in those bottles which were open during heating irrespective of whether the milk had been deaerated prior to heating or not. Intermediate results were obtained with milk which was heated either in open bottles that were closed immediately after heating or in evacuated bottles that were opened immediately after heat treatment. Since oxidation-reduction systems are present in milk these results are to be expected.

Table 2. Effect of deaeration and heati
the Eh of milk as determined b
trisulphonate

Treatment	pH
Bottle evacuated rapidly to 25 in. Hg	6.20
" " at 25 in. Hg for 25 min.	6.20
" " at 25 in. Hg for 25 min. and opened after heating	6.20
Bottle open during heating and closed after it	6.40
Bottle evacuated at 25 in. Hg for 25 min. and opened before heating	6.40
Bottle heated open	6.40

A further series of experiments was made to determine the effect of the degree of heat treatment. It was rather unexpected to find that an increase of 7°C in heat treatment had apparently no effect on Eh (Table 3). In later work this result was confirmed electrometrically.

The results given in Tables 2 and 3 can be considered as having an accuracy of only ±10%. Since atmospheric oxidation did not always give a full return of colour it is probable that some of the dye was destroyed during heating.

If evacuated bottles were shaken there was an increase in the colour of the dye but this returned to the original value within 10 min. When air was admitted into the headspace there was a rise to 90-100% full colour. It was not found possible to reverse this rise in Eh by subsequent deaeration of the milk.

(b) <u>The effect of variation in heat treatment on the colour, acidity and reducing powers of milk</u>. In order to examine the effect of heat on milk, six levels of heat treatment were chosen for study:-

(i) pasteurization at 63°C for 30 min.

(ii) homogenization, after heating to 80°.

Sterilization of homogenized milk at

(iii) 105.5°C for 15 min. (4 lb./sq.in. steam pressure)

(iv) 110°C for 15 min. (6 lb./sq.in. steam pressure)

Table 3. The effect of heat
as determined by po

Heat treatment	Open/ Evacuated
108.5°C for 15 min.	O E
115.5°C for 15 min.	O E

Bottles were either evacu
and heated while still closed (E

(v) 114°C for 15 min. (8 lb./sq. in steam pressure)

(vi) 117°C for 15 min. (12 lb./sq. in. steam pressure).

The effect of evacuation of the bottles was studied at each level of heating. Evacuation was carried out after pasteurization and homogenization but before the sterilization process. The results considered here were obtained on 220 ml. quantities of milk heated in 8 oz. bottles.

(i) Pasteurization and homogenization. A comparison of the effects of the two lowest levels of heat treatment (pasteurization and homogenization) on the acid-ferricyanide-reducing powers, titratable acidity and pH was made using milk from two Ayrshire cows (Table 4). Neither treatment affected the acid-ferricyanide-reducing substances in the milk. However, homogenization caused a slight fall in the titratable acidity with a consequent rise in pH. The oxidation-reduction potential of homogenized milk appeared to be rather more positive than in pasteurized milk (Table 5), but there was no sharp distinction between the potentials of milks which had received these two heat treatments. Deaeration of the milk before measurement was made under nitrogen, caused a fall of 260 to 461 mV when compared with the Eh of the same milk measured in air. When considered in conjunction with the results of Eh measurements made on sterilized milk this wide

Table 4. The
ti

Source of milk	Pasteurized	
	Acid-ferri-cyanide-reducing substances as extinctions	Titrata acidi (ml. N/ NaOH/1(mill
Cow 3	0.059	1.3
Cow 4	0.044 0.072	1.4 1.4

Table 5. The effect of deaeratio
potential of pasteur

Source of milk	Open - measureme made in a
Pasteurized Commercial HTST (i)	+23
(ii)	+9
Cow 1. 63°C for 30 min.	-35
Homogenized Farm 1. (i)	+85
(ii)	+20
Cow 2.	+30

variation was attributed to differences in the time interval between processing and measurement.

(ii) Sterilization. The effect of the degree of heat treatment used on certain properties of the sterilized milk was studied by heating for 15 min. at various temperatures. The effect of heating in "open" and "evacuated" bottles was determined at the same time. The results are presented in relation to two milks. Milk from farm 2, which had a Friesian herd, gave results that were representative of several experiments in which the milk from normal Ayrshire herds was used. Results for milk from farm 1, which had an Ayrshire herd, are included since it gave abnormal results in pH and titratable acidity.

The changes in acidity caused by the heat treatment are shown in Fig. 2. The pH of normal homogenized milks was about pH 6.60-6.75, falling to pH 6.30-6.20 after the highest heat treatment (117.5°C). The pH of the milk in "open" bottles was usually 0.1 units higher than that of the milk in the "evacuated" bottles. The titratable acidity increased as the level of heat treatment was raised. After heating at 117.5°C for 15 min. there was usually an increase of about 0.6 ml. N/9 NaOH/10 ml. milk. The titratable acidity was always greated in "evacuated" than in "open" bottles. The other milk reported had a high initial pH of 6.88 and a low titratable acidity of 1.15 ml. N/9 NaOH/10 ml. milk after homogenization. During heat treatment the pH fell and the acidity

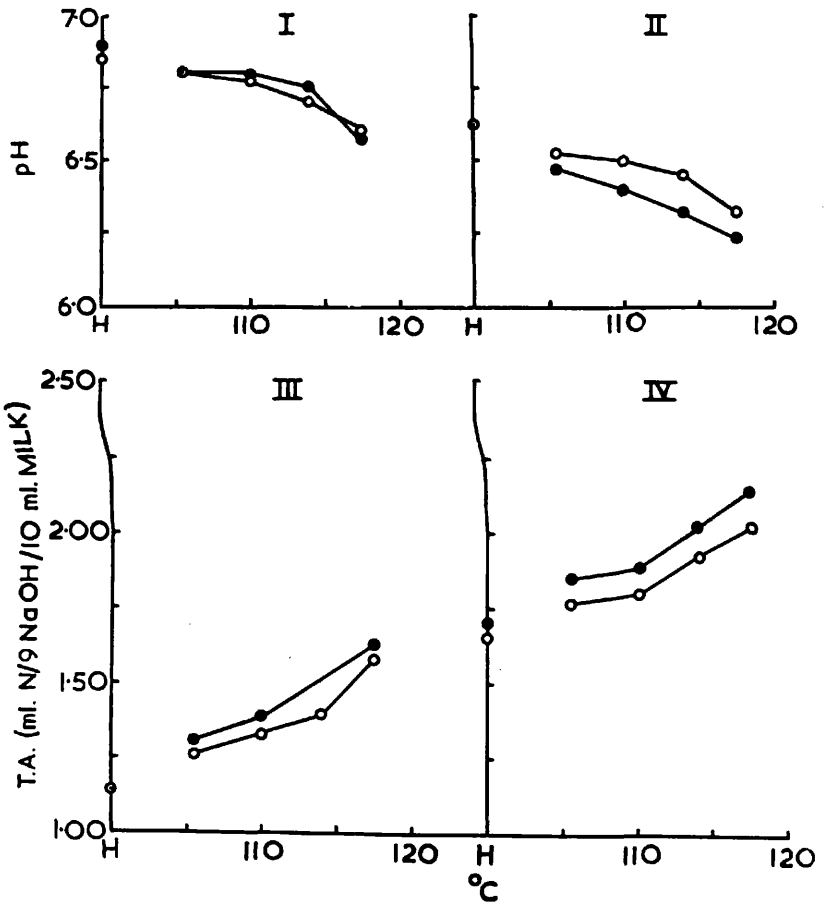


Fig. 2. The relation of pH and titratable acidity of milk in "open" and "evacuated" bottles to heat treatment for 15 min. at various temperatures. I & III abnormal milk from farm 1. II & IV normal milk from farm 2. H milk homogenized only. O "open" bottles, ● "evacuated" bottles.

increased but not at the same rate as in a normal milk. There was very little difference in pH and titratable acidity between the milk heated when "open" and the same milk heated when "evacuated".

The effect of the degree of heat treatment on the colour of the sterilized milk as measured by a tintometer is shown in Fig. 3. In addition to the colour of the milk itself, Clegg & Lomax (58) have suggested that the yellow colour of the Aschaffenburg turbidity filtrate is also affected by the degree of heat treatment which the milk has received. The results for the colour of the filtrate are therefore also shown in Fig. 3. With milk from farm 2, the colour of the milk as measured in the tintometer and by the yellowing of the turbidity filtrate increased with the temperature of heating. At the lower temperatures, 105.5° and 110°C, there was no difference between milk heated in "open" and "evacuated" bottles. At higher temperatures the colour increased more rapidly in the "evacuated" bottles. In the more alkaline milk from farm 1 the rate of browning in "evacuated" bottles was greater than that shown by the normal milk. The colour formation in "open" bottles was the same at 114° and 117.5°C for both milks but was lower in the abnormal milk when this was heated at 105.5° and 110°C.

The effect of heat treatment on the reducing properties of milk as measured by the acid ferricyanide test and by the oxidation-reduction potential is shown

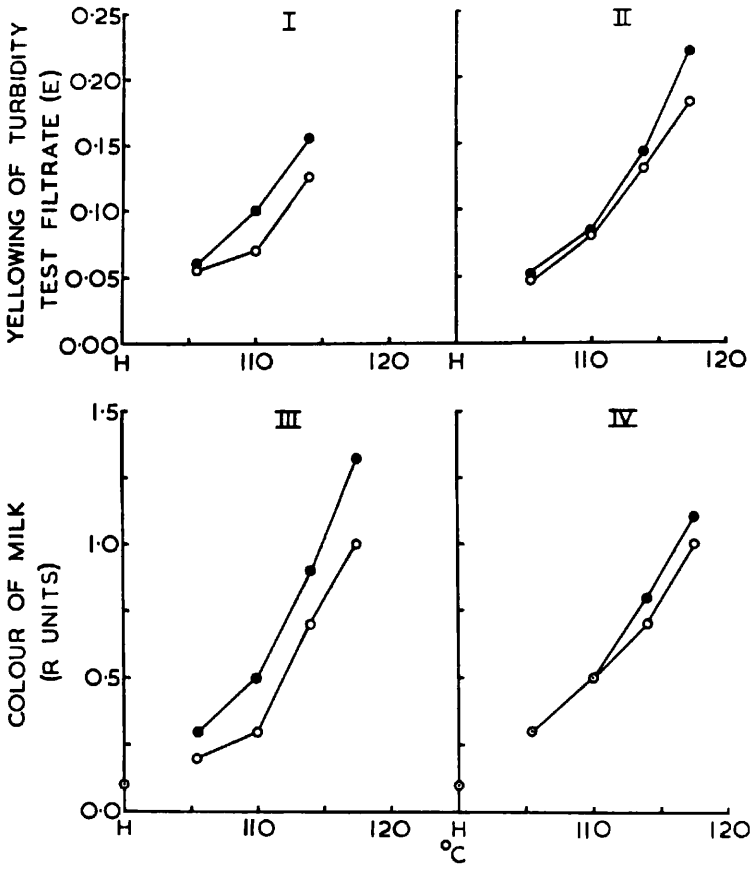


Fig. 3. The relation of the yellowing of the turbidity test filtrate and the colour of the milk in "open" and "evacuated" bottles to heat treatment for 15 min. at various temperatures. I & III abnormal milk from farm 1. II & IV normal milk from farm 2. H milk homogenized only. O "open" bottles, ● "evacuated" bottles.

in Fig. 4. There appeared to be little difference in the production of acid-ferricyanide-reducing substances between the normal and the more alkaline milk in the "evacuated" bottles. However, the reducing substances formed in the abnormal milk heated in "open" bottles was less than in the normal milk that had been subject to the same treatment. While there was a marked increase in acid-ferricyanide-reducing substances due to increased heat treatment this had little or no effect on the Eh. The average value for both the normal and the abnormal milks heated in "evacuated" bottles was -290mV. However, in milk heated in "open" bottles the increase in reducing substances was reflected by a slight fall in the Eh over the range of heat treatment. This change was rather more marked in the abnormal milk where the Eh fell from +50 mV to -11 mV over the range of heating. Corrections for pH, which have not been made, would give a more negative result at the lower pH values. The increased effect of the heat-induced reducing substances in the abnormal milk may have been due to a low solids-not-fat content of the milk which could be indicated by the low titratable acidity. A low protein content would also account for the greater depression of the acid-ferricyanide-reducing substances in the "open" bottles of the abnormal milk.

As previously stated, the milk was heated in 220 ml. quantities in 8 oz. bottles. When larger

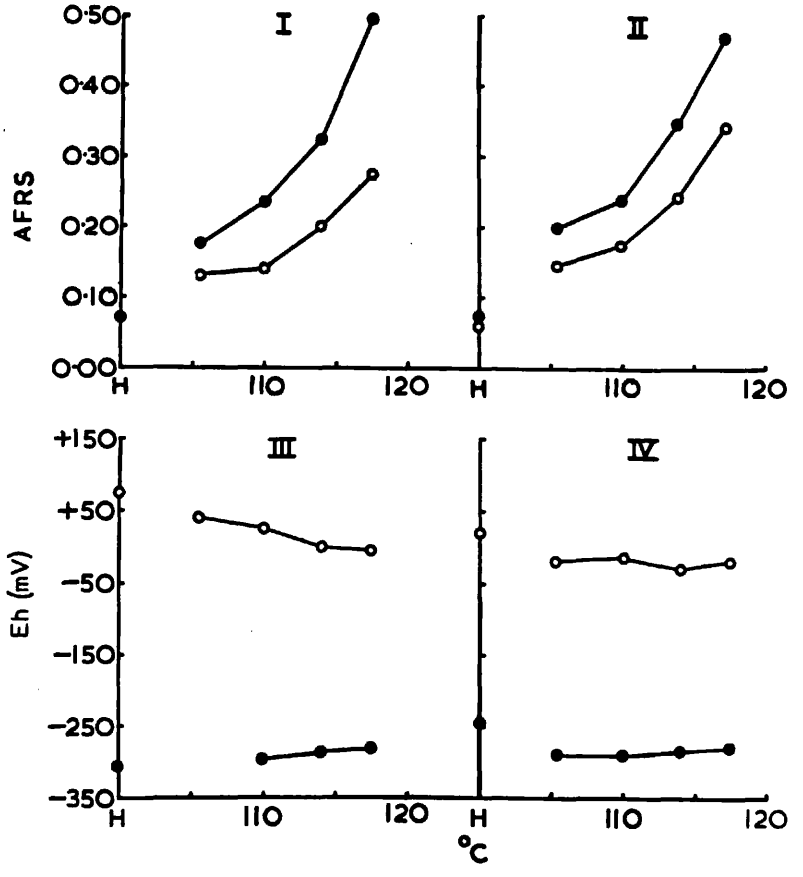


Fig. 4. The relation of acid-ferricyanide-reducing substances (AFRS) and Eh of milk in "open" and "evacuated" bottles to heat treatment for 15 min. at various temperatures. I & III abnormal milk from farm 1. II & IV normal milk from farm 2. H milk homogenized only. O "open" bottles, ● "evacuated" bottles.

volumes of milk were heated, i.e. 440 ml. milk in 16 oz. bottles or 1 litre in 40 oz. bottles, greater differences were obtained in acidity, colour of the milk and reducing properties (with the exception of Eh) between the "open" and "evacuated" bottles. This was therefore investigated.

(c) The source of variation between "open" and "evacuated" bottles. When chemical tests were first made on freshly produced laboratory sterilized milk, erratic results were obtained for the titratable acidity and acid-ferricyanide-reducing substances. For example, two crown-capped bottles from each of two batches of laboratory-produced sterilized milk gave for acid-ferricyanide-reducing substances extinction values of 0.742 and 0.702 for one batch and 0.852 and 0.759 for the other. As such results were well outside the experimental error for the method, some other source of difference was sought, and it was found that because it was not possible to fill and cap the bottles with sufficient speed to prevent the cooling of the bottles or the milk, the vacuum in the headspace of the laboratory produced bottles varied within a batch.

It was considered that this variation in vacuum might affect the chemical changes induced in the milk and so this point was studied. Larson, Jenness & Geddes (10) examined the effect of heating milk protein sols in air and under nitrogen on the value of reducing substances as obtained by the acid ferricyanide

test. Their results showed that higher values were obtained when the sols were heated under nitrogen than when heated in air. Larson et al. suggested that the effect might not be the same in whole milk due to the presence of lactose. Greenbank & Wright (28) showed that there was a greater fall in oxidation-reduction potential when milk was deaerated before heating than if the milk was heated in an open vessel or in a closed vessel without prior deaeration. These workers made no chemical measurements of reducing properties but assumed that the results obtained by Larson et al. (10) gave sufficient proof that the greater fall of potential in deaerated milk was due to sulphydryl groups. However, Harland, Coulter & Jenness (27) found that it was not possible to correlate any one chemical test for reducing substances with the oxidation-reduction potential of the milk although they confirmed that the effect of heating milk under nitrogen was the same as that for milk protein sols giving greater reducing powers than when heated in air or oxygen. Although the results of Harland et al. (27) and Greenbank et al. (28) were obtained on milks heated at temperatures below 100°C, it seemed reasonable to presume that a similar effect would operate at higher temperatures.

Since it was not practicable to heat milk under nitrogen at temperatures above boiling point, a bottle with a perforated cap and rubber diaphragm was used in

the present investigations. The vacuum was obtained by inserting through the diaphragm a hypodermic needle which was attached to a water pump. Using this method, it was shown that the acid-ferricyanide-reducing values for duplicate bottles gave good agreement. Differences of the order previously obtained with capped bottles (p.30) were found between bottles which had been heated with a vacuum in the headspace and those which had been heated "open" (Table 6).

The heat treatment gave rise to two sources of error in the ferricyanide test. In "open" bottles lower results than expected were obtained in milks which had "bumped" during heating. In "evacuated" bottles, the metal caps sometimes slackened during heating with a partial or total loss of vacuum. As a result, a low acid-ferricyanide-reducing value was obtained. This is shown in Table 6 with three bottles heated at 114°C for 15 min.

It was demonstrated that provided the vacuum was maintained during heating reproducable results were obtained with titratable acidity, pH, browning of the milk and yellowing of the Aschaffenburg turbidity test filtrate. In these tests the reaction was always more extensive in those bottles which had been evacuated before heating. The titratable acidity was greater, the pH lower and the milk showed more discolouration when the milk was heated in an "evacuated" bottle than when heated in an "open" bottle. From these results

Table 6. The effect of deae
heating on the aci
substa

Heat treatment	Vacuu
105.5°C for 15 min.	absent (i
	(ii
	present (i
	(ii
114.0°C for 15 min.	absent
	lost during
	present

it would appear that brown discolouration occurred more readily in the absence of oxygen in liquid whole milk. This is the opposite of the results obtained by Henry, Kon, Lea & White (59) in the examination of milk powders. These authors found that browning was more extensive in milk powders packed in air than in nitrogen. There are two possible explanations, i) that there are two different types of browning reactions, one which occurs more readily in the absence of oxygen in liquid milk and another which occurs more readily in the presence of oxygen in milk powders, ii) that although "open" and "evacuated" bottles were heated together, the heat capacity of the two types of bottles is different and that this results in a slight difference in heat treatment. From the consideration of the results of Lea & Hannan (12) who studied the reaction of casein and glucose at different levels of humidity, the first possibility seems improbable. It was shown experimentally that the second explanation was more likely.

To study this question the rate of heating and cooling of water was measured in an "open" and an "evacuated" bottle using two thermistors. The leads from one of the thermistors were sealed into the rubber diaphragm of a bottle cap making it possible to measure the temperature inside the evacuated bottle (Fig. 5). The heating and cooling curves for the two bottles are shown in Fig. 6. The heating curve was

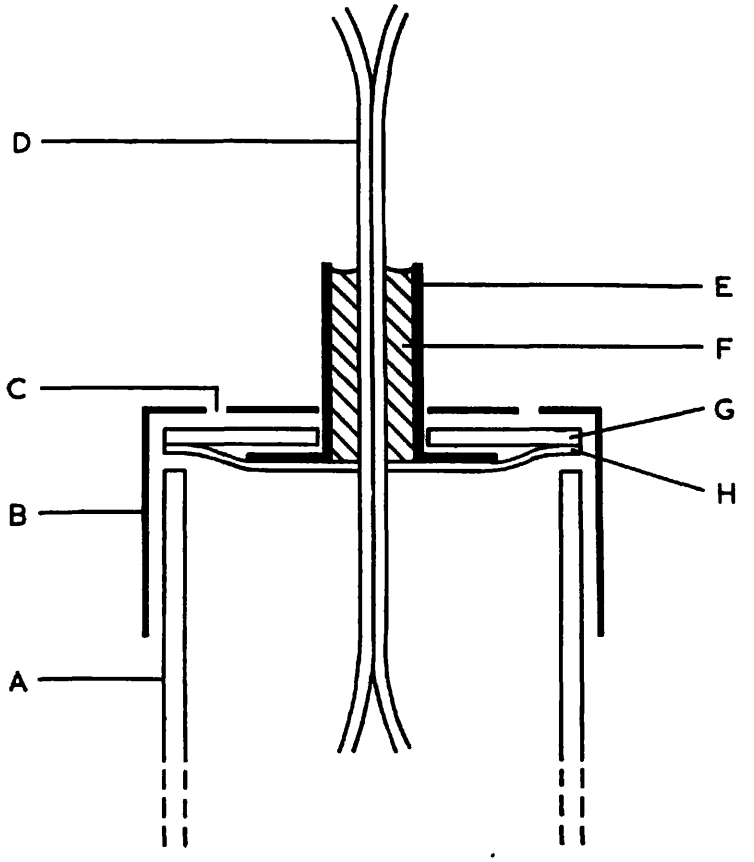


Fig. 5. Air-tight insertion of leads through a rubber diaphragm. A neck of the bottle, B perforated metal cap, C additional small holes for the insertion of a hypodermic needle through the diaphragm, D leads to thermistor, E bicycle tyre valve seating, F Araldite D, G rubber diaphragm, H rubber sheeting stuck on to G.

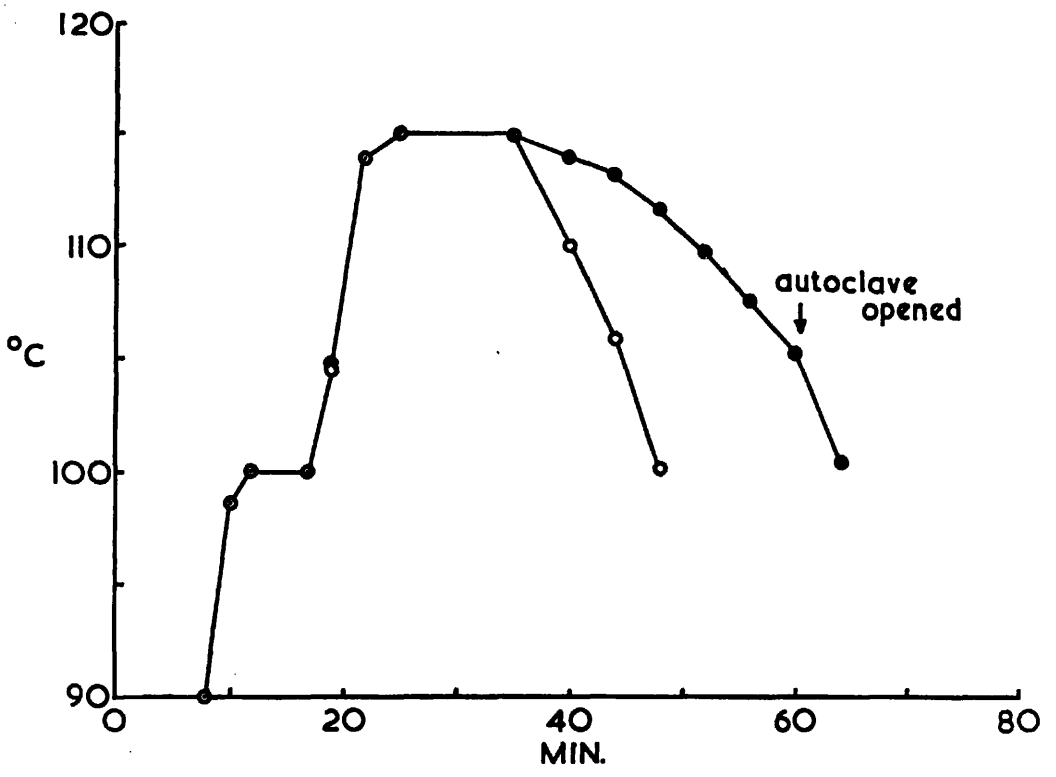


Fig. 6. Heating and cooling curves for water in "open" and "evacuated" bottles. O "open" bottles, ⊙ "evacuated" bottles.

the same for both bottles but the rate of cooling was very much slower in the "evacuated" bottle. During the time required for the water in the "open" bottle to fall to a temperature of 100.2°C that in the "evacuated" bottle had only fallen to 111.8°C. This difference in the rate of cooling may be sufficient to account for the more extensive changes found in the milk heated in "evacuated" bottles.

In preparing Figs. 2-4 it was assumed that the milk in "open" and "evacuated" bottles received equal heat treatment at each temperature. When each variable was plotted against temperature separate curves were obtained from milks from "open" and "evacuated" bottles. This could result either from the additional heat treatment received by the "evacuated" bottles due to the slower rate of cooling (Fig. 6) or from the effect of deaeration before heating. This was resolved by plotting one variable against any variable other than temperature. If the differences between milk in "open" and "evacuated" bottles were due only to the effect of heat treatment on the two variables being compared, a single curve should result. When the titratable acidity was plotted against the yellowing of the Aschaffenburg turbidity test filtrate the values for milks from "open" and "evacuated" bottles gave a single curve (Fig. 7 I) therefore these two functions varied only with the degree of heat treatment. If either or both of

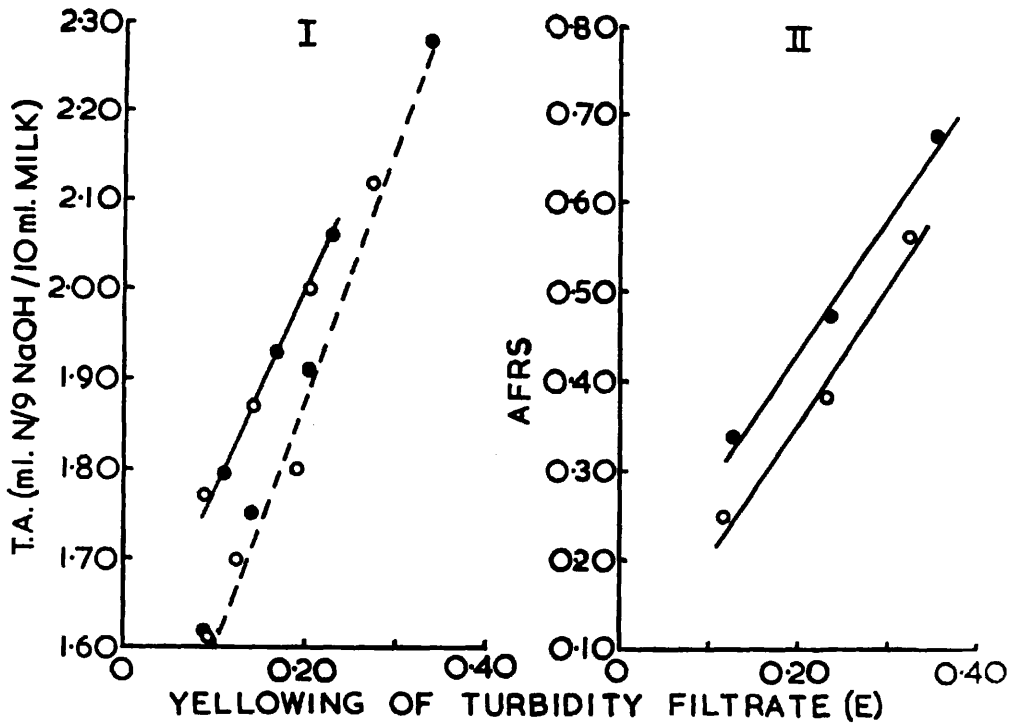


Fig. 7. The relation of the yellowing of the Aschaffenburg turbidity test filtrate to the titratable acidity and acid-ferricyanide-reducing powers of milk heated in "open" and "evacuated" bottles at different temperatures. I titratable acidity (TA) related to yellowing of the filtrate for two different samples of milk. II acid-ferricyanide-reducing substances (AFRS) related to yellowing of the filtrate for a third milk. O "open" bottles, ● "evacuated" bottles.

the variables being compared reacted differently in "open" and "evacuated" bottles then two curves should result. This was demonstrated with a third milk when acid ferricyanide reduction was compared against the yellowing of the turbidity filtrate (Fig. 7 II). Since it has been shown that the yellowing of the turbidity filtrate varied only with the degree of heating of the milk, it follows that the production of acid-ferricyanide-reducing substances is not the same in the two types of bottles. The higher values obtained with milk heated in "evacuated" bottles are consistent with the views expressed by other workers (10, 27) that heating milk in the absence of oxygen has a protective action for the reducing groups. By this method it can be shown that the colour of the milk, the titratable acidity and the yellowing of the turbidity filtrate increase with the degree of heat treatment while the acid-ferricyanide-reducing substances and Eh are affected by deaeration prior to heat treatment.

(d) The oxidation-reduction potential of heated milk. Among others Jackson (60) and Hartman, Garrett & Button (61) have measured the oxidation-reduction potential of raw and pasteurized milk mainly in an attempt to establish the source of the "oxidized" flavour of milk. Since these measurements were made at low temperatures in order to restrict bacterial growth, a long holding period was required before the electrodes came into equilibrium with the system.

Tobler (51) showed that by making the measurements at 50°C constant values could be obtained in a relatively short time (about 3 hr.). Tobler made most of his measurements on milk heated in open vessels but he noted that lower values were obtained if heating was carried out under anaerobic conditions. Other workers have shown that deaeration gives a marked shift of the Eh in a negative direction (27, 61).

The following experiments were done to find how the treatment before and during sterilization and conditions of storage affected the Eh of heated milk.

The oxidation-reduction potential obtained with commercially high temperature-short time (HTST) pasteurized milk is shown in Fig. 8, where curves for deaerated and for normal milk are compared, the measurements having been made under nitrogen. The initial plateau during the 10th-140th min. was presumably due to the oxygen dissolved in the milk. The removal of air from the headspace of the bottle before the Eh of the milk was measured did not appear to affect the duration of this stationary period but resulted in lower Eh values after 150 min. In this example a difference of 80 mV was obtained between the normal and deaerated milks. In the same way the Eh attained in homogenized milk depended on the extent to which the constituents of the milk were subject to atmospheric oxidation before deaeration. The time-potential curve for two samples of homogenized milk is

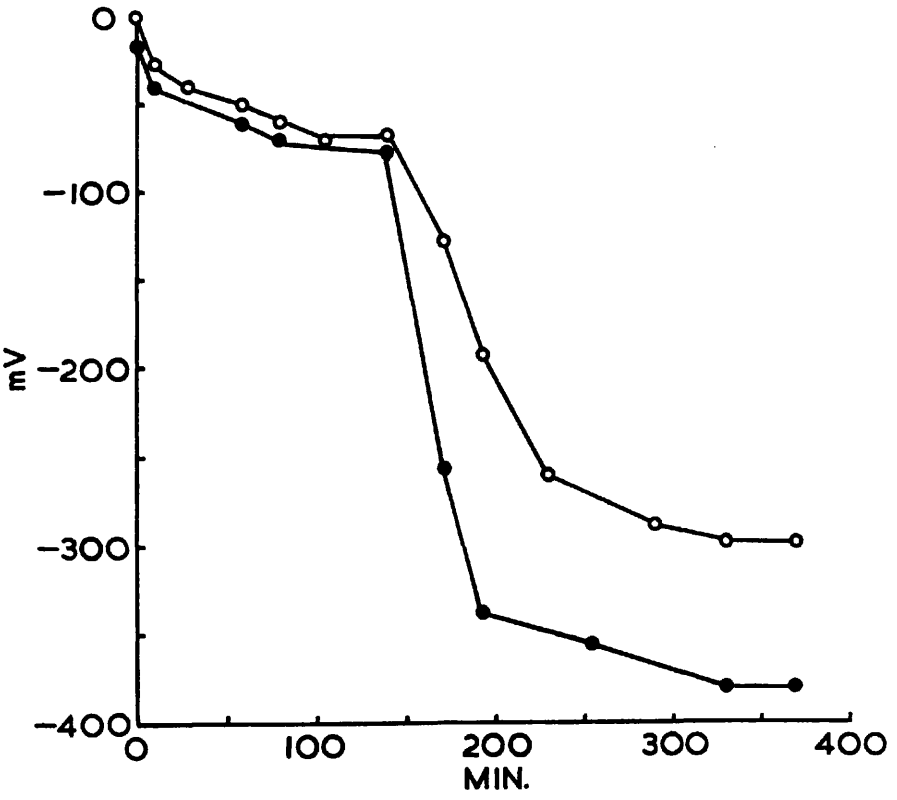


Fig. 8. Potential-time curve for normal and deaerated commercially HTST pasteurized milk measured under nitrogen at 50°C. O normal milk, ● milk deaerated at 25 in. Hg for 30 min. before measurement.

given in Fig. 9. With the first sample (Fig. 9, I) a constant value of -242 mV was reached in 120 min. whereas with the second sample (Fig. 9, II) only 80 min. were required to give a constant value of -300 mV.

The reaction of the poising capacity of milk towards atmospheric oxidation was shown by making Eh measurements in air followed by nitrogen and also by the reverse procedure. The potential of the sterilized milk measured in air was relatively high, but milk from the "evacuated" bottles gave a lower potential than the milk from the "open" bottles. This is shown in Fig. 10 for milk from one source. When the air was replaced by nitrogen, the potential in the milk from the "open" bottle showed a very slight positive drift, but this is not obvious in Fig. 10, I because of the small mV scale. After a period of 40 min. the Eh of the milk from the "evacuated" bottle showed a slow fall indicating a poor poising capacity, a constant value not being obtained in 240 min. When the reaction of the same milk was measured first under nitrogen and then in air (Fig. 10, II) an equilibrium value was obtained quite rapidly with milk from the "evacuated" bottle. The absence of a plateau at the beginning of the curve for the "open" bottle was assumed to indicate that there was no free oxygen present in the sample since the bottle had been stored closed for 7 days before being tested. However, milk from the "open" bottle showed the same

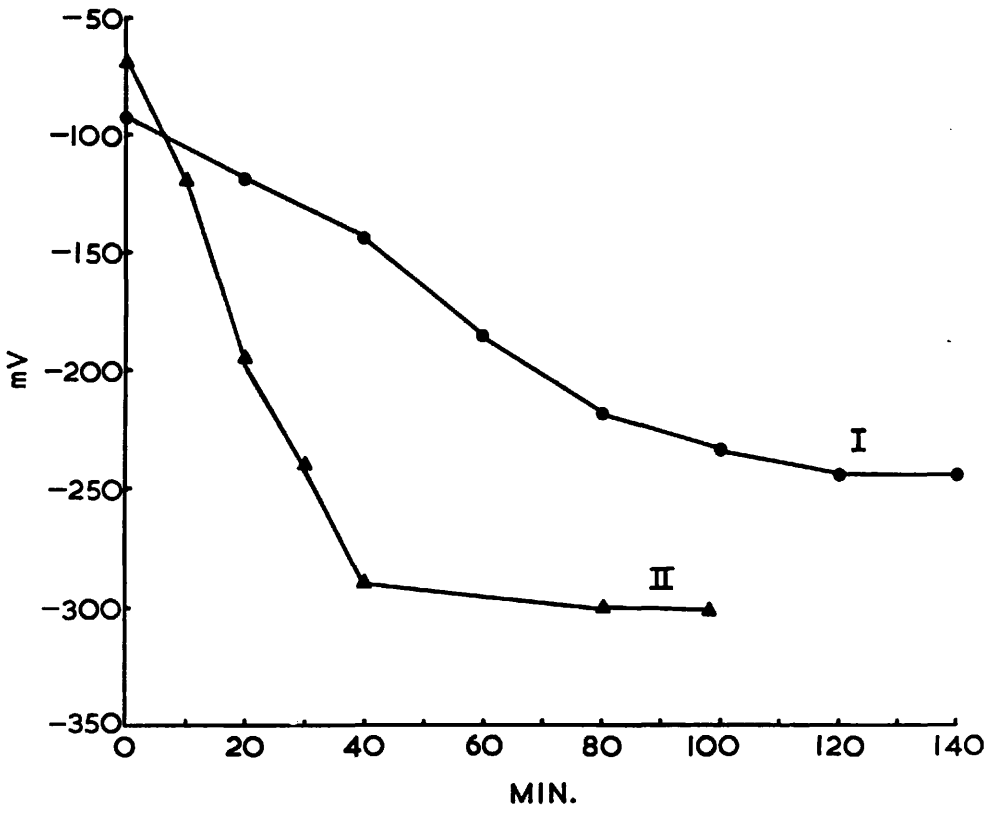


Fig. 9. Potential-time curve for homogenized milk deaerated at 25 in. Hg for 30 min. before measurement under nitrogen at 50°C. I milk from farm 2. II milk from farm 1.

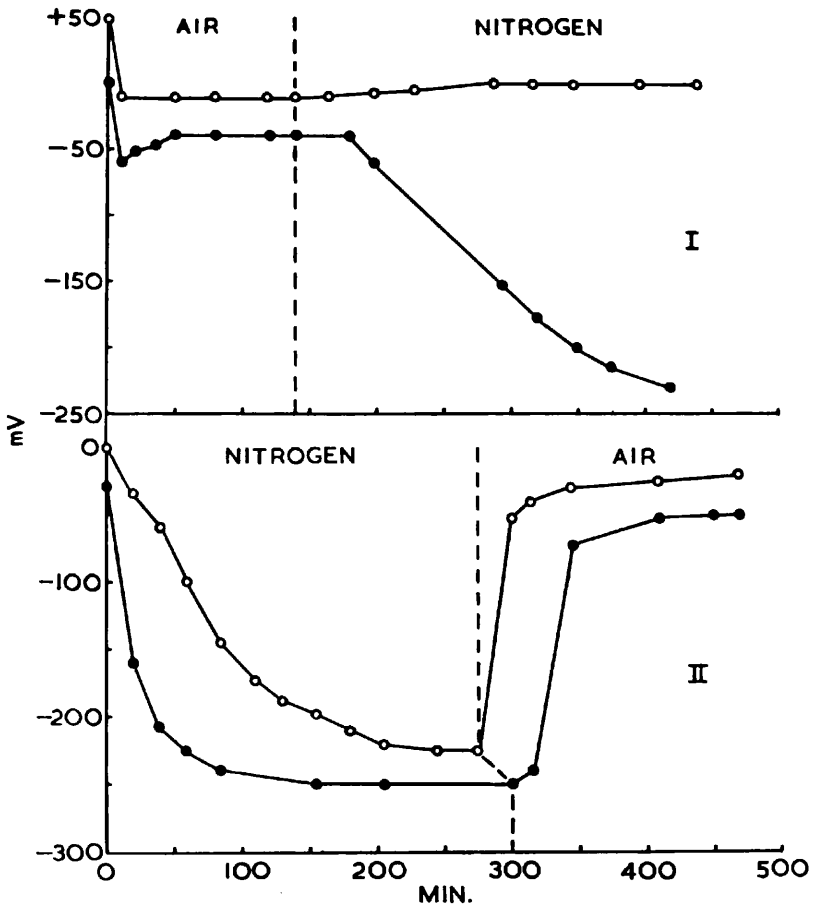


Fig. 10. Potential-time curve for milk sterilized by methods A & D and measured at 50°C, I under air and then under nitrogen, II under nitrogen and then under air. Milk from cow 2 sterilized by method A (●) and D (○).

poor poising capacity as was exhibited by milk from the "evacuated" bottle (Fig. 10, I) when measured under nitrogen after having been held in air. When the flow of nitrogen was stopped, there was an immediate rise in potential in the milk from the "open" bottle while there was an appreciable lag (15 min.) before the potential was affected in the more strongly poised milk.

The curves shown in Fig. 10 were obtained with milk from one cow (cow 2) and are typical of milks which did not readily cause a prolongation of the lag phase of bacterial growth. With milks which did show this inhibitory effect a different picture was obtained. This is illustrated by the Eh curve for milk from farm 1 heated in an "open" bottle and measured first under air and then under nitrogen (Fig. 11). The initial sharp fall followed by a slight rise was shown by all milks with a capacity to resist atmospheric oxidation when Eh measurements were made in air. When air was replaced by nitrogen, there was an immediate slight fall in potential and then a steady period lasting for 70 min. Thereafter the curve fell sharply, being similar in gradient to the curve for milk from an "evacuated" bottle measured under nitrogen (Fig. 10, II). While milk from cow 2 heated in an "open" bottle showed no resistance to atmospheric oxidation, milk from farm 1 treated in the same way showed considerable poising capacity.

Heat treatment over the range 104.5-117.5°C for

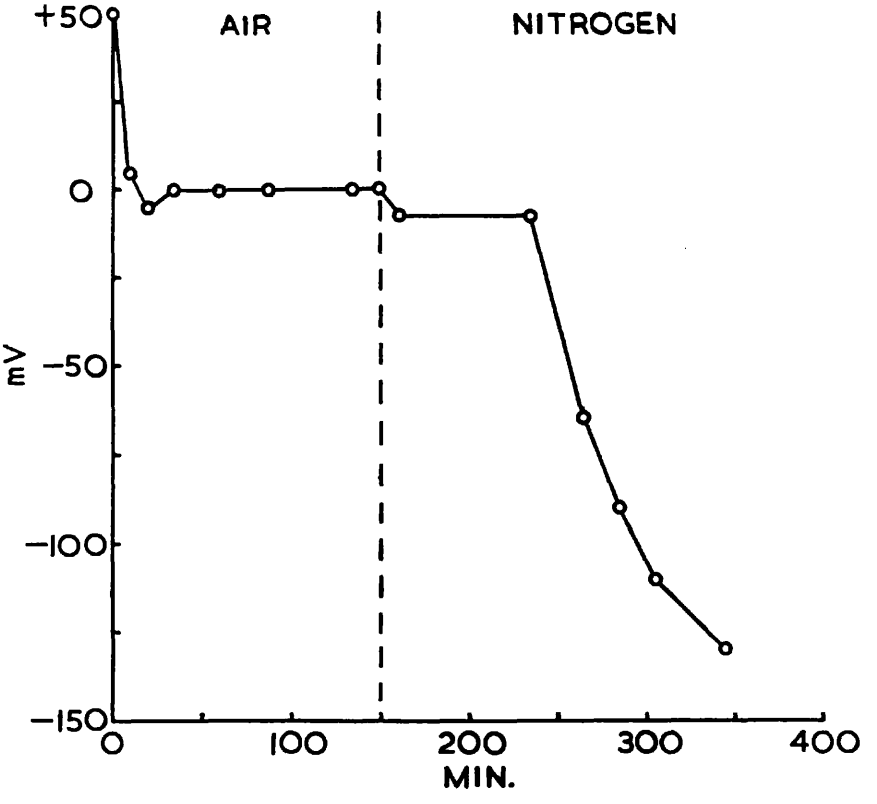


Fig. 11. Potential-time curve for milk from farm 1 sterilized in an "open" bottle and measured first under air and then nitrogen at 50°C.

15 min. did not appear to affect the time required to obtain a constant value for the potential. The four curves shown in Fig. 12 were obtained with samples of the same milk heated for 15 min. at different temperatures. The curves are very similar to that shown for homogenized milk in Fig. 9, II. The fall in the value of the initial reading as the degree of heat treatment increased cannot be regarded as important because considerable variation was possible owing to the fact that the initial reading was taken within 5 min. of the measuring cell being placed in the water bath during which period the readings fell very rapidly.

(e) The chemical reactions of some commercial samples of sterilized milk. Bottles of commercially sterilized milk were obtained from two sources at weekly intervals over a period of 6 months. The batch process of sterilization was used at plant A. The combination of an ultra-high temperature-short time heating unit and a continuous system of sterilization gave three products at plant B, i.e. normal sterilization (S), ultra-high temperature-short time treatment (UHT), and UHT followed by normal sterilization (UHT-S).

The average values for acidity, reducing powers and colour of the milk from the four processes are given in Table 7. As would be expected, the values obtained on milk from plant B show that the colour of the milk, acidity and reducing powers rose as the

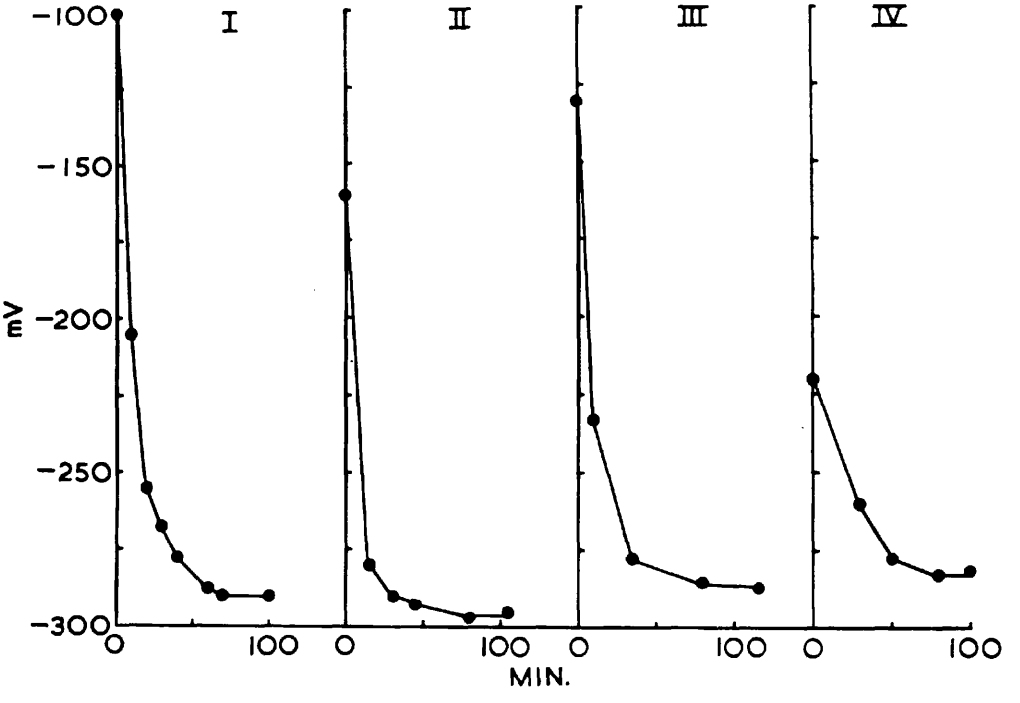


Fig. 12. Potential-time curves for milk from farm 2 sterilized in "evacuated" bottles for 15 min. at different temperatures. I at 104.5°, II at 110°, III at 114°, IV at 117.5°. Measurements made under nitrogen at 50°C.

Table 7. Average results of chemical

in milk by commercial

Test	Plant A	
Colour Red units	0.45	(
Yellow units	0.65	(
Yellowing of turbidity test filtrate (Spekker absorptiometer drum readings)	0.087	
pH	6.64	(
Titratable acidity (ml. N/9 NaOH/10 ml. milk)	1.78	1
Eh (mV)	-238	-
Acid-ferricyanide-reducing substances (Spekker absorptiometer drum readings)	0.235	(
No. bottles	22	

degree of heat treatment was increased. Contrary to the results obtained with the milk sterilized in the laboratory (p.29), the Eh became progressively though slightly more positive with increasing heat treatment. The heat treatment used in the batch process (plant A) may be considered to have been more severe than the UHT-S treatment judging by the colour of the turbidity filtrate, titratable acidity and acid-ferricyanide-reducing powers, but less severe if pH, Eh and the colour of the milk are considered.

The Eh measurements made on commercially sterilized milk showed good agreement between duplicate bottles but differences in the gradient of the potential-time curve were taken to indicate variations in the processing (Fig. 13). The results shown in Fig. 13 were obtained on duplicate samples of two different batch processed milks from plant A.

Differences in the gradient of the curves obtained from milk treated by the three methods used at plant B, showed that the poising capacity of the milk decreased as the degree of heat treatment increased (Fig. 14). Milk which had received the UHT treatment had the most negative potential and the best poising capacity, giving results similar to those obtained on milk heated in "evacuated" bottles under laboratory conditions. When the UHT treatment was followed by normal sterilization, there was a considerable reduction in the poising capacity of the milk. Measurements

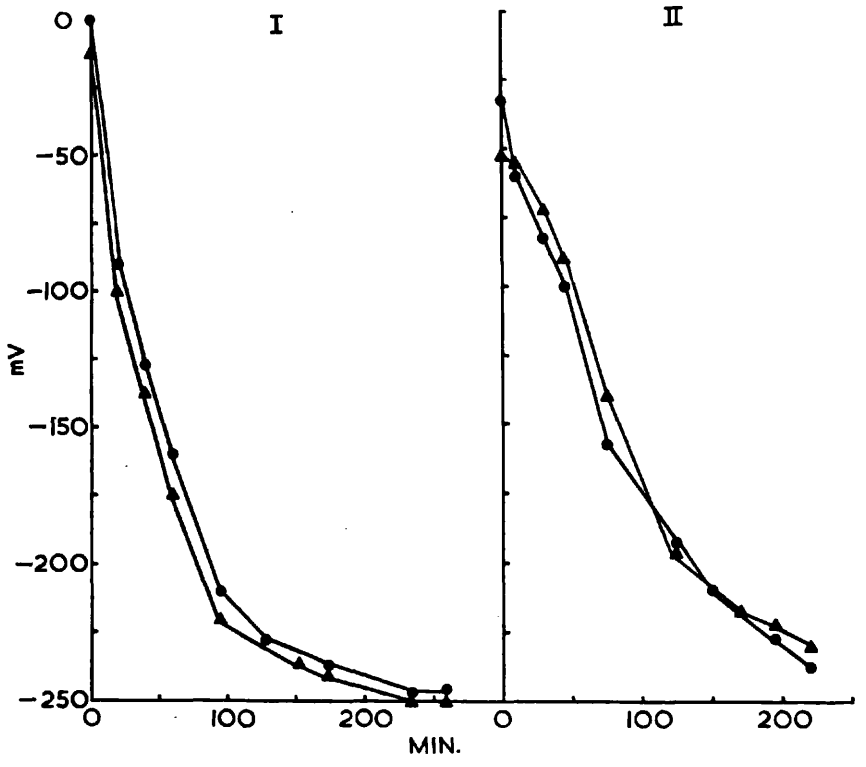


Fig. 13. Potential-time curves for duplicate samples of two milks sterilized commercially at plant A. I milk processed on 2/12, II milk processed on 9/12. Measurements made under nitrogen at 50°C.

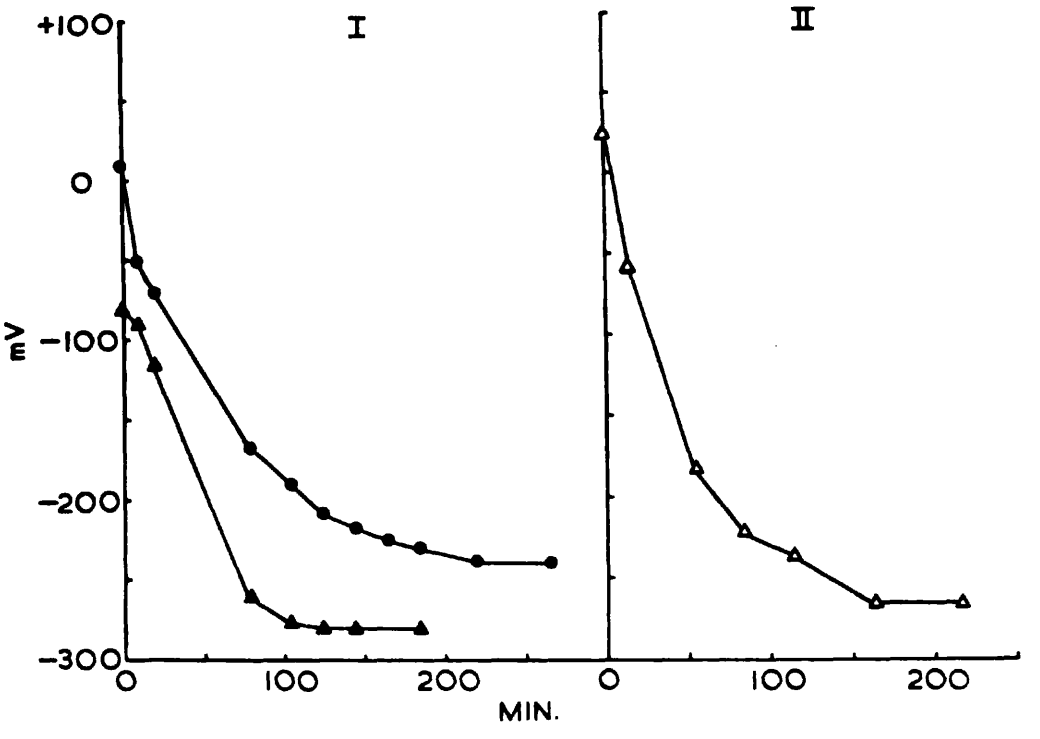


Fig. 14. Potential-time curves for UHT, S and UHT + S milk from plant B measured under nitrogen at 50°C. I UHT (▲) and UHT + S (●), II S (△).

made under nitrogen on UHT and UHT-S milk which had been processed on the same day gave curves (Fig. 14, I) which were very similar to the curves obtained when milk sterilized in the laboratory in "open" and "evacuated" bottles was examined under nitrogen (Fig. 10, II).

The difference in buffering capacity between UHT treated and milk sterilized at plant A is shown in Fig. 15. The flow of nitrogen through the measuring cell was stopped after the establishment of a constant value. UHT treated milk showed a resistance to atmospheric oxidation similar to that obtained in milk heated in "evacuated" bottles (Fig. 10, II) whereas there was an immediate rise in the potential of the batch sterilized milk when air was admitted to the measuring cell (Fig. 15, II).

With the exception of UHT milk and excluding Eh from consideration, these reactions show changes equivalent to those obtained with milk sterilized under laboratory conditions at 104.5-110°C for 15 min. A heat treatment falling within this range (107.5°C for 15 min.) was used to investigate the effect of heat-induced changes in milk on the growth of bacilli (p.70).

3. Discussion

The changes in acidity, acid-ferricyanide-reducing powers and the colour of the milk due to the heat treatments were followed in parallel to give a

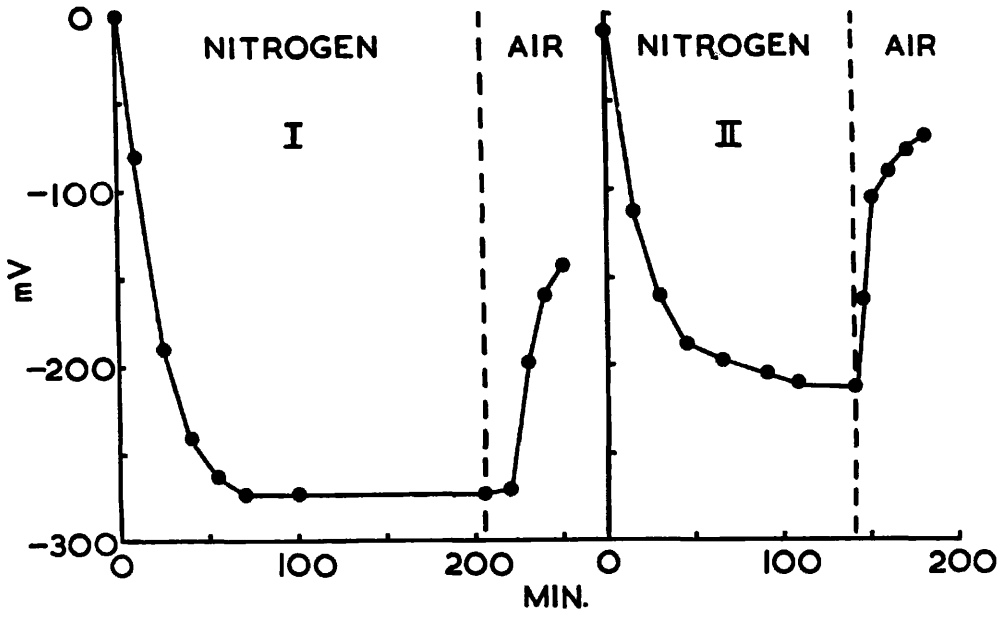


Fig. 15. Potential-time curve for UHT milk from plant B and batch sterilized milk from plant A measured at 50°C under nitrogen and then under air. I UHT milk from plant B, II milk from plant A.

comparison of the changes caused by the different heat treatments and to associate these changes with the effect of the heat treatment of the milk on the bacterial growth (see next section). The results agreed in general with the results obtained by other workers who had studied these changes individually. That is to say, the yellowing of the turbidity filtrate, the acid-ferricyanide-reducing powers, the titratable acidity and the brown discolouration of the milk increased and there was a fall in pH as the degree of heat treatment was raised. The changes were more marked in milk which had been heated in "evacuated" bottles. This was attributed to the different rates of cooling between the "open" and "evacuated" bottles. However, deaeration followed by heating milk under vacuum did result in a constant increase in acid ferricyanide reducing powers compared with milk which had been heated in an "open" bottle.

While the chemical tests under consideration were useful for comparing treatments, they did not all appear to agree when comparison was made between different processes. The results obtained with the three types of milk from plant B showed the chemical changes expected as the severity of the heat treatment was increased. When milk processed at plant A was compared with the milk from plant B, titratable acidity, yellowing of the turbidity filtrate and the acid-ferricyanide-reducing substances suggested that the

treatment used at A was more severe than UHT-S while Eh, pH and the brown discolouration of the milk indicated the reverse.

The method of measuring the Eh of milk by dye reduction used originally in the present work, gave some indication of values lower than those reported by Wilson (57), but, since milk is not itself strongly poised, the poising action of the dye gave erroneous results. The electrometrical method gave very much lower results than those reported by Tobler (51) who originally described the method. This is of little importance in itself since different procedures were used for heating the milk. Furthermore, it is probable that by suitable adjustments in the processing technique and by the use of pure nitrogen during measurements, still lower values could be obtained for the Eh of heated milk than were obtained in the present study. Tobler has also concluded that there is a fall in the Eh of milk as the temperature of heating is raised but that the length of time for which heating is continued, at any temperature, does not affect the level of Eh. Although only one time of heating was used in this work, the Eh of deaerated milk as determined appeared to remain constant at -290 mV over a range of 80°-117.5°C.

The value of 290mV obtained for deaerated milk heated under vacuum indicates that anaerobic conditions have been established although it is more

positive than the potential of raw milk measured under nitrogen. Harland, Coulter & Jenness (27) have attributed the Eh of heated milk mainly to the ratio of ascorbic to dehydro-ascorbic acid. The rate of the heat degradation of ascorbic acid is known to be reduced when heating is carried out in the absence of oxygen. Townley & Gould (62) showed that only one third as much volatile sulphide was formed from milk heated in sealed cans as when the milk was heated in open vessels or when there was a leakage of pressure in the cans. Thus heating of the milk under vacuum has a protective action also for thiol groups. These results could be used to explain the better poising capacity of milk heated under vacuum.

Only the oxidation-reduction potential was found to remain constant with heat treatment and yet show a difference between the "open" and "evacuated" treatment thus corresponding with the inhibitory action of the milk towards the growth of small numbers of bacilli. Milk in common with all biological fluids, has a poor capacity for poising the oxidation-reduction potential. Thus the shape of the curve and the time required by the electrodes to attain an equilibrium Eh value may be used to obtain information of poising capacity of the milk. When measurements were made under nitrogen, a sigmoid curve was shown by milk (e.g. Fig. 8), whether raw or heated, which had been in recent contact with the air. If time was allowed for

the oxygen to be taken up by the milk before measurement, the initial plateau was eliminated from the curve. The length of time required for the electrodes to give a constant reading was inversely proportional to the poising capacity of the milk. All milks did not show an equal resistance to atmospheric oxidation. It will be shown in the following section that some milks are capable of inhibiting the growth of small numbers of bacilli. A greater ability to inhibit bacterial growth was associated with those milks which showed a greater ability to resist atmospheric oxidation.

4. Summary

a) The changes in acidity, reducing powers and colour of the milk due to heat treatment within the range 104-117°C for 15 min. have been studied. The titratable acidity, acid-ferricyanide-reducing powers, the yellowing of the Aschaffenburg turbidity test filtrate and the brown discolouration of the milk increased and there was a fall in pH as the degree of the heat treatment was raised.

b) The changes caused by the heat treatment were more marked in milk heated in bottles with an airtight seal and a vacuum in the headspace than when the milk was heated in an open bottle. This was considered to be due to differences in the degree of heat treatment due to the slower rate of cooling of the milk in the

sealed bottles.

c) The oxidation-reduction potential of the milk was studied by an electrometric method. Wide differences in Eh values were obtained by variations in the treatment of the milk before and after heating. Milks heated in closed bottles with a partial vacuum in the headspace gave a much more negative oxidation-reduction potential than those heated in open bottles. There were differences in the poising ability of different milks.

SECTION IV

INVESTIGATIONS INTO THE GROWTH RATE OF SOME MESOPHILIC SPORE-FORMING BACILLI IN PASTEURIZED AND STERILIZED MILK

1. Introduction

Although as explained on p. 30 the milks in "open" and "evacuated" bottles were given the same apparent heat treatment, there was a difference in the time-temperature conditions in the two treatments due to the slower cooling of the "evacuated" bottles which resulted in these bottles having a slightly longer heat treatment. An attempt was made to study the possible effect of these changes and the effect of deaeration of the milk on the growth of organisms surviving "sterilization".

Bulk milk from Ayrshire farms was tested for bacterial spores capable of resisting heating at 80°C for 10 min. From thirty-six samples only six were found to contain resistant spores. Milk from three of these sources was investigated during the months of July to September 1953. The milk was homogenized and filled into bottles which were either left "open" or crown-capped using a hand capper kindly lent by the Crown Cork Company Ltd. After heating at 100°C for 30 min. or at 108.5°C for 15 min. (5 lb./sq. in. steam pressure), the bottles were incubated at 22°, 37° or 56°C and inspected daily for visual spoilage. The counts at 37°C on the homogenized milk ranged from 10 to 150 organisms/ml. However, the flora which

survived heating was so heterogeneous that although the results of the chemical investigations showed differences between the milks, no conclusions could be drawn as to the effect of these changes on the bacterial growth.

Since the natural microflora of raw milk cannot be controlled, the main experimental work was carried out with pure cultures of mesophilic spore-forming bacilli. For these experiments the use of the crown-cap was discontinued in favour of a perforated screw cap which was more suitable for controlling the degree of vacuum in the headspace and permitted samples to be taken during incubation without removing the cap. Details of the apparatus used are given on pp. 10, 16.

2. The selection and use of test species

Four main factors were considered in the selection of the species used for this work. These factors were as follows.

i) The species should have been isolated from "sterilized" milk or there should be a report of such an isolation by other workers.

ii) There should be variation between species in nutritional requirements. Since it has been shown that the requirements of the genus Bacillus are relatively simple (63, 64), the ability to utilize NH_4^+ as sole nitrogen source has been taken as the criterion of synthetic ability. Ammonia is formed when milk is heated (8) therefore an organism capable

of utilizing NH_4^+ should not be inhibited through the destruction of essential amino acids.

iii) There should be variation between species in oxygen requirements. The limited oxygen supply in the bottles should favour the growth of facultatively anaerobic species.

iv) There should be variation between species in optimum growth temperature. Previous investigations into the spoilage of sterilized milk in the laboratory have been made using incubation temperatures ranging between 24° and 37°C (43, 46, 65, 66), so it was of interest to investigate how far growth was affected by the temperature of incubation in relation to the optimal growth temperature.

The oxygen requirement, ability to use NH_4^+ and the optimum growth temperature of the organisms that were selected for use are given in Table 8.

The two strains of B. subtilis, B. licheniformis 8 and B. cereus 201 give typical biochemical reactions according to the classification of Smith, Gordon & Clark (52). B. subtilis D1/2M showed dormancy in nutrient and 1% starch agar whereas strain 6 did not. On primary isolation, B. cereus 201 was capable of hydrolysing starch weakly but has since lost this ability, B. brevis 58 was most closely related to the type species of B. brevis but produced acetyl methyl carbinol. B. circulans 152 produced acetyl methyl carbinol at 45° but not at 30°C. This organism also

Table 8. The oxygen requirement, abil
NH_4^+ and optimum growth tem
the test species of B

Organism	Utilization of NH_4^+	Oxygen requirem
B. subtilis 6	+	Aerobe
" D1/2M	+	Aerobe
B. licheniformis 8	+	Facultat anaerob
B. cereus 201	-	Facultat anaerob
B. brevis 58	-	Aerobe
B. circulans 152	-	Facultat anaerob
" 154	+	Facultat anaerob

formed spores at the higher but not at the lower temperature.

The survival rate of bacterial spores in "sterilized" milk has been reported to be of the order of 1 spore/100 ml. milk (46). Accordingly, inocula of this order were used as far as possible. At the beginning of every experiment to ensure that each bottle received an inoculum of viable spores, plates were inoculated at the same time as the bottles. Typical figures for the plate counts are given in Table 9. They show that this method of inoculation was suitable for all the cultures. There was some variation between plates but no obvious trend during the process of inoculation. It is very unlikely that any bottle failed to receive one or more viable spores.

3. <u>The effect of the removal of air from the headspace before sterilization on growth at 37°C in the presence or absence of oxygen</u>

a) <u>Adjustment to the headspace</u>. The presence or absence of a vacuum in the headspace of a bottle before and after heat treatment presents four possible combinations of environmental conditions which might result under commercial conditions. These conditions were simulated by sterilizing the milk in screw cap bottles with rubber diaphragms in the caps. The vacuum was obtained before heating by evacuating with a hypodermic needle through the rubber diaphragm of the

Table 9. Colony counts on plates
of delivery from a h

Culture number		Plate count/ml. deliv				
		1	2	3	4	5
6	i	6	6	5	4	1
	ii	9	6	7	2	5
8	i	10	6	4	8	9
	ii	6	6	6	7	8
D1/2M	i	5	3	3	7	2
	ii	5	6	5	8	6
58	i	7	5	9	12	5
	ii	10	4	9	7	7
201	i	6	6	10	2	6
	ii	3	5	4	2	7
154	i	7	14	8	10	11
	ii	13	9	11	12	6

cap. After sterilization the headspace was filled either with commercial nitrogen (A) which contained not more than 0.5% oxygen or with air (B). Bottles which had been sterilized when open to the atmosphere were either filled with commercial nitrogen after removal of the air (C) or closed with air in the headspace (D). These treatments represent:-

A. Milk which was sterilized and held under partial vacuum, i.e. the bottle remained correctly sealed during and after sterilization. In practice, it was necessary to replace the vacuum with an inert gas to permit inoculation and sampling.

B. Milk which was sterilized in an efficiently sealed bottle but in which the seal became ineffective after sterilization with the subsequent entrance of air into the headspace.

C. Milk which was sterilized in bottles capped without establishing a vacuum but in which the seal later became closed.

D. Milk sterilized and held in the presence of air.

Pasteurized milk (E) was used to give a comparative estimate of the relative inhibitory effects of heat treatment. Uninoculated controls for the pasteurized milk and for the milk sterilized in open bottles were included where the corresponding inoculated milks were used. Experiments in which the controls showed contamination are not reported.

For simplicity the treatments will be referred to as A, B, C, D and E. A folded sheet has been included for reference at the end of the thesis (p.103).

b) Effect of the composition of the headspace on growth rate. The first series of experiments were made using single cow milk samples and the four species of bacteria B. subtilis 6, B. licheniformis 8, B. cereus 201 and B. brevis 58. It will be seen from Table 8 that B. subtilis 6 and B. brevis 58 were aerobes and B. licheniformis 8 and B. cereus 201 were facultative anaerobes. B. subtilis 6 and B. licheniformis 8 were capable of utilizing ammonium salts. In these experiments single bottles for each treatment, containing about 440 ml. milk (16 oz. bottles), were inoculated at a level of 10^{-2} spores/ml. milk and incubated at 37°C.

The growth rate of B. subtilis 6 in two experiments is illustrated in Fig. 16. No marked difference between heat treatments was shown in the first experiment (Fig. 16 I). However, there was a tendency for the viable count to fall more rapidly in milks held under nitrogen (A & C) than under air (B & D). A similar result was obtained when the growth rate was re-examined in milk from the same cow (cow 2). The response was more variable in a second experiment (Fig. 16 II) when the growth rate was examined in milk obtained from another cow (cow 1). In this experiment little difference was shown between treatments B, C and

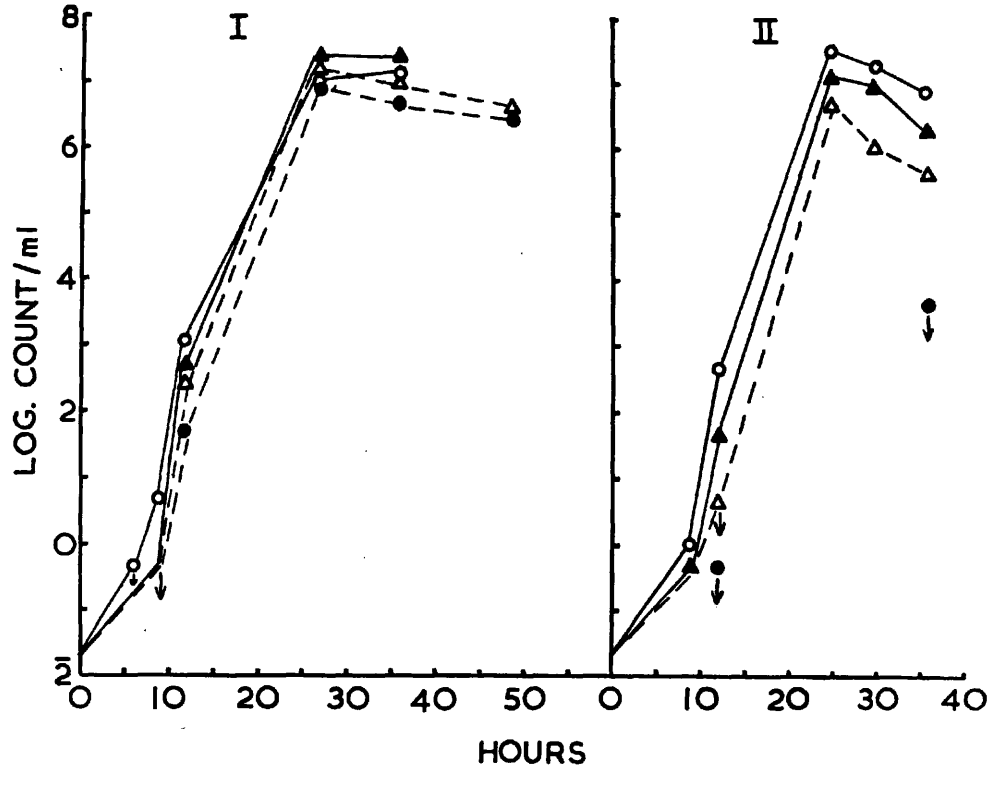


Fig. 16. Growth rate of B. subtilis 6 at 37°C in milk treated by methods A, B, C and D. I milk from cow 2, II milk from cow 1. Treatments A ●---●, B ▲——▲, C Δ---Δ, D O——O. ↓ count below this value.

D but growth was less than log $\bar{1}.70$ and log 3.70/ml. after incubation for 12 and 36 hr. respectively in milk receiving treatment A. The level of inoculum was very similar in the two experiments and therefore could not in itself account for the difference in the growth rate in treatment A. Furthermore it should be noted that although the differences between treatments were not great in the first experiment, growth was initiated most readily in milk D and least readily in milk A. In other words, growth was detected first in milk which had been heated in an "open" bottle and incubated with air in the headspace, and it was detected last in milk which had been heated in an "evacuated" bottle and incubated with nitrogen in the headspace. Irrespective of treatment the rate of growth and the maximum population were similar in all bottles, any differential effect occurring during the lag phase.

B. licheniformis 8 gave growth curves similar to those obtained in the first experiment with B. subtilis 6 using the same level of inoculum (Fig. 17). Once again, the growth in milk A occurred least readily. Growth occurred most readily in milk D in the second experiment but approximated to that in A in the first experiment. No explanation can be given for the slow rate of growth in D compared with that in C in this experiment. This slow rate of growth was not shown in later work with this organism. The sources of milk

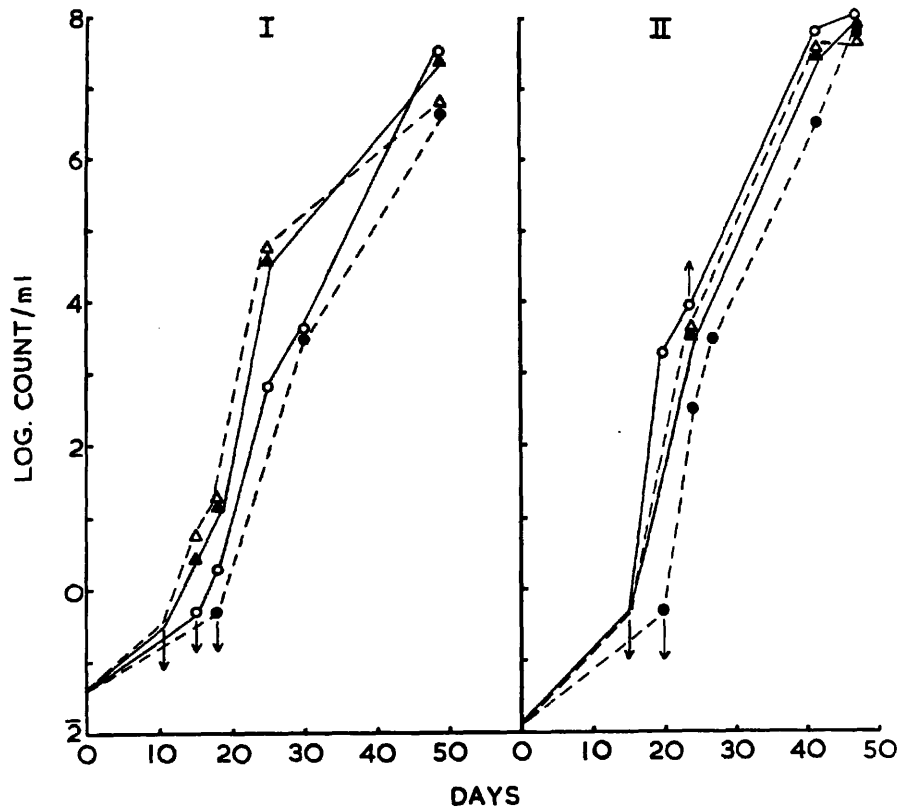


Fig. 17. Growth rate of B. licheniformis 8 at 37°C in milk treated by methods A, B, C and D. I milk from cow 3, II milk from cow 4. Treatments A ●---●, B ▲——▲, C Δ---Δ, D O——O. ↑↓ count above or below this value.

for these two experiments were the cows 3 and 4.

Milk from cow 1 was used to examine the growth rate of B. cereus 201 (Fig. 18). This organism showed marked variation in growth rate according to the treatment of the milk. In the first experiment the growth in milk A was delayed for about 10 hr. compared with the growth in milk given treatments C, D and E. Treatment B was not included in this experiment. Once again, the rate of growth and the maximum population were similar in milk given treatments C, D and E. The variation in the length of the lag phase between treatments was even more marked in the second experiment. Here, the growth in both treatments A and B was retarded for nearly 20 hr. compared with the growth in treatments C, D and E.

The rate of growth of B. brevis 58 was very much slower than that of the three strains already mentioned using the same level of inoculum. Using higher inocula, 10 and 100 spores/ml. milk respectively in two experiments, the results shown in Fig. 19 were obtained. The growth curves were similar to those obtained with B. cereus 201 in that there was some evidence of delayed growth occurring in milks A and B. The inhibition was somewhat less in the second experiment than in the first and it is possible that this was due to the use of the higher inocula. The milk used was from cow 5.

c) Spore-formation in sterilized milk. It is

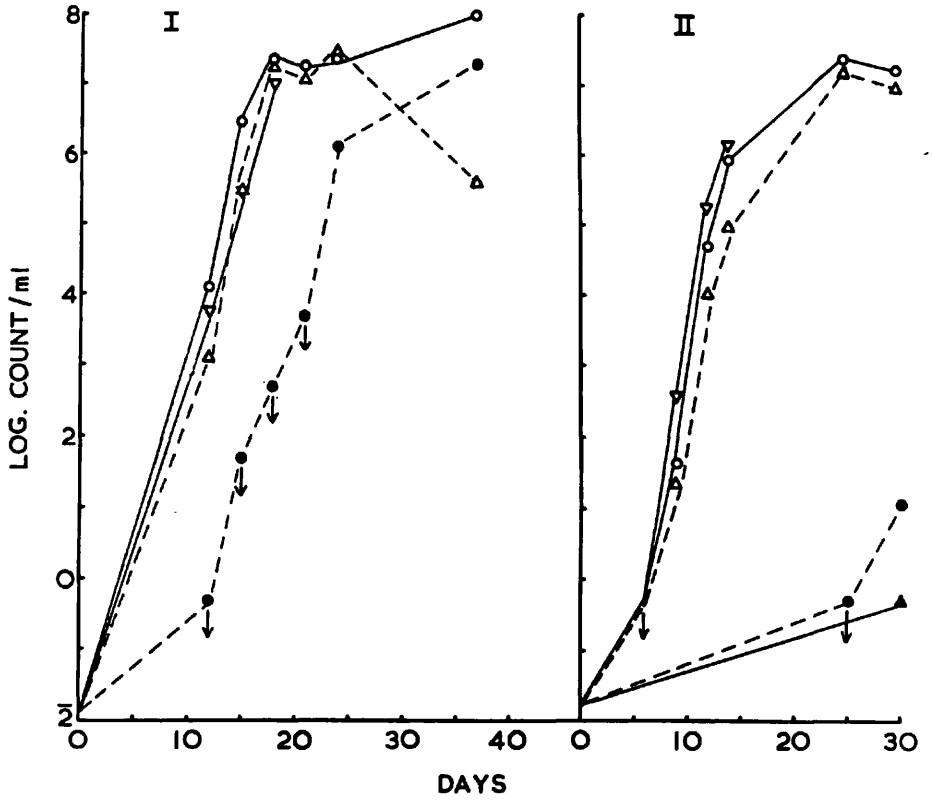


Fig. 18. Growth rate of B. cereus 201 at 37°C in milk treated by methods A, B, C, D and E. Milk used in I and II from cow 1. Treatments A ●---●, B▲——▲, C △---△, D O——O, E ▽——▽. ↓ count below this value.

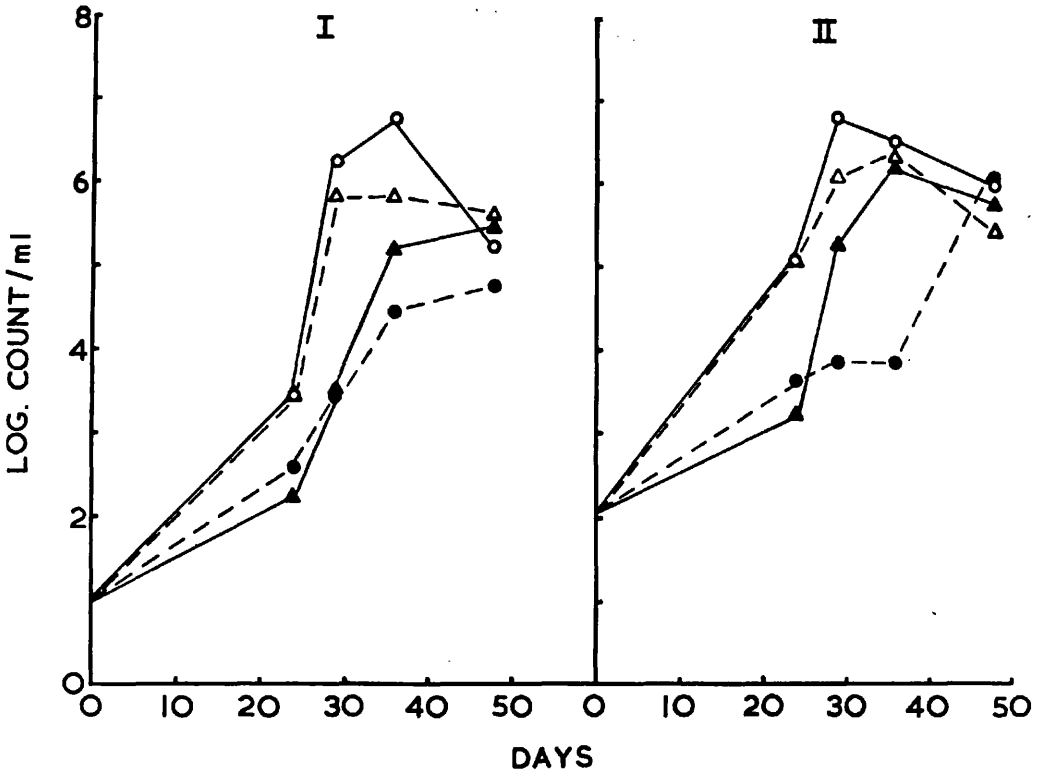


Fig. 19. Growth rate of B. brevis 58 at 37°C in milk treated by methods A, B, C and D. Milk used in I and II from cow 5. Treatments A ●---●, B ▲——▲, C △--△, D ○——○.

generally accepted that spore formation is even more sensitive to external conditions than is spore germination or vegetative growth (67). Therefore, environmental factors which are insufficient to affect germination may still be detected by their influence on spore formation. Spore formation in milk has been reported by Grinsted & Clegg (42) who followed the heat resistance of a high inoculum of spores in autoclaved litmus milk. They found that spore formation was not extensive enough to be detectable in a smear.

During the initial examination of the growth rates from very small inocula some information was obtained on the spore formation of B. subtilis 6, B. licheniformis 8, B. cereus 201 and B. brevis 58 (Table 10). These four organisms showed similar behaviour in that with each strain spore formation was greatest in milk D and least in milk A. The results of the first two experiments with B. subtilis 6 and the experiment with B. licheniformis 8 are of particular interest. No difference was observed in the growth rates of any of the milks A, B, C or D, yet the spore formation followed the same general pattern of inhibition shown by all the organisms studied, ie. spores were formed most readily in milk given treatment D and least readily in milk given treatment A.

Spore formation in this genus has been considered to be an aerobic process (68). Comparison

Table 10. Spore formation of B. subtilis 6, B. licheniformi
B. cereus 201 and B. brevis 58 in sterilized mi
treated by methods A, B, C and D

Culture	Expt.	Incubation (hr.)	Spore count/ml. of milk			
			A	B	C	
6	a	18	<0.5	<0.5	<0.5	
		24	15	3	300	
		37	>1,000	>1,000	>1,000	>1,
	b	12	<0.5	<0.5	<0.5	
		27	4	352	138	
		36	344	>1,000	500 ap.	>1,
		49	>1,000		>1,000	
	c	12	<0.5	<0.5	<0.5	
		25		49	2	>10,
		27		420	47	
		30		640	117	
		33		760	500	
		36	<0.5	1,130	860	
8	a	30	<0.5	<0.5	<0.5	
		42	<0.5	<0.5	0.5	
		48	22	9	110	
201	a	14	<0.5	<0.5	<0.5	
		25			7	
		30	<0.5	<0.5	28	>1,
	b	14	<0.5	0.5	<0.5	
		26	1	9	2,700	
		30	81,000	3,800	88,000	
58	a	0	11	13	11.5	
		24	9.5	3.5	3.5	
		29	4.5	6	3.5	
		36	8	8	3	
		48	15.5	15	500 ap.	>1,
	b	0	127.5	133.5	126	
		24	55	59	37	
		29	62.5	54	34	
		36	60	41	43.5	
		48	74	383	1,000 ap.	>1,

of the two pairs of milks B and D incubated under air and A and C incubated under nitrogen showed that Eh is also of importance. Spore formation was greater in the milks of higher Eh (C and D) than in the milks of lower Eh (A and B) irrespective of the gas in the headspace. It must be presumed that the variation in the ability of B. subtilis 6 to form spores in milks B and C in the second two experiments (b and c) depended on the poising capacity of the particular milk.

These results agree in general with the statement by Grinsted & Clegg (42) that spore formation is not usually extensive in sterilized milk. However, spore formation was detected in smears in other experiments in the present investigation.

d) Discussion. These experiments were made to determine whether the conditions which could arise in commercial sterilized milk would influence the growth of mesophilic spore-formers. From the results obtained it seemed evident that a degree of inhibition may occur under certain conditions in the milk from at least some cows. The inhibition which takes the form of a prolongation of the lag phase, was associated with treatment A and to a less extent with treatment B. That is to say, the inhibition was associated with "evacuated" bottles in which the milk was deaerated before heat treatment. Thus without any further consideration of chemical change it was possible to exclude organic peroxides as a source of inhibition

since it is not likely that peroxides would be formed under these conditions.

Since this work was done on milk which had received only one level of heat treatment (115.5°C for 15 min.), it was possible that the inhibition might be associated with the more extensive chemical changes produced in the milk in "evacuated" bottles. If this was correct, milks which had received a lower level of heat treatment would be expected to be less inhibitory and it should be possible to demonstrate inhibition in "open" bottles which had received a higher level of heat treatment.

The two organisms which have shown the most marked inhibition are B. cereus 201 and B. brevis 58. While the former organism is a facultative anaerobe and the latter an aerobe, neither is capable of utilizing NH_4^+ as sole nitrogen source. B. subtilis 6 which showed more inhibition in one experiment is able to utilize NH_4^+ but is an aerobe. From these results it would seem as if inhibition was more likely in those organisms which were unable to utilize NH_4^+ but that aerobic species which possessed this ability might also show some inhibition. It should be noted that although milk is described as being incubated under air, in fact the supply of oxygen was limited through the use of an airtight seal. Furthermore there would be a decrease in the concentration of oxygen during incubation owing to the uptake of oxygen by the

reducing groups of the milk.

A further point which emerges from these results is shown in the experiments with B. brevis 58 (Fig. 18 & p.54). From this work it appears that inhibition may be related to the size of the inoculum. This is of importance since the level of inoculum which it was found necessary to use in these experiments was higher than that reported for commercially sterilized milk.

The cows from which milk was obtained for this work, were all Ayrshire from farm 1 which had been chosen for their freedom from mastitis and their ability to produce not less than 1.5 gal. milk at the evening milking. Some were in their first lactation but others including cows 1 and 2 had had more than one lactation. In the experiments described in this section a prolonging of the lag phase was obtained only in milk from cows 1 and 5 and not from cows 2, 3 and 4. Other work not reported here showed that inhibition was also obtained in milk from the cows 6 and 7. To eliminate any variation in the results attributable to the use of single cow samples of milk, further experiments were made using bulk farm milk.

No difference in growth was obtained between pasteurized milk (E) and milk heated in "open" bottles (treatments C and D) as is shown with B. cereus 201 Fig. 18). Since difficulty was found in obtaining pasteurized milk in a sterile condition the study of growth in pasteurized milk was discontinued.

The results of spore counts made during these experiments showed that spore formation was sensitive to adverse Eh to a greater extent than spore germination. The inhibitory action was similar to that exerted on spore germination and was distinct from the limitation of oxygen.

In conclusion, these experiments suggest that the growth of some mesophilic spore-formers may be delayed in milk which has been deaerated before sterilization and that the inhibitory effect may become more pronounced as the size of the inoculum is decreased.

4. The effect of the size of the inoculum on the growth of spore-forming bacilli in sterilized milk

a) Introduction. Since the results of the experiments with B.brevis 58 suggested that the retardation of growth was influenced by the size of the inoculum, it was of primary importance to determine at what level of inoculum evidence of inhibition could be most conveniently demonstrated for each culture.

Because of the amount of work involved, it was found necessary to examine only single bottles of the two extreme treatments (A and D) at each level of inoculum, to use single plates and a wide range of dilutions for the count. Bulk farm milk from farm 1 and to a less extent from farms 4 and 5 was used. B. subtilis 6 and B. licheniformis 8 which showed

little sensitivity to the treatment of the milk, were inoculated into 40 oz. bottles which contained approximately 1 litre of milk. With the exception of B. cereus 201 the remaining cultures were examined in 8 oz. medical flats containing approximately 220 ml. milk. B. cereus 201 was examined in milk from cow 6, the milk being held in 16 oz. bottles containing approximately 440 ml. milk which had been given treatments B and D. As in the previous experiments, the milk was sterilized at 115.5°C for 15 min. (10 lb. steam pressure) and the incubation was carried out at 37°C until the milk became unstable to 80% alcohol or for 14 days if no growth could be demonstrated.

The growth from each bottle was checked for purity. Three colonies were picked and identified by biochemical tests. Stained smears were examined from milks showing no growth in 1 ml. after incubation for 14 days.

b) Effect of inoculum size. As already shown (p.52) the length of the lag phase of B. subtilis 6 was not affected appreciably by the treatment of the sterilized milk in two out of three experiments when an inoculum of 0.05 spores/ml. milk was incubated at 37°C. When the inoculum was reduced to 0.005 spores/ml. there was marked inhibition of growth in milk receiving treatment A on incubation at 37°C. No organisms were detected in 1 ml. of milk A after incubation for 14 days whereas growth was well established at 15 hr.

(25×10^5/ml.) in milk which had been given treatment D. This experiment was made using about 1 litre of milk in 40 oz. bottles. It might be argued that the larger volume of milk required a longer time for the temperature to rise to 115.5°C and to cool down again, involving a greater degree of heat treatment than was given to the smaller bottles, and that this might be the cause of the inhibition in milk A. However the growth in milk D did not differ from that obtained previously (p.52) and it will be shown later (p.70) that similar results can be observed in milks subjected to a wide range of sterilization temperatures.

The effect of the size of the inoculum on the growth of a second strain of B. subtilis (D1/2M) was similar to that obtained with the first strain. As shown in Fig. 20, growth in milk given treatment A was slower than in milk given treatment D at all levels of inoculum from 0.02 to 3 spores/ml. milk. The effect was most marked with the smallest number of spores (Fig. 20, I), no growth being detected in 1 ml. of milk A during the first 21 hr. while in milk D the count had already risen to 5×10^3/ml. milk. The difference in time taken to cause instability to 80% alcohol in the two milks A and D decreased as the inoculum was increased. This work was done with milk from farm 1. Similar results with inocula of 0.03 and 0.3 spores/ml. were obtained in another experiment using milk from farm 5.

An inoculum of 0.02 spores/ml. showed no delay

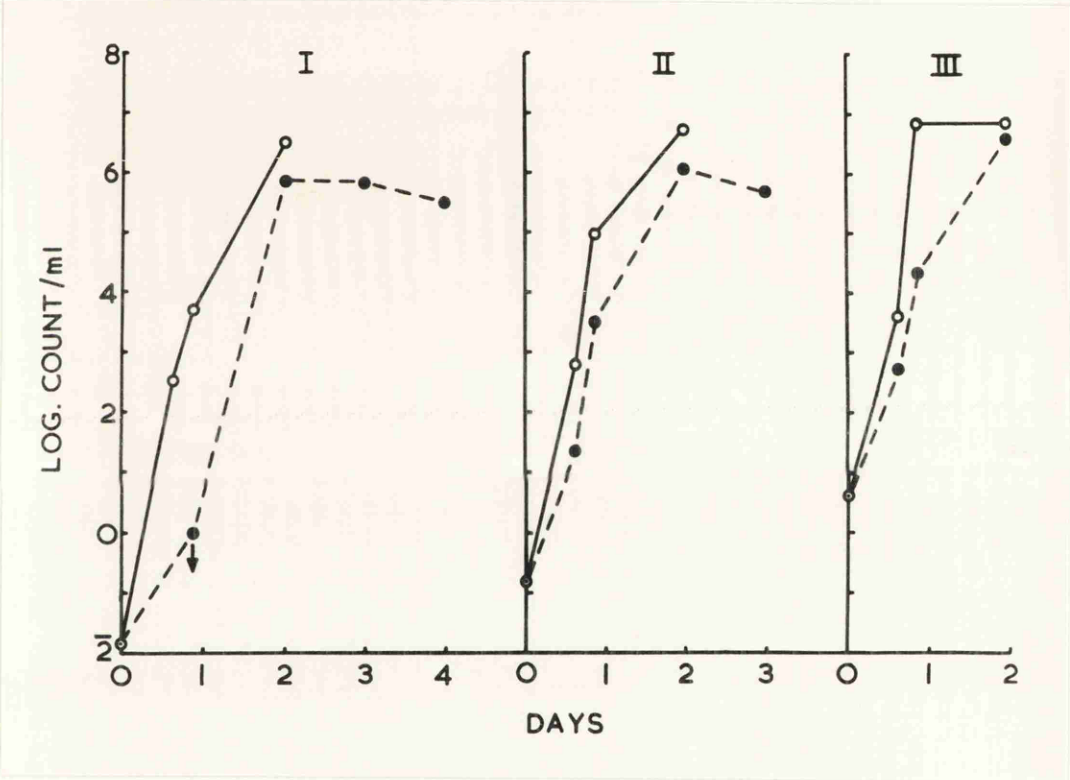


Fig. 20. The effect of the size of the inoculum on the growth of B. subtilis D1/2M at 37°C in milk treated by methods A & D. Inoculum: I 0.02 spores/ml., II 0.2 spores/ml., III 3 spores/ml. Milk from farm 1. Treatments A ●---●, D O——O. ↓ count below this level.

in the growth of B. licheniformis 8 in milk which had received treatment A (p. 53). When the inoculum was reduced to 0.006 spores/ml. no growth of this organism was obtained in milk A after incubation for 15 days whereas growth was rapid in milk D, giving a count of 5×10^7/ml. in 2 days.

As previously stated, the effect of inoculum size on the growth of B. cereus 201 was examined in milk from a single cow sample. The results given in Fig. 21 show that even when the milk had received treatment B instead of A (i.e. when the headspace of an "evacuated" bottle was filled with air instead of nitrogen) there was progressively greater inhibition as the inoculum was decreased. There was no inhibition in milk B when an inoculum of 50 spores/ml. was used whereas the lag phase was increased by 7 hr. at an inoculum of 0.03 spores/ml. milk.

The growth curves shown in Fig. 22 were obtained with B. brevis 58 using three levels of inocula in milks which had received treatments A and D. Once again the lag phase was most pronounced in milk A at the lowest level of inoculum. It should be noted that while the treatment of the milk had no effect on the logarithmic growth of this organism, the maximum population was considerably lower in milk A than in milk D at all levels of inoculum (10^4 as compared with 10^7/ml.). Since the logarithmic phase was not affected, the lower maximum population was established

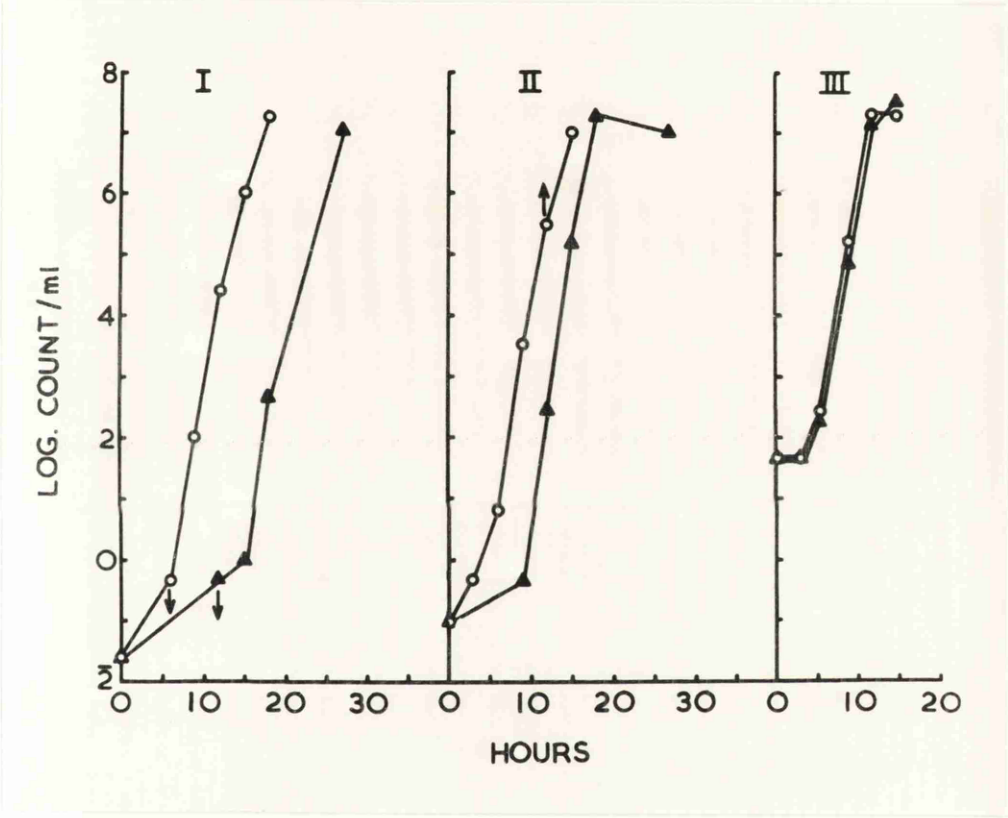


Fig. 21. The effect of the size of inoculum on the growth of B. cereus 201 at 37°C in milk treated by methods B and D. Inoculum. I 0.03 spores/ml., II 0.1 spores/ml., III 50 spores/ml. Milk from cow 6. Treatments B ▲—▲, D O—O. ↑↓ count above or below this level.

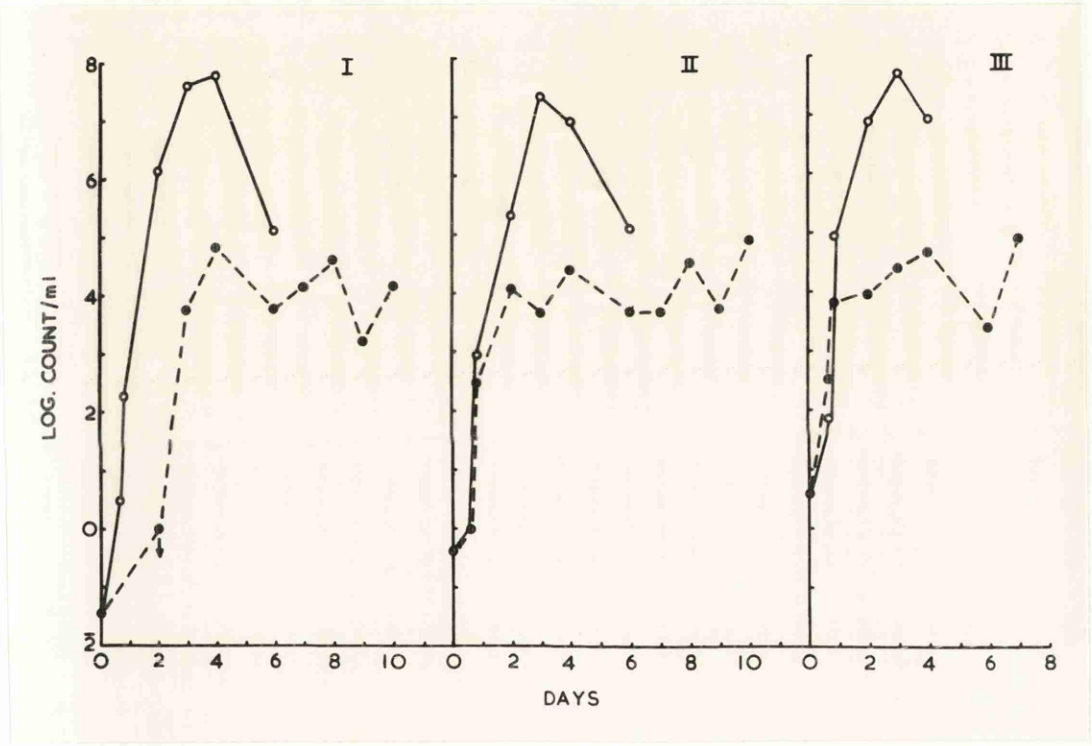


Fig. 22. The effect of the size of inoculum on the growth of B. brevis 58 at 37°C in milk treated by methods A and D. Inoculum: I 0.04 spores/ml., II 0.4 spores/ml., III 4 spores/ml. Milk from farm 1. Treatments A ●---●, D O——O. ↓ count below this level.

sooner in milk A than in milk D except where the lower inoculum delayed the onset of growth. Incubation for 6-8 days was required at the level of 10^4/ml. before the milk became unstable to alcohol. This bacillus required an incubation period of 3-4 days at 37°C to attain a maximum population of 10^7/ml. in milk D. There was no stationary phase in milk D the organism proceeding directly into the decline phase, whereas in milk A there was a prolonged stationary phase.

The behaviour of the two strains of B. circulans (152 & 154) was quite different to that of the species of bacilli already considered in that growth occurred more readily in milk A than in milk D. The growth curves at 37°C for B. circulans 152 using inocula of 0.2, 2 and 44 spores/ml. milk are given in Fig. 23. At the lowest level of inoculum growth was absent in both milks A and D. Growth from an inoculum of 2 spores/ml. was detected in milk A after incubation for 3 days. No growth was detected in milk D having the same inoculum after incubation for 14 days (Fig. 23, I). With an inoculum of 44 spores/ml., growth occurred in both milks but was detected first in milk A (Fig. 23, II). For this experiment milk from farm 1 was used. Very similar results were obtained in milk from farm 5 with inocula of 3, 30 and 300 spores/ml. In both experiments there was a tendency for the viable count to decrease in milk D during the first 48 hr. of incubation. Similar results were obtained with

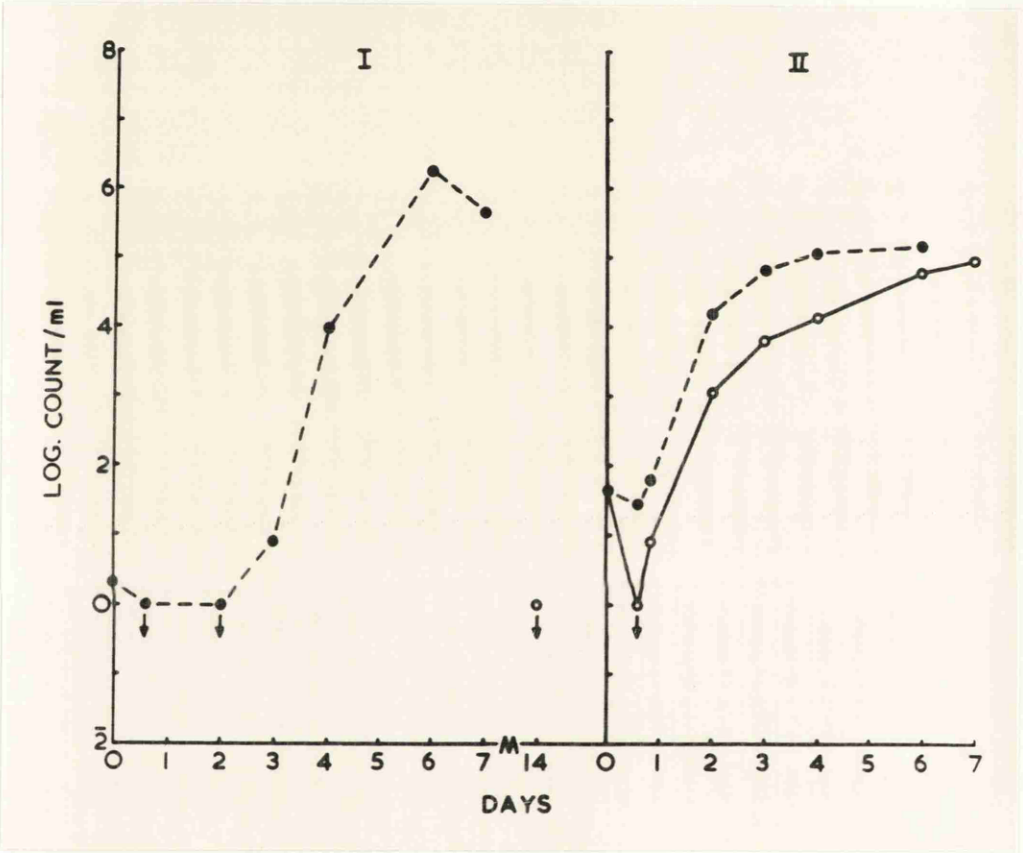


Fig. 23. The effect of the size of inoculum on the growth of B. circulans 152 at 37°C in milk treated by methods A and D. Inocula: I 2 spores/ml., II 44 spores/ml.; 0.2 spores/ml. gave no growth at 14 days in both milks A and D. Milk from farm 1. Treatments A ●---●, D O——O. ↓ count below this level.

B. circulans 154 but it was necessary to use very much lower levels of inocula to demonstrate inhibition. The growth curves obtained using inocula of 0.01, 0.2 and 9 spores/ml. in milk from farm 1 are shown in Fig. 24. At the level of 0.01 spores/ml. milk, growth occurred only in milk A (Fig. 24, I). Growth occurred in both milks A and D at the two higher levels of inocula but in D only after a lag of ½ to 1 day. There was no perceptible delay in the onset of growth in milk A. There was no evidence of a decrease in viable count during the lag phase with this strain of B. circulans.

c) Effect of optimum growth temperature in relation to the size of inoculum. The results discussed so far relate to growth during incubation at 37°C. Three species of bacilli, B. subtilis 6, B. licheniformis 8 and B. circulans 154 were used to examine the effect on rate of growth in milks A and D of lowering the incubation temperature to 30°C. B. subtilis 6 and B. circulans 154 were examined at the same time in milk which was from farm 1. B. licheniformis 8 was tested separately in milk from farm 3. In each case the milk was sterilized in 8 oz. bottles containing approximately 220 ml. milk.

Spores of B. subtilis 6 to give a final concentration of 0.03 and 0.2 spores/ml. were inoculated into duplicate bottles of milk sterilized by treatment A and treatment D. One set of bottles was

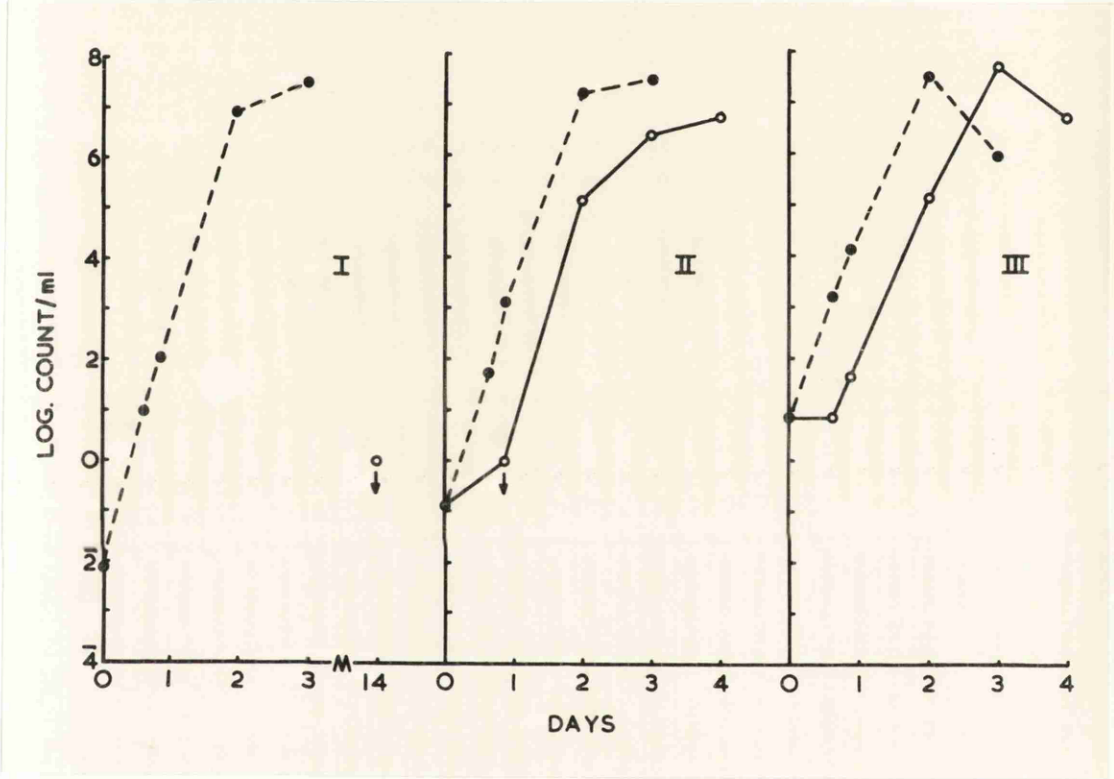


Fig. 24. The effect of the size of inoculum on the growth of B. circulans 154 at 37°C in milk treated by methods A and D. Inoculum: I 0.01 spores/ml., II 0.2 spores/ml., III 9 spores/ml. Milk from farm 1. Treatments: A ●---●, D O——O. ↓ count below this level.

incubated at 37°C and the other at 30°C. The growth curves are shown in Fig. 25. At neither level of inoculum was there any evidence of inhibition of growth at 37°C in milk A other than the rather longer period taken for milk A to become unstable to 80% alcohol. At 30°C where growth was somewhat slower than at 37°C, there was a lag period of about 21 hr. in milk A compared with milk D at the lower level of inoculum. Similarly with B. licheniformis 8 a lag in the onset of growth was shown (Fig. 26) during incubation at 30°C but not during incubation at 37°C using an inoculum of 0.025 spores/ml. The optimum growth temperature was 45°C for both B. subtilis 6 and B. licheniformis 8.

With B. circulans 154 inoculated at the same level into milk of the same batch as was used for B. subtilis 6 (Fig. 27) the inhibition of growth in milk D was complete at 37°C but was reduced to about 21 hr. at 30°C which was the optimum growth temperature for this strain.

These results suggest that any inhibition of growth under unfavourable conditions (milk A for B. subtilis 6 and B. licheniformis 8 and milk D for B. circulans 154) would be least at the optimum growth temperature for the particular strain and would become more marked as the incubation temperature departed from this value. Further evidence of this is given in a later section (p.73) in which growth is compared on incubation at 37° and 22°C.

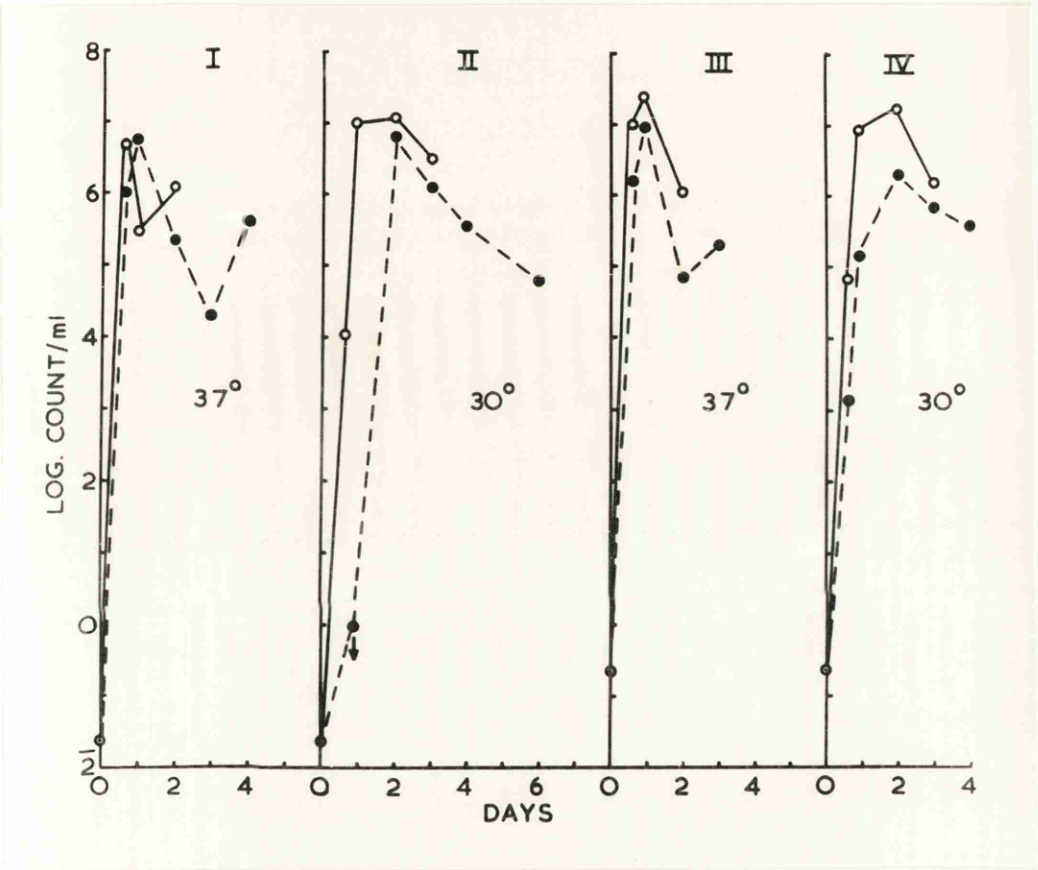


Fig. 25. The effect of incubation temperature and inoculum size on the growth of B. subtilis 6 in milk treated by methods A and D. Incubation temperature: I & III 37°C, II & IV 30°C. Inoculum: I & II 0.03 spores/ml., III & IV 0.2 spores/ml. Milk from farm 1. Treatments A ●---●, D O——O. ↓ count below this value.

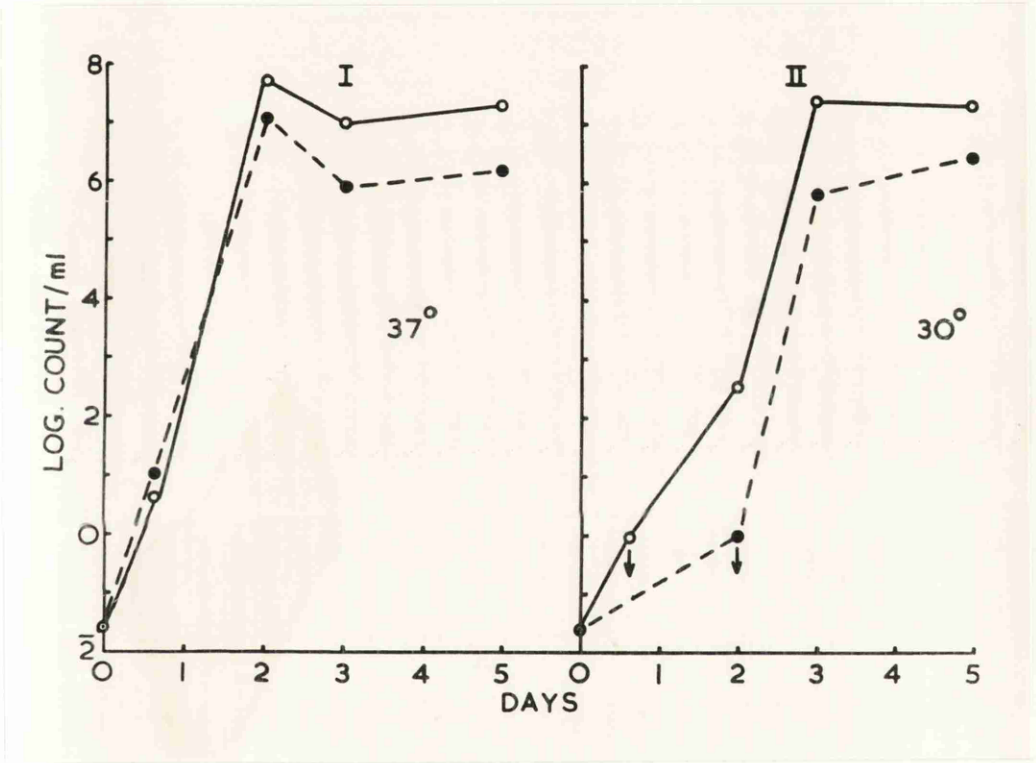


Fig. 26. The effect of incubation temperature on the growth of B. licheniformis 8 in milk treated by methods A and D. Incubation temperature: I 37°C, II 30°C. Milk from farm 3. Treatments A ●---●, D O——O. ↓ count below this level.

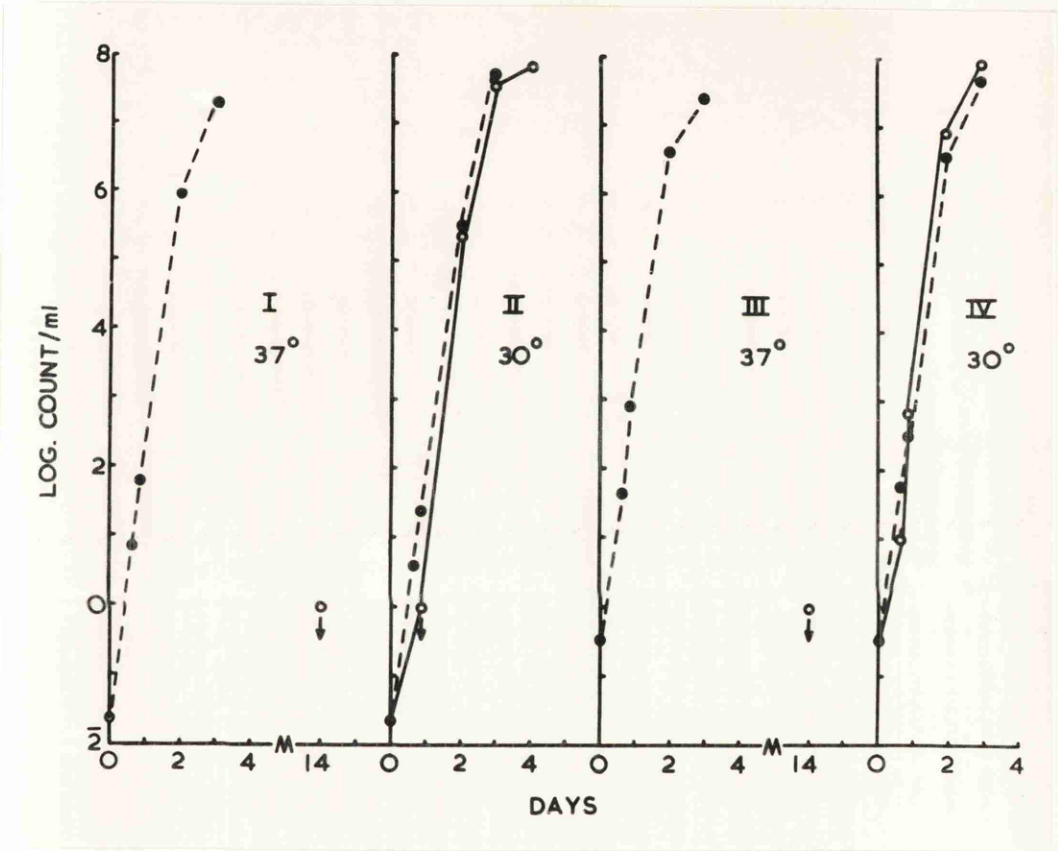


Fig. 27. The effect of incubation temperature and inoculum size on the growth of B. circulans 154 in milk treated by methods A and D. Incubation temperature: I & III 37°C, II & IV 30°C. Inoculum: I & II 0.02 spores/ml., III & IV 0.3 spores/ml. Milk from farm 1. Treatments: A ●---●, D O——O. ↓ count below this level.

d) Spore germination. The size of inoculum required to show an inhibitory effect varied from about 0.005 spores/ml. for B. subtilis 6 and B. licheniformis 8 to about 50 spores/ml. for B. circulans 152. This suggested that there might be species differences in the ability of the spores to germinate. The small inocula which were used to demonstrate inhibitory effects were unsuitable for the measurement of spore germination. For this purpose it was necessary to use larger numbers of spores which would not normally show any differences in growth rate associated with treatments A and D of the milk. It was most fortunate therefore that the particular batch of milk used (from farm 3) in these experiments was the only milk tested which showed particularly marked inhibitory properties after treatment A. Even with the large inocula used (about 5,000 spores/ml. milk) differences in growth rate were shown between milks given treatments A and D.

All the test cultures, with the exception of B. subtilis D1/2M, were studied in this batch of milk but only representative results i.e. those obtained with B. subtilis 6, B. brevis 58 and B. circulans 154, are presented (Figs. 28, 29, 30).

With B. subtilis 6 (Fig. 28) over 90% of the spores had germinated (as determined by loss of heat resistance) after incubation for 9 hr. at 37°C in both milks A and D. While the germination rate was only

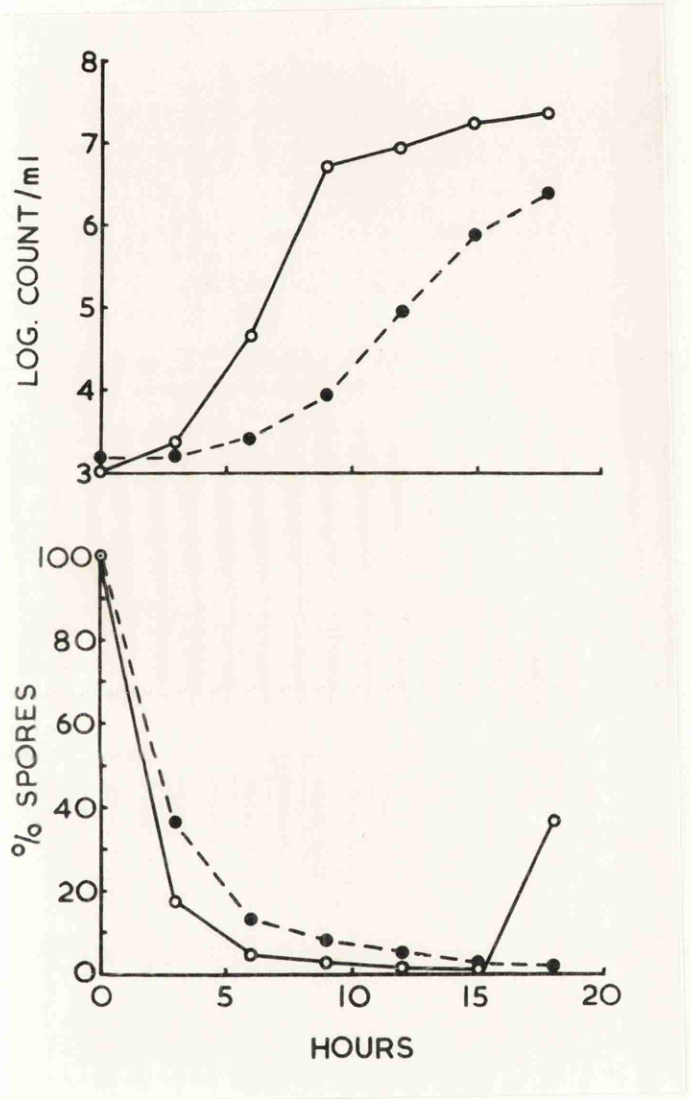


Fig. 28. The spore germination and growth rate of B. subtilis 6 at 37°C in milk treated by methods A and D. Treatments: A ●---●, D O——O.

slightly slower in A than in D there was a marked difference in the total viable count at 9 hr. being 54×10^5/ml. in milk D but only 9×10^3 in milk A. Thus there was evidence of a possible delay in germination and a definite prolongation of the lag phase with this organism. Nearly complete germination was also obtained with B. licheniformis 8 though the rate was slower than that of B. subtilis 6 and there was no difference in the viable count between the two treatments.

The germination of B. brevis 58 (Fig. 29) showed like that of B. subtilis 6, rapid germination in both milks. After incubation for 9 hr. 65% of the spores had germinated and there was no further increase in germination with further incubation. Although two-thirds of the spores had germinated in 9 hr., vegetative growth was not detected in milk D until 15 hr. and in A until 25 hr. at 37°C.

B. circulans 154 showed a different type of response (Fig. 30). Germination was rapid in milk D, nearly 70% in 3 hr., with an equally rapid vegetative growth detectable at 6 hr. Growth in milk A was detected after incubation for 9 hr., i.e. only slightly later than in milk D, but the rate of germination was very slow in milk A, only about 10% for the first 15 hr. and increasing slowly with time. This type of response in which germination occurred in milk D but was slow in milk A was also obtained with B. cereus 201 and B. circulans 152. However, the germination of

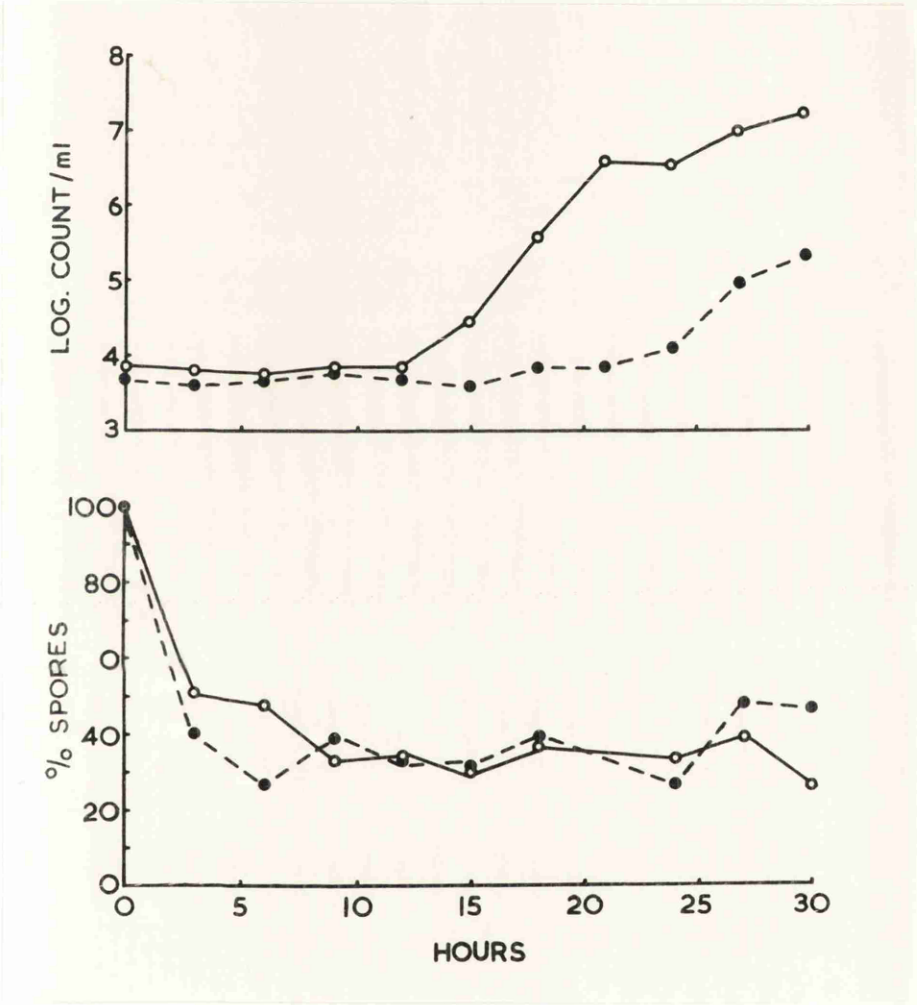


Fig. 29. The spore germination and growth rate of B. brevis 58 at 37°C in milk treated by methods A and D. Treatments: A ●---●, D O——O.

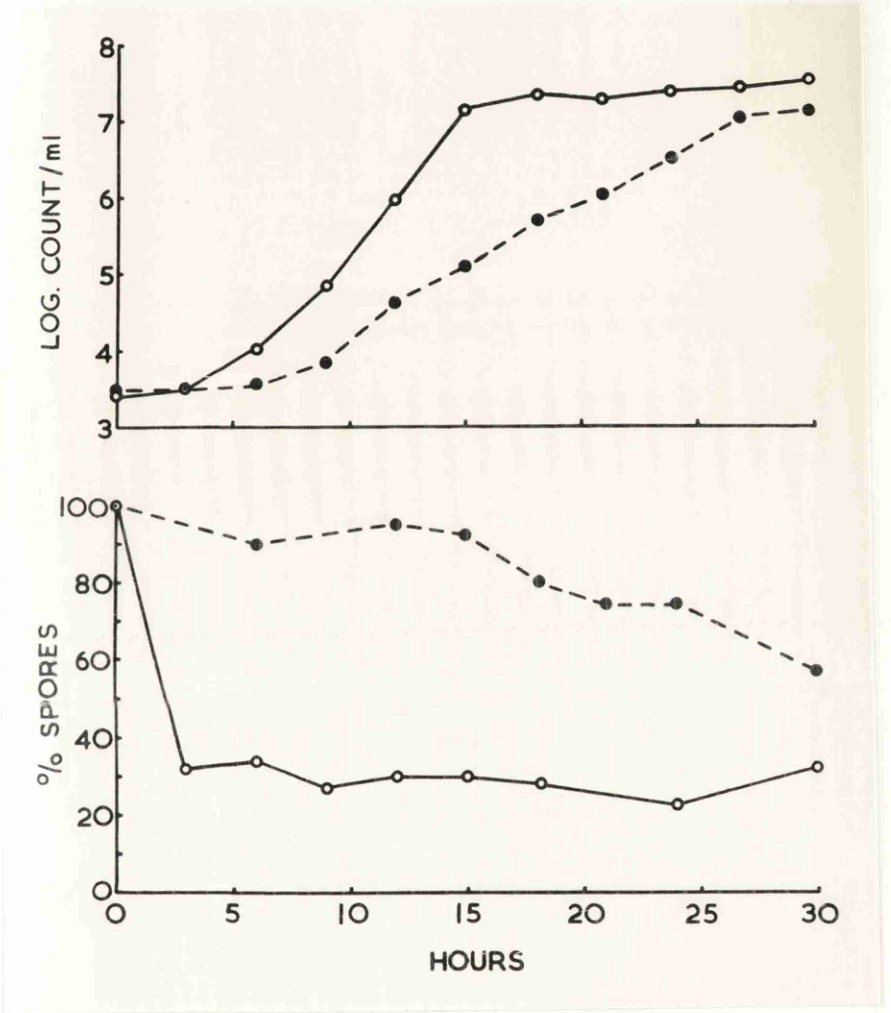


Fig. 30. The spore germination and growth rate of B. circulans 154 at 37°C in milk treated by methods A and D. Treatments: A ●---●, D O——O.

strain 152 in milk D was accompanied by a fall in the total viable count, and vegetative growth was inhibited in both milks A and D during the experimental period.

These results show that at this level of inoculum (5,000 spores/ml.) the two treatments of the milk (A and D) had little effect on the germination rate of the spores of B. subtilis 6, B. licheniformis 8 and B. brevis 58. Once germinated, the cells of B. subtilis 6 and B. licheniformis 8 showed no lag in milk D whereas B. brevis 58 showed a distinct lag phase. The transition of the cells from the lag phase to the logarithmic phase (the period of active multiplication) appeared to be much more sensitive to the treatment of the milk than the germination process, showing a longer lag in milk A than in milk D. The second group of organisms, B. circulans 152 & 154 and B. cereus 201, showed no delay in the germination of the spores in milk D, vegetative growth occurring rapidly in B. cereus 201 and B. circulans 154 though not in strain 152. The germination of the spores of this group in milk A was very much restricted. In spite of the low germination rate, vegetative growth occurred at a relatively early stage although the lag phase was longer than in milk D. The germination and growth rate of B. circulans 154 showed an unexpected reversal of its behaviour with low inocula where growth was inhibited in milk D. It illustrates the difficulties of applying results obtained with high inocula to the

behaviour of very low inocula. The converse is also true.

e) Discussion. The size of the inoculum has been shown to be of importance in determing whether or not spores will give rise to vegetative growth in sterilized milk. For most of the organisms studied, inhibition of growth from very small inocula was obtained in milk given treatment A, i.e. heated in "evacuated" bottles and incubated with nitrogen in the headspace. A study of the germination rates of a relatively high inoculum showed that inhibition may occur at two points, either germination being restricted or the lag phase prolonged. However, the reversal of the inhibitory effect of B. circulans 154 at high and low inocula indicates that results obtained with higher inocula should not be applied too strictly to the behaviour of low inocula.

There was no evidence that the ability to utilize NH_4^+ as sole nitrogen source had any influence on the growth of these organisms in sterilized milk. While B. subtilis 6 and B. licheniformis 8 gave similar growth and germination patterns, another species capable of utilizing NH_4^+ (B. circulans 154) gave results similar to that with B. cereus 201. Furthermore B. brevis 58, incapable of utilizing NH_4^+, had more in common with the B. subtilis group than with the B. circulans-B. cereus group. The results reported in this section did not confirm the suggestion

of the initial experiments that the oxygen requirement of the organism influenced its ability to germinate and grow in sterilized milk.

Inhibition of growth in sterilized milk tended to be least at the optimum growth temperature for the strain and to be more marked at lower or higher incubation temperatures. This might be expected since growth will be slower due to the temperature effect and more liable to reveal any adverse influences. B. subtilis 6 and B. licheniformis 8 with optimum growth temperatures of 45°C showed little evidence of inhibition with an inoculum of 0.05 spores/ml. at 37°C, but with the same inoculum they showed a delay in growth when incubated at 30°C. B. circulans 154 with an optimum temperature of 30°C showed greater inhibition at 37° than at 30°C.

5. Growth in relation to the degree of heat treatment of the milk

Milk sterilized at 115.5°C (10 lb./sq. in. steam pressure) for 15 min. was used throughout this work except when investigating the effect of the degree of heat treatment. For this, the time of sterilization was kept constant at 15 min. and the temperature (steam pressure) varied. The inoculum used for each organism was at a level known to show inhibitory effects in milk heated at 115.5°C for 15 min. Incubation was carried out at 37°C.

B. subtilis 6 was examined in 1 litre quantities of milk (40 oz. bottles) from farm 1, using an inoculum of 0.004 spores/ml. milk. The growth in milk A was completely inhibited over a period of incubation of 14 days at 37°C. After treatment D, the growth in milk heated at the three temperatures was rapid and the milk became unstable to 80% alcohol after incubation for 2 days at 37°C. Thus no difference was detected in the growth of the bacillus when the same milk, under treatments A and D, was heated at 107°, 112.5° and 117.5°C (4, 8 and 12 lb./sq. in. steam pressure) for 15 min.

At the same level of inoculum, B. subtilis D1/2M gave results similar to those given by strain 6 with milks from a different source (farms 4 and 6). For this bacillus, the milk heated at 117.5°C was not the same as that heated at 107° and 112.5°C. Growth occurred readily in milk D, causing instability towards 80% alcohol after 2-5 days. There was complete inhibition of growth over a period of 14 days in milk A at each level of heat treatment.

Similar results were obtained with B. licheniformis 8 with an inoculum of 0.006 spores/ml. in milk from two different sources (farms 3 and 4) which had been heated at 107.5° and 115.5°C (4 and 10 lb./sq. in. steam pressure) for 15 min.

Two temperatures, 108.5° and 121°C (5 and 15 lb./sq. in. steam pressure) were used to study the

growth of B. cereus 201. Although incubation was not continued for so long as with the three previous strains, the same pattern of growth was obtained. At an inoculum of 0.01 spores/ml. milk, the growth in milk which had received treatment D caused instability towards 80% alcohol within an incubation time of 27 hr. Growth was not detected in milk A after incubation for 84 hr. There was some evidence, with this and other organisms, that the sterilization of milk for 15 min. at temperatures of 121°C or higher produced changes which adversely affected bacterial growth in milk D.

With B. brevis 58 the general picture was the same as for B. subtilis, B. licheniformis and B. cereus showing rapid growth in milk D and a delay or inhibition in milk A (Fig. 31). However, B. brevis 58 was more sensitive to an increase in the degree of heat treatment. This organism was able to render unstable to 80% alcohol milk D which had been heated at 107.5°C after incubation for 3 days at 37°C, while incubation for 5-7 days was required for milk heated at 112.5° or 117.5°C. Similarly instability was observed in from 11-13 days in milk A that had been heated at 107.5°C, and in 17-18 days in milk A that had been heated to 112.5°C. Growth was completely inhibited in milk A which had been heated at 117.5°C.

It has been shown that B. circulans 154 was inhibited in milk D but not in milk A when the level of heat treatment was 115.5°C for 15 min. This also held

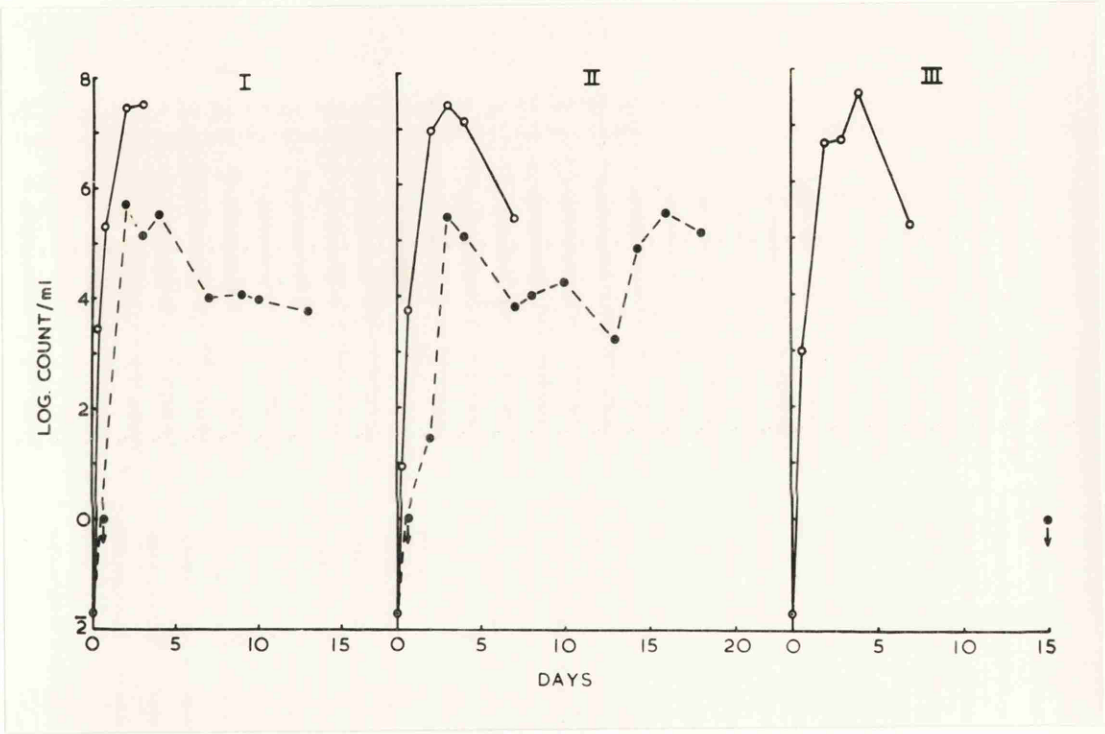


Fig. 31. Growth rate of B. brevis 58 at 37°C in milk which had received different degrees of heating and treated by methods A and D. Heat treatments: I 107.5°C for 15 min., II 112.5°C for 15 min., III 117.5°C for 15 min. Milk from farm 5. Treatments: A ●---●, D O——O. ↓ count below this level.

for milk heated at 107.5°, 112.5° and 117.5°C. At each level of heat treatment vegetative growth in milk A was rapid and caused instability of the milk towards 80% alcohol after incubation for 3 days. Growth was completely inhibited in milk D over a period of 14 days.

It is evident from the results obtained with these organisms that the influence of the chemical changes induced in milk at levels of heating between 107.5° and 117.5°C for 15 min. is small compared with the effect of treatments A and D. It has been shown in Section III (p.29) that only the oxidation-reduction potential showed a difference between milks heated in "open" and "evacuated" bottles which was not affected by heat treatments within this range. Therefore it seems likely that the oxidation-reduction potential is closely associated with the inhibition of the growth of bacilli.

6. A comparison of growth rates in sterilized milk incubated at 37° and 22°C

Because the growth of bacilli is more rapid at 37° than at 22°C, most of the present work has been done at the higher temperature. However, it was important to consider what happens to milk held at 22°C, i.e. at a temperature near to that at which commercial sterilized milk might be held in the period between heat treatment and consumption. It has been suggested (p.65) that any departure from the optimum growth

temperature results in a more marked inhibition of the organism. The following experiments confirm this view.

The methods were the same as were used in previous sections. The milk was sterilized at 115.5°C for 15 min. Instability of the milk towards 80% alcohol was taken as the end point of observations.

The growth of B. subtilis 6 from an inoculum of 0.005 spores/ml. milk was observed on single bottles for each treatment incubated at 37° and 22°C (Fig. 32). At 37°C the rate of growth of this bacillus was similar to that previously reported. Growth was rapid in milk D, causing instability towards 80% alcohol after incubation for 2 days whereas no growth was detectable in milk A after a period of 14 days. As was to be expected, the rate of growth in milk incubated at 22°C was slower. Vegetative growth was detected in milk D on the second day of incubation and instability of the milk towards 80% alcohol was noted on the seventh day. There was complete inhibition of growth in milk A over a period of 32 days at 22°C.

B. subtilis D1/2M gave results very similar to those obtained with strain 6. Growth was rapid in milk D, instability towards 80% alcohol being noted on the third days at both incubation temperatures. In milk A, growth was inhibited over periods of 15 and 30 days on incubation at 37° and 22°C respectively.

In the experiment with B. cereus 201, triplicate

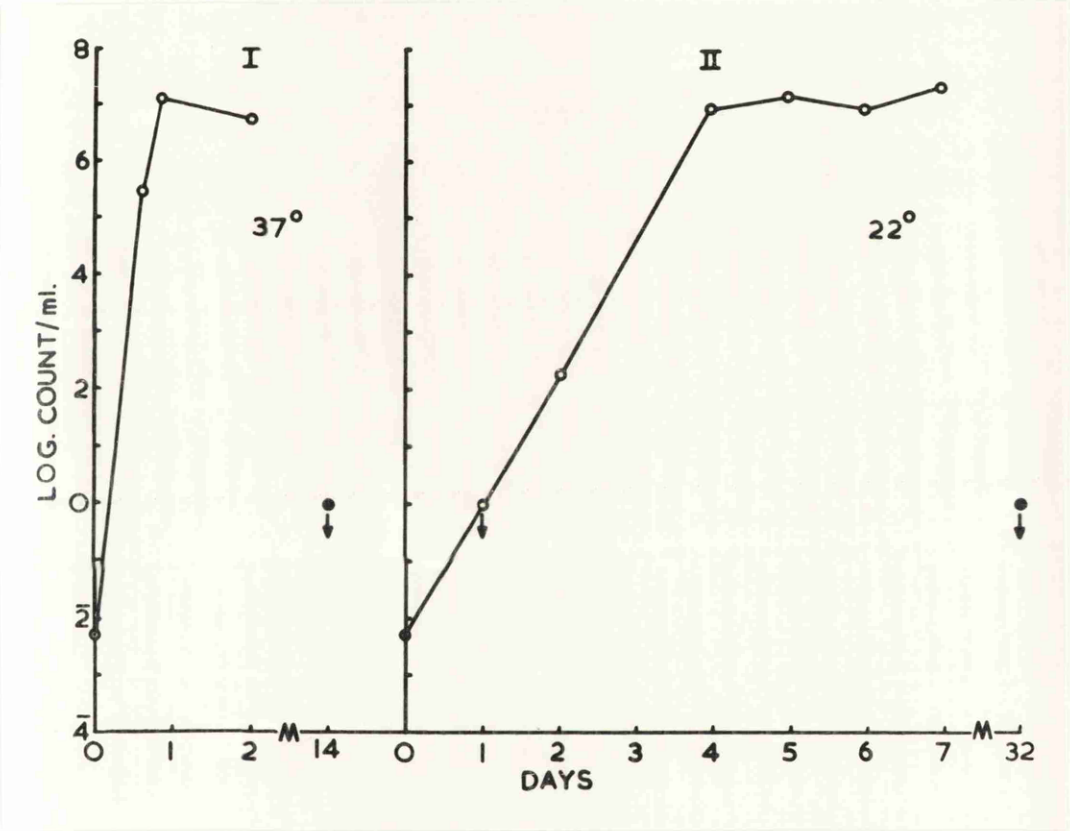


Fig. 32. Growth rate of B. subtilis 6 at 37°C and 22°C in milk treated by methods A and D. Milk from farm 1. Incubation temperatures: I 37°C, II 22°C. Treatments: A ●---●, D O——O.
↓ count below this level.

bottles were inoculated to give 0.08 spores/ml. milk. On incubation at 37°C the growth rate of this organism in milk A varied in the three replicates, but tended to show only a slight delay in growth compared with milk D. The development of instability of the milk towards 80% alcohol was delayed by one day in the three bottles which had received treatment A. At 22°C, growth in all three "open" bottles (D) had caused instability of the milk towards 80% alcohol by the seventh day of incubation. No growth was detected in any of the three "evacuated" bottles (A) over a period of 32 days.

With B. brevis 58 four bottles of milk for each treatment A and D were inoculated with 0.19 spores/ml. and duplicate bottles of each treatment incubated at 22° and at 37°C. At the higher temperature, there was rapid growth in milk D but the onset of vegetative growth was delayed for 2-4 days in milk A (Fig. 33). There was a marked difference between milk treatments in the time required to produce instability of the milk towards 80% alcohol. For milk in the two "open" bottles incubation for 4-5 days was required, but about four times as long was required for the "evacuated" bottles. At 22°C growth occurred in milk D after incubation for 7-11 days. The development of instability of the milk towards 80% alcohol required further incubation for 11 days, instability being noted on the 18th and 22nd days for the two "open" bottles. Growth was not detected in the two "evacuated" bottles

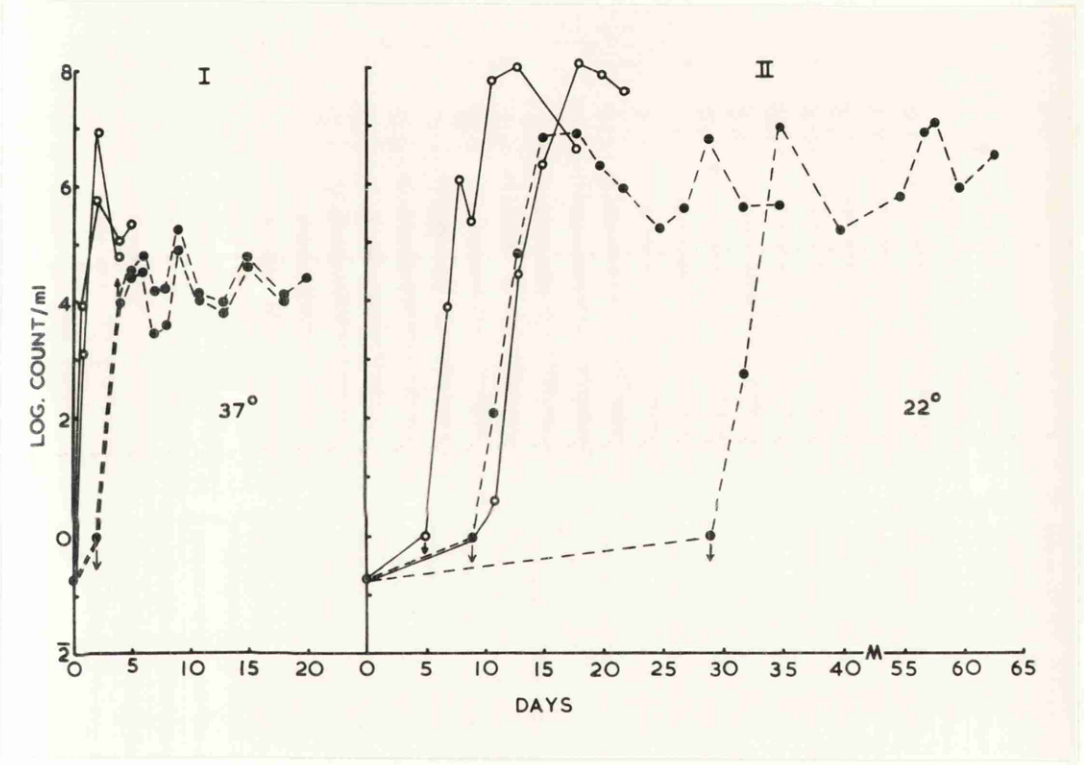


Fig. 33. Growth rate of B. brevis 58 at 37°C and 22°C in milk treated by methods A and D. Milk from farm 8. Incubation temperatures: I 37°C, II 22°C. Treatments A ●--●, D O——O. ↓ count below this level.

until the 11th and 32nd day of incubation respectively and instability of the milk towards 80% alcohol was noted on the 35th and 63rd day.

Thus, the inhibition shown on incubation at 37°C was brought out more clearly on incubation at 22°C. This inhibition combined with the slower growth of the bacilli resulted in a much longer "life" of the milk which had been heated in "evacuated" bottles (treatment A) when considered in relation to the time of storage prior to consumption.

7. The stability of sterilized milk towards 80% alcohol as affected by the growth of bacilli

Morgan & Chalmers (69) proposed the use of a test for stability towards 80% alcohol as a keeping quality test for sterilized milk. They suggested that after incubation of the milk for 27 hr. at 37°C the absence of a precipitate on mixing equal volumes of milk and 80% alcohol would indicate a keeping quality of 3 weeks for the milk when held at atmospheric temperature. Neutral red was included with the alcohol to indicate changes in the reaction of the milk caused by the organisms present. Morgan & Chalmers suggested that this idea should be given further trial but no subsequent work has been published on the relation of alcohol stability to keeping quality in sterilized milk. Other workers have used 80% alcohol to indicate the presence or absence of bacterial growth

(40, 70). While Hermier & Mocquot (70) note that a negative alcohol test is not necessarily indicative of bacteriological sterility the other workers do not state that they made this reservation.

During the work on growth rates reported in previous sections, the stability of the milk towards 80% alcohol was tested each time a sample was taken. Although many hundreds of tests were made, they were carried out with the object of establishing an arbitrary end point for observation rather than of investigating the suitability of alcohol stability as a keeping quality test for sterilized milk. However it has been possible to obtain some information on factors which influence the time required for some bacilli to cause instability of the milk towards 80% alcohol.

The numbers of positive and negative alcohol tests obtained at different levels of count for the individual strains of bacilli examined are given as histograms in Fig. 34. Records of negative tests were more numerous than positive tests since the milks were usually discarded shortly after the first positive test. These results do not take into account the treatments of the milk, the size of the inoculum or the time of incubation but all refer to tests on milk which had been sterilized at 115.5°C for 15 min. and incubated at 37°C. With the exception of B. brevis 58 and B. circulans 152 positive alcohol tests were not obtained with milk containing fewer than 10^5 bacteria/ml.

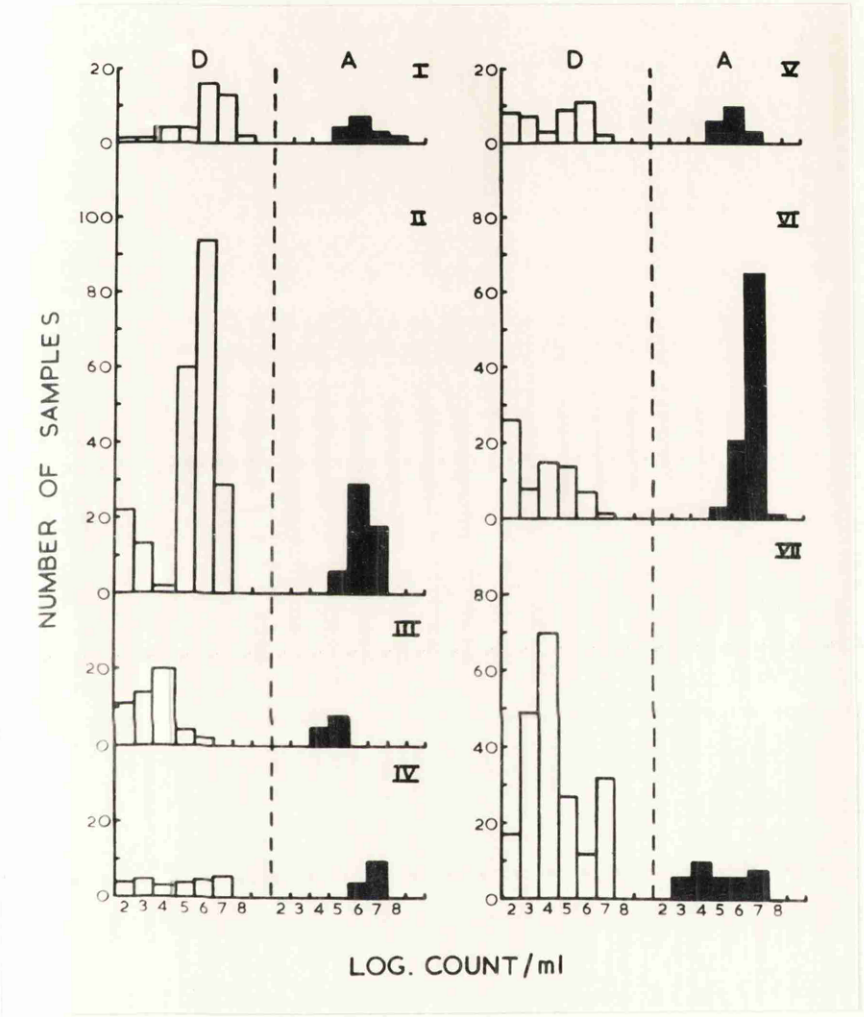


Fig. 34. The relation of colony count/ml. milk to the positive and negative 80% alcohol test at 37°C. I B. subtilis 6, II B. licheniformis 8, III B. circulans 152, IV B. circulans 154, V B. subtilis D1/2M, VI B. cereus 201, VII B. brevis 58. ☐ negative test, ▨ positive test.

Considered in relation to the treatment of the milk (Fig. 35) it was shown that the alcohol-positive low-count milks were usually those associated with inhibitory conditions i.e. treatment A for B. brevis 58 and treatment D for B. circulans 152.

The results given in Fig. 34 showed that milks of high bacterial content, 10^6-10^7/ml., were frequently not detected by the 80% alcohol test until these high counts had been maintained for several days. From an examination of the growth curves given earlier in this work it was evident that the end point of the experiment (i.e. the point at which the milk became unstable to 80% alcohol) normally occurred at the end of the logarithmic phase or even later. For most of the strains studied the stationary phase was maintained for periods of days before a positive alcohol test was obtained. The reduction of the maximum population that occurred when B. brevis 58 and B. circulans 152 were incubated in inhibitory milk necessitated an even longer period at the maximum population to obtain a positive alcohol test. The relation between the positive alcohol test and the time at which this occurred during the growth cycle is shown in Table 11. With B. cereus 201 and B. circulans 154 the milk usually became unstable to alcohol before the maximum population had been attained but with other strains the maximum population had been established for a variable period before the test became positive. In the

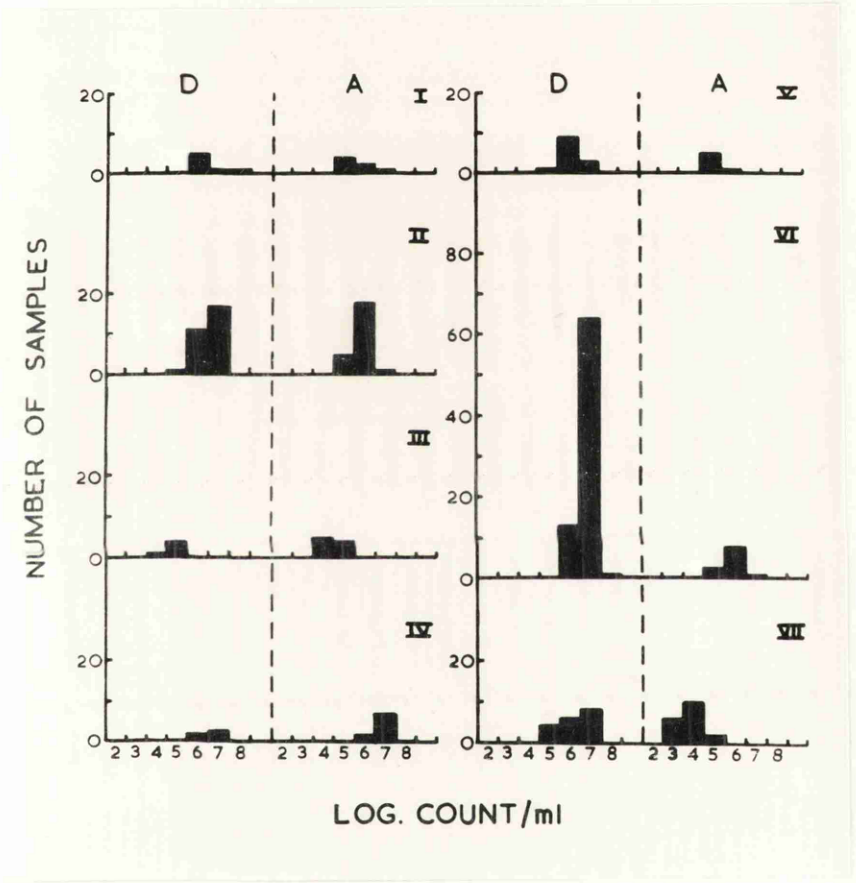


Fig. 35. The relation of milk treatments A and D to count/ml. milk at which a positive 80% alcohol test was obtained at 37°C. I B. subtilis 6, II B. licheniformis 8, III B. circulans 152, IV B. circulans 154, V B. subtilis D1/2M, VI B. cereus 201, VII B. brevis 58. A positive tests in milk A, D positive tests in milk D.

Table 11. The relation between the numbe
giving a positive alcohol test
bacterial growt

Culture	Number of experiments giving positi				
	Before maximum population attained	After maximum popul			
		Period of incubat give positive establishment of ma (day			
		≯0.5	1	2	
6	1	2	5	1	
D1/2M	10		16	2	
8	4		2	10	
201	49	2	1		
152			2	1	
154	11		6		
58			5	4	

greater proportion of the experiments using B. licheniformis 8 and B. brevis 58 an incubation period of 3 or more days was required after the maximum population was reached in order to obtain a positive alcohol test. This corresponded with the relatively large number of high-count alcohol-negative tests obtained with these two strains (Fig. 34).

The alcohol test suggested by Morgan & Chalmers made use of an incubation period of 27 hr. at 37°C in an attempt to detect poor keeping quality of the same milk at atmospheric temperatures. The time required to give a positive alcohol test at 37°C for milks A and D containing variable numbers of spores is shown in Fig. 36. Under the experimental conditions used in the present investigation a positive alcohol test was usually obtained within 27 hr. when B. subtilis 6 was incubated in milk D. B. cereus 201 was the only organism which gave a positive test within 27 hr. in milk of both treatments. However B. cereus is a species which does not commonly survive the commercial sterilization process (4, 40, 41).

Experiments, using inocula of 4×10^{-3} to 600 spores/ml. milk, showed that within this range the size of the inoculum did not appreciably affect the incubation time at 37°C required to give a positive alcohol test in non-inhibitory milk (milk A for B. circulans and milk D for the other organisms) (Fig. 36). As the inoculum was increased the

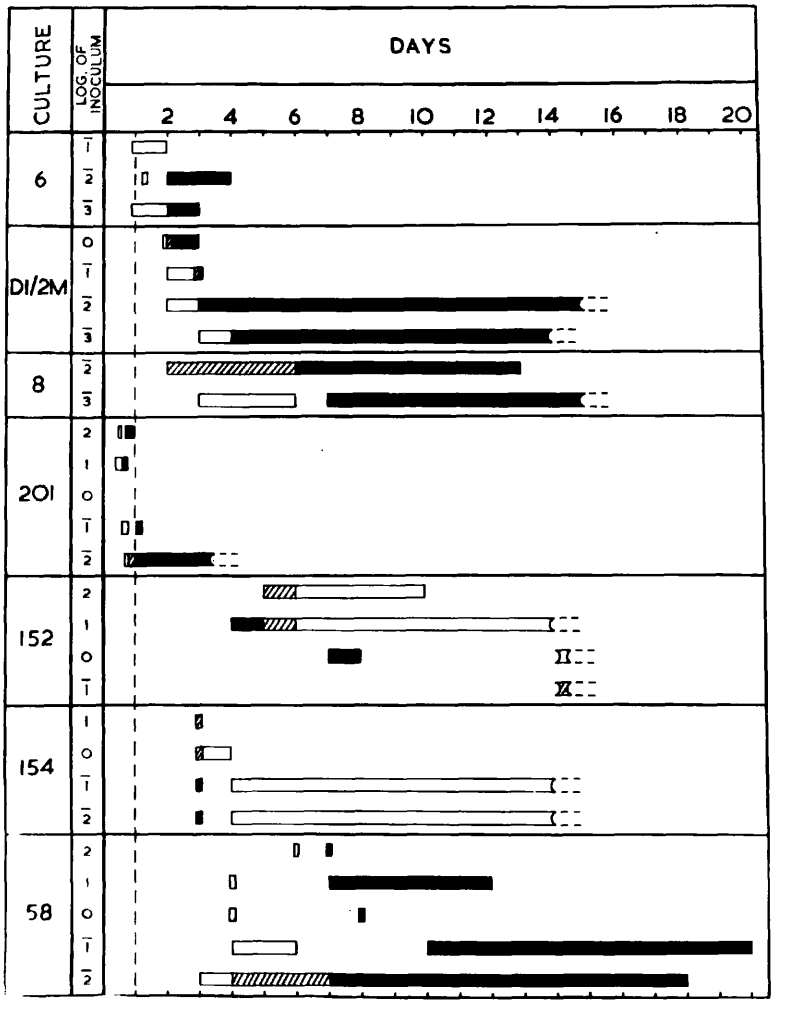


Fig. 36. Incubation time required to give a positive 80% alcohol test at 37°C in milk treated by methods A and D. ■ results in milk A, □ results in milk D, ▨ overlap between results in milks A & D. —〔-- incubation for a longer period required to obtain a positive test.

incubation time required for inhibitory milks approached that for the non-inhibitory milks. A direct comparison of the alcohol test after 27 hr. at 37°C and the keeping quality of the same milk at 22°C has only been made using one level of inoculum for each strain. The results (Table 12) showed that with B. cereus 201 a positive Morgan & Chalmers test in a milk sterilized in an "evacuated" bottle may be obtained in milk which would keep for at least 14 days and probably for more than 4 weeks at 22°C. The negative tests at 37°C obtained with B. licheniformis 8 and B. brevis 58 indicated correctly that the milk would have a keeping quality of 3 weeks or longer at 22°C. However, the results obtained with the two strains of B. subtilis (6 and D1/2M) showed that a negative Morgan & Chalmers test could be associated with a keeping quality of a week or less. This species commonly survives the commercial sterilization of milk and although only two strains of B. subtilis were examined the fact that negative tests could be associated with a poor keeping quality detracts from the usefulness of the Morgan & Chalmers test as a method of controlling the bacteriological quality of sterilized milk.

Table 12. The relation between the
in milk held at 37° for
at 22°C

Culture	Milk treatment	Alcohol	
		27 hr. at 37°C	Da
6	D	+	
	A	−	
D1/2M	D	−	
	A	−	
8	D	−	
	A	−	
201	D	+	
	A	+	>1l
58	D	−	
	A	−	

SECTION V

DISCUSSION

The results of the investigations into the growth of bacilli in sterilized milk presented in Section IV have shown that the sterilization process may produce conditions which are inhibitory for the growth of small numbers of spores. The inhibition took the form of a prolongation of the lag phase of growth. The duration of the lag phase could be altered by varying the size of the spore inoculum or the temperature of incubation. Inhibition became more marked as the size of the inoculum was decreased and as the difference between the optimum growth temperature for the strain and the incubation temperature increased. Under the experimental conditions used, this inhibition was related to the treatment of the milk before and during heat treatment rather than to the degree of heat treatment. With the possible exception of B. brevis 58, there was no evidence of inhibition becoming greater as the degree of heat treatment of the milk was increased within the range 107.5°-117.5°C for 15 min.

Within the range of heat treatments studied, the results reported in Section III showed that the acid-ferricyanide-reducing substances, the titratable acidity, the yellowing of the Aschaffenburg turbidity test filtrate and the browning of the milk increased and the pH became lower as the degree of the heat

treatment was raised. The results are in accordance with those obtained by other workers who usually studied only one change at a time (25, 27, 53, 58). In the present work all the tests were done on each sample and can therefore be directly compared with one another. Differences in chemical reaction were noted between milks heated in "open" and "evacuated" bottles, but with the exception of the protective action of heating under a vacuum on the acid-ferricyanide-reducing substances, these differences were attributed to a difference in the rate of cooling between the two types of bottles. The "open" and "evacuated" treatments also had a marked effect on the oxidation-reduction potential of the milk giving values of about -20 mV and -290mV for milk heated in "open" and "evacuated" bottles respectively. When the degree of heat treatment was raised from 104.5° to 117.5°C for 15 min. the potential of the milk heated in "open" bottles fell from -10 mV to -30 mV but the potential of milk heated in "evacuated" bottles was not affected. The poising capacity of milks towards atmospheric oxidation was always better in milk which had been deaerated than in milk which had not. There was, however, a variation in the poising capacity between milks from different sources. Since it was shown that the Eh of milk was affected by the treatment before and after heating, the oxidation-reduction potential results reported in this thesis can be considered only

in relation to the experimental technique used in this work. It is possible that with suitable adjustments of the experimental procedure still lower values may be demonstrated in bottles of sterilized milk.

Four treatments (p.103), involving the presence or absence of a vacuum in the headspace of the bottles during sterilization of the milk and the presence or absence of air in the headspace during incubation, were used to examine the growth of bacilli in sterilized milk. The results presented in Section IV showed that by the use of a suitable inoculum and incubation temperature, a prolongation of the lag phase could be demonstrated in milk A with B. subtilis 6 & D1/2M, B. licheniformis 8, B. cereus 201 and B. brevis 58 and in milk D with B. circulans 152 & 154. The limitation of the oxygen supply did not appear to account for more than part of the inhibitory effect since a prolonged lag phase could be demonstrated with B. cereus 201 in milk B. Furthermore, spore-formation, normally considered an aerobic process (68), was not usually as extensive in milk B as in milk D. Since the inhibitory action of the milk was not affected by the degree of heat treatment it seemed possible that heat-induced degradation products were not the source of inhibition. Organic peroxides, shown to inhibit the germination of spores (71), are unlikely to be the source of inhibition for B. circulans since this organism was able to germinate in milk D. If peroxide

formation was occurring a more positive potential would be expected in milk D than was obtained experimentally. There appeared to be some association between the Eh and poising capacity of the milk used for the examination and the degree of inhibition shown by the organism. Neither the Eh of the milk nor the inhibitory effect of the milk on the bacterial growth was affected by the degree of heat treatment used in the sterilization process. The milks which showed a good poising capacity towards atmospheric oxidation were more inhibitory than those with a poor poising capacity.

Workers using members of the genus Bacillus and organisms of other but non-sporing genera have shown that the ability of inocula to grow at adverse potentials decreases as the size of the inoculum is reduced (72, 73). It could therefore be postulated that whereas the potential of milk A was too negative for the growth of small inocula of B. subtilis 6 & D1/2M, B. licheniformis 8, B. cereus 201 and B. brevis 58, the potential of milk D was within the range permitting growth. Wood, Wood & Baldwin (73) have found the optimal growth Eh value for B. megaterium to be 0 mV, a value not very much more positive than those obtained in milk D. The results obtained with B. circulans are not inconsistent with this theory. It is known that this species is capable of growth under very much more anaerobic conditions than other members of the

genus (74). Therefore it is possible that while the spores of B. circulans germinate at a relatively positive Eh (i.e. in milk D), a very much more negative potential is required for initiating vegetative growth. This type of Eh requirement has been shown with Clostridium tetani by Knight & Fildes (75). The effect of increased inhibition at lower inocula observed in these experiments and also by other authors, can only be explained on the hypothetical assumption that organisms have an ability to alter their local surroundings, the ability varying between strains. Some organisms must possess this ability to a marked degree since, for example, B. subtilis 6 and B. licheniformis 8 are capable of growth in milk A from inocula of 5 spores/400 ml. milk although not from an inoculum of 5 spores/1,000 ml. milk. The carry-over of growth-stimulating substances from the original suspension does not account for the inhibition of the smaller inocula since inhibition could be demonstrated at different levels using the same suspension inoculated into milk from different sources. Having assumed that organisms possess the ability to adjust their environment, it is reasonable that an inhibitory effect associated with Eh should be greatest in milks which have a good oxidation-reduction poising capacity.

The variation in the capacity of milks from different sources to resist oxidation and hence the variation in ability of the milks to inhibit bacterial

growth may be associated with the β-lactoglobulin content of the milk. Nitrogen analyses of milk from cows 1 and 2 and from farm 3 were done by the Technical Chemistry Department of this Institute. The analyses showed that the casein number was high for cow 2 but low for cow 1 and farm 3, i.e. in the last two milks a higher percentage of the total nitrogen in the milks was present as serum proteins, proteoses and non-protein nitrogen. This may be correlated with the fact that inhibitory effects could be shown readily with milk from cow 1 and farm 3 but not with milk from cow 2. Aschaffenburg & Drewry (76) have shown that there are two genetically determined types of β-lactoglobulin in the milk of cows. Type A is associated with a lower casein number than type B. The frequencies of the two genotypes were shown to vary between breeds. Type B was the more common type but A had a gene frequency of 0.40 and 0.31 for Friesian and Ayrshire cows respectively. It has been found that β-lactoglobulin is the source of the volatile sulphides that are formed when milk is heated (10), and also that the destruction of sulphide groupings is considerably decreased when milk is heated under vacuum (62). Therefore the resistance of milk towards atmospheric oxidation is likely to be greatest in those milks which have a high lactoglobulin content and which are heated under vacuum. Under the experimental conditions used, hydrogen sulphide and other volatile

sulphides formed during heating would escape from bottles treated by methods C and D but not from bottles treated by methods A and B. It has been shown that under some circumstances H_2S will inhibit the growth of small inocula of some bacilli (77). The presence of H_2S in milk will give a low potential and therefore it was not possible in the present work to differentiate between the effects of H_2S and of Eh.

It has obviously been impossible to determine with small inocula the stage of growth at which inhibition occurs. By the use of suitable conditions it has been possible to demonstrate with all the strains used, a delay in the transition from the germinated spore to an actively proliferating state. With some cultures, such as B. cereus 201 and B. circulans, it has been possible also to demonstrate inhibition of germination. However, the apparent reversal of inhibition of B. circulans 154 at high and low levels of inocula shows that caution is required in applying the results of germination studies to the growth of very small inocula. This apparent reversal of results obtained with B. circulans 154 is not necessarily inconsistent with the theory that this species requires a more negative potential for vegetative growth than for spore germination. If a sufficiently large number of spores germinate under conditions unfavourable for vegetative growth (i.e. in milk D), the ability of the germinated cells to modify

the environment could permit vegetative growth to occur after incubation for a shorter period than would be required to obtain vegetative growth under conditions which delayed the germination of the spores (in milk A).

Certain objections might be raised to the application of these results to the growth of organisms in commercially sterilized milk. For example, it has been shown that the "cleanliness" of spores affects their germination requirements (78). As a result "clean" spores have been defined as spores whose germination requirements are not altered by further washing. Unwashed spore suspensions have been used in these experiments. However, since the spores in milk will have come from a variety of sources and can hardly be considered "clean", it was considered sufficient that the suspensions used for inocula should be obtained under standard conditions. A more serious objection is that the spores did not undergo heat treatment before examination of the growth rate. Curran & Evans (79, 80) have shown that the spores of some Bacillus species obtained under laboratory conditions may require "heat-activation" before being capable of germination in media which were considered optimal. With some cultures heat-activation was demonstrable only when incubation was carried out at 15°C and not at 22°C or higher temperatures. These and other authors have shown that the spores which survive sterilization are not activated by further

heat-shock (38, 80, 81, 82). In the experiments reported in this thesis heat-activation could not be demonstrated with any of the cultures when incubation was carried out at 37°C. Since, under experimental conditions which have appeared to be non-inhibitory, all these organisms showed rapid growth in sterilized milk, there was no evidence that the spores required further activation. As this work involved the growth of such small inocula it was considered that the use of a freshly heated spore suspension would not give sufficient control over the size of the inoculum. For this reason, the control gained by the use of a stable spore suspension which had not been freshly heated outweighed the objections which might be raised to the use of unheated spores.

It has been commonly assumed that the spores surviving sterilization are damaged by the heat treatment. This has resulted in the use of supplemented media and the advocation of sub-optimal incubation temperatures for the growth of the surviving spores. This assumption of heat damage which is difficult to prove or disprove, is not necessarily correct. The assumption has been based on the fact that workers have found that as lethal agents, such as heat treatment, reduce a bacterial or spore population, more closely defined conditions are required for the subsequent growth of the surviving cells. However, experiments reported in this thesis have shown that a

medium (sterilized milk) capable of supporting growth from a given inoculum of unheated spores may show inhibitory effects when the size of the inoculum is reduced. It is possible therefore that the effect of "heat-damage" is a reflection of the presence of unsuspected inhibitors in the growth medium. Furthermore, the results of Williams & Reed (83) purporting to demonstrate the beneficial use of sub-optimal incubation temperatures for the growth of heat damaged cells are equivocal. They showed that incubation temperatures of 24-27°C were most suitable for the recovery of the heated spores of Cl. botulinum. It was noted by Williams & Reed that although an incubation temperature of 37°C was commonly used for this organism, the optimum growth temperature for Cl. botulinum was given in reference books and by some other workers as being less than 37°C and probably about 30°C. In spite of this variation in opinion Williams & Reed neither gave optimum growth temperatures for the strains used nor did they give control results with unheated spores. Therefore it is possible that these authors merely demonstrated that the optimum growth temperature for an organism is most suitable for the recovery of small numbers of spores, a conclusion that would be in agreement with the results presented in this thesis.

Apart from the objections which can be raised to the technical procedures, the experiments presented in

Section IV do reproduce the results which have been reported for the spoilage of commercially sterilized milk. Hiscox & Christian (38) state that a "good vacuum" obtained in bottles will control some forms of spoilage but not others. The results presented here have shown that a "good vacuum" controls the growth of some species but does not affect B. circulans. Grinsted & Clegg (42) considered that since all the species of Bacillus which they examined grew at 15°C on milk-starch agar slopes, any spores surviving sterilization would cause spoilage in milk. The results presented by Burton, Akam, Thiel, Grinsted & Clegg (46) do not appear to support this view. Burton et al. obtained counts of 1-5 mesophilic organisms/ 100 ml. milk which had received in-bottle sterilization at 113°C for 35 min. Thirteen other bottles from the same crate, incubated unopened, were classed as sterile after incubation at 24°C for 10 weeks. This was demonstrated with samples processed over a period of 4 months. Since each bottle contained approximately 600 ml. milk it is most unlikely that more than a few were bacteriologically sterile. Curran & Evans (80) have shown that when spores are held under conditions which permit germination but not growth the spores die fairly rapidly. This appears to have happened when Burton et al. incubated unopened bottles at 24°C. The results obtained here in which growth was inhibited in milk that had received treatment A on incubation at

22°C are similar to those of Burton et al. The fact that oxidation-reduction potentials of -200 mV to -280 mV have been demonstrated in commercially sterilized milk support the contention that correctly produced commercially sterilized milk is inhibitory for the growth of bacilli. The autosterilization of spores in sterilized milk held at atmospheric temperature offers a method for the control of bacterial growth in this product.

The detection of bacterial growth in sterilized milk is a problem which has not been referred to directly. The Morgan & Chalmers 80% alcohol test has been proposed as a rapid method for the detection of growth. While the suggested incubation period of 27 hr. is not considered to be sufficient, a period of 2 or 3 days should be satisfactory in detecting the rather slower growing organisms which are also capable of giving a poor keeping quality. This longer incubation period would give an indication of potential trouble although the remainder of the batch stored normally at atmospheric temperatures would probably show a degree of autosterilization during this period.

It should be noted also that very occasionally during the winter, bacteriologically sterile sterilized milk has been unstable to 80% alcohol.

The oxidation-reduction potential of sterilized milk is sufficiently low to permit the growth of clostridia. Although no members of this genus were

detected during the course of the experiments which have been reported nor has their presence been suspected, the possibility of the growth of clostridia must be considered in examining the spoilage of sterilized milk.

SECTION VI

SUMMARY OF RESULTS

1. Chemical investigations into the changes in milk due to laboratory sterilization

a) The brown discolouration of milk, the yellowing of the Aschaffenburg turbidity test filtrate, the titratable acidity and the acid-ferricyanide-reducing powers increased and the pH fell as the degree of heat treatment was increased within the range 104.5°-117.5°C. The chemical changes were more marked in milk which had been heated in "evacuated" bottles than in "open" bottles but this was attributed to different rates of cooling for the two types of bottles.

b) Provided that the method of processing remained constant, the oxidation-reduction potential was not affected by heat treatment at or below 117.5°C. A value of about -290 mV was obtained when the potential of milk heated in "evacuated" bottles was measured at 50°C under nitrogen. There was variation in the capacity of different milks to resist atmospheric oxidation. It is suggested that a good poising capacity against oxidation may be associated with a high lactoglobulin content of the milk.

c) In addition to the experimentally sterilized milk, investigations were made into the heat-induced changes in some bottles of milk sterilized commercially

by the batch, continuous and ultra-high temperature processes. The oxidation-reduction potential of these milks was more negative than -200 mV. The lowest potential and the best powers of resisting atmospheric oxidation were shown by milk which had received the ultra-high temperature process.

2. The growth of bacilli in laboratory sterilized milk

a) The most marked inhibitory effect on the growth of B. subtilis 6 & D1/2M, B. licheniformis 8, B. cereus 201 and B. brevis 58 from small inocula, shown in milk heated in the range 107.5-117.5°C for 15 min. was not related to the degree of heat treatment but to the presence or absence of a vacuum in the headspace of the bottle during heating. The inhibition in milk which had been deaerated before sterilization was constant over the range of heat treatments investigated when compared with growth in the same milk which had been heated in "open" bottles or pasteurized.

b) The inhibitory effect was associated with a low oxidation-reduction potential and a good poising capacity against oxidation in the milk and was manifested by a prolonging of the lag phase.

c) The degree of inhibition became more pronounced as the size of the inoculum was decreased. For any one level of inoculum, inhibition was least marked at the optimal growth temperature for the organism.

d) B. circulans 152 & 154 gave similar results with regard to size of inoculum, temperature of incubation and degree of heat treatment of the milk, but this species showed inhibition in "open" and not in "evacuated" bottles, i.e. at high but not low Eh.

e) The production of instability of the milk towards 80% alcohol was taken as the end point for all experiments. This test was found not to be suitable as a means for detecting the growth of the seven strains of bacilli used in this work when the incubation period was less than 2-3 days at 37°C even when large numbers of bacilli were present at the time of testing.

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SUMMARY OF THE METHODS OF TREATING THE MILK

Treatment	Type of milk	Gas in headspace
A	"evacuated"	N_2
B	"	air
C	"open"	N_2
D	"	air
E	pasteurized	air

Type of milk

"open"	sterilized in open bottles
"evacuated"	deaerated at 25 in. Hg for 30 min. and sterilized with a vacuum in the headspace of the bottle.

SUMMARY OF THE METHODS OF TREATING THE MILK