

SPORE REMOVAL BY BACTOFUGATION AND ITS
EFFECT ON ULTRA HIGH TEMPERATURE
STERILIZATION ON MILK

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ABSTRACT

SPORE REMOVAL BY BACTOFUGATION AND ITS EFFECT ON ULTRA HIGH TEMPERATURE STERILIZATION OF MILK

By Manuel José Torres-Anjel

The removal of bacterial spores by bactofugation and the resulting effect on sterilization efficiency and milk spoilage were studied. Also, other means were used to attempt to reduce the resistance of spores to heat. Spores were cultured in a solid medium (Modified Fortified Nutrient Agar) after heat shock of the spore inoculum. The obtention of Bacillus subtilis, Bacillus cereus and Bacillus stearothermophilus spores by this method was very successful. Spore counts were performed by a modified agar plate technique and by a modified membrane filter technique. In both cases a standard methods agar specially modified for spores was used. Heat resistance of the most important organism in this investigation (B. subtilis A₁) was studied by fraction negative tests in a thermoresistometer.

Similar results were obtained for reconstituted skim milk and autoclaved whole milk as substrates and the four different subculture media, dextrose-tryptone-starch broth, ultra-high temperature (UHT) sterilized milk, and aerobic and anaerobic litmus milk. A D_{121} value of 0.435 to 0.625 min and a z value in the ultra high temperature range of 121.1 to 143.3 C (250 to 290 F), of 12 C (21.5 F) were found for

B. subtilis A₁. A D₁₂₁ value of 0.010 min was found for B. cereus 7. Temperature-survivor curves for 4.0 sec showed that changes in the temperature in the UHT range were numerically more significant than changes in initial population of spores in relation to spoilage probability. The higher the temperature the greater this effect.

Heat shock of 80 C for 15 min did not stimulate a massive germination of B. subtilis A₁ in milk. A penicillin-penicillinase system technique was tried to determine counts of primary, non-germinated spores but without success.

A commercial bactofuge was used for the spore removal trials. The removal of spores from milk with a single bactofugation was more effective at a flow rate of one-third compared to the normal capacity of the machine (99.9 vs. 98.0%). Single bactofugation at the slower flow rate gave approximately the same removal percentage as double bactofugation at the faster flow rate. More than two bactofugations were unnecessary. Milk losses in the sludge were approximately four times greater when the one-third flow rate was used compared to the normal rate. The sludge contained practically no fat. Milk temperature within the range of 71.1 to 82.2 C (160 to 180 F) was adequate for efficient removal of spores by bactofugation.

When the temperature of sterilization by UHT was reduced to 132.2 C (270 F) and 137.8 C (280 F), with initial populations of spores of $>10^2$ to $>10^4$ /ml prior to

bactofugation, spoilage was >90% for the non-bactofuged milk and <10% for the bactofuged milk. Thus a small reduction in the common UHT treatments is possible when the initial number of spores is substantially reduced by bactofugation, or at the same temperature the spoilage probability will decrease accordingly.

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To Amparo, naturally

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INTRODUCTION

Sterile milk and sterile milk products are increasing in importance in areas where long shelf life under limited or no refrigeration is required. These types of products may contribute to the solution of protein and other nutrient scarcity problems, particularly of infants and children in developing countries.

Of the several sterilization processes, continuous sterilization at ultra high temperatures (UHT) for very short times coupled with aseptic packaging seems to have a very promising future. The high temperatures used in the process are principally to assure the destruction of bacterial spores. Only minor changes occur in nutritional properties during UHT sterilization of milk products. Sterilization temperatures are responsible for some of the undesirable changes in UHT sterilized milk. The flavor can be improved compared to pasteurized products. The objectional cooked flavor in UHT treated milk is reduced compared to retort sterilization but is not completely eliminated.

This research was initiated to study means of removing bacterial spores from milk or reducing their inherent heat resistance. Special attention was given to the removal of spores from milk by bactofugation. Concurrently the influence of both standard and substandard UHT processing

was investigated. Bactofugation has been used in the removal of bacterial cells by several investigators, but literature on the specific application for the removal of spores is very limited. No literature was found on the effects of bactofugation as a pretreatment to sterilization. The results of these findings could apply to any milk sterilization process, or to any fluid milk product, fluid imitation product or other liquids subjected to sterilization.

Centrifugal removal of microorganisms has been called bacterial ultracentrifugation, bacterifugation and bactofugation. These terms have been respected in the literature review.

LITERATURE REVIEW

Bactofugation

Bactofugation as a Process

Simonart and Debeer (63) reported on "ultracentrifugation" as a method to improve the microbiological quality of milk. They suggested that the difference in size between the colloidal particles of milk (maximum 200 μ) and the bacterial cell (1 to 2 μ and more) is sufficient to separate the latter from the former by an adequately regulated centrifugal force.

In the early stage of their experiments they used a Sharples high speed centrifuge with a stainless steel clarifying bowl 1H with an interior diameter of 4.4 cm. It was operated at 30,000 rpm. The capacity of the machine was 6 liters/hr. In the experiments they used milk with normal flora and artificially contaminated milk containing Streptococcus lactis, Escherichia coli, Micrococcus aureus, Proteus vulgaris, Pseudomonas fluorescens, Bacillus subtilis, and Bacillus mycoides. The authors concluded "in general the flagellated bacteria (P. fluorescens, P. vulgaris and E. coli) are less easily eliminated than the non-flagellated bacteria, which, on the other hand, constituted the group that most easily agglutinates." The ideal centrifugal force was around 10,000 x g. For spores

the removal was >98% while for bacterial cells it was generally <90%. Simonart and his coworkers at the University of Louvain in Belgium continued to improve the laboratory process and to convert it into a commercial process (64, 65, 67, 72, 73). Most of their findings were summarized by Simonart (62) in a lecture given at the Netherlands Institute of Dairy Research (N.I.Z.O.), Ede. The term supercentrifugation instead of ultracentrifugation was used in this work to describe the semi-industrial centrifugation of milk (8,000 to 20,000 x g, 45 to 200 liters/hr) that promotes the removal of a high proportion of bacterial cells. But "the separating power of the centrifuge decreases after the bowl has been running for 15 to 20 min. However, when a hole of 0.35 mm is drilled in the side wall of the bowl, the separating power could be kept at a satisfactory level indefinitely."

Simonart (62) described also the industrial centrifugal-pasteurization process (6,000 liters/hr, 9,000 x g) at 72 to 76 C (161.6 to 168.8 F), which had an efficiency of about 91% in the removal of bacterial cells. He applied the term bacterifugation to this process. The "bacterifugation effect" (called bactofugation effect by the manufacturers of the commercial equipment) according to his description, "gives, as a percentage of the bacterial population of the raw milk, the sum of the bacteria eliminated by the centrifugal force and those killed by the thermal treatment."

A report from Russia on the removal of bacteria from milk by high speed centrifugation (49) utilizing 12,000 to 14,000 rpm and a throughput of 70 liters/hr showed at 30 to 40 C (86 to 104 F) 85.5% removal at the highest speed and 79% at the lowest. When the throughput was lowered, a maximum removal of 96.5% was obtained. A regular clarifier operating at 8,000 rpm removed 46% of the bacteria. The work by Surkov and Schmidt (79) was of interest since it was the only work available referring to the theoretical basis of bactofugation. They explain that the determining factor of the process is expressed by the equation:

$$T_1 = T_2 \quad (1)$$

in which T_1 stands for the time of the passing of the liquid through the centrifuge rotor and T_2 for the time needed for the sedimentation of particles (bacteria) in the rotor. From this equation may be derived the following formula (6):

$$\frac{T_1 \cdot l (R^2 - r_o^2)}{V} = \frac{9 (R - r_o) n}{2 \rho \Delta W^2 r_{\text{mean}}} \quad (2)$$

l stands for the length of the centrifuge (cm)

R stands for the inner radius of the rotor (m)

r_o stands for the inner radius of the liquid layer (m)

V stands for the capacity of the centrifuge (m³/sec)

η stands for the viscosity of the liquid
($\text{m}^2/\text{sec.}$)

ρ stands for the size of the sedimented particle
(m)

Δ stands for the difference in the density of the
dispersed phase and that of the dispersing
medium (kg^2/m^2)

W stands for the angular velocity of the rotor
rotation (r/sec)

$$r_{\text{mean}} = \frac{R + r_o}{2} [M]$$

By grouping the construction, biological and regime
factors, the equation (2) will read:

$$[1 \times r_{\text{mean}}^2] \times [\rho^2] \times \left[\frac{\Delta}{\eta} \times V \times n\right] \approx 0.0725$$

construction factors
biological factors
regime factors

For milk the following relation of $\frac{\Delta}{\eta}$ and the temperature
($^{\circ}\text{C}$) is valid:

$$\frac{\Delta}{\eta} = 0.29 \, t$$

Then:

$$[1 \times r_{\text{mean}}^2] \times \rho^2 \times [V \times t \times n^2] \approx 0.25$$

Using the same centrifuge the construction factors
remained unchanged. The biological factor was not regu-
lated and was determined by the microflora of milk. The

authors described the characteristics of size and shape of several microorganisms, and commented that the milk plasma containing microorganisms is not a mono component system, which levels down the summary values of the curves of bacteria distribution in the medium. These authors also commented very pertinently that bacteria are living beings and are in motion, the intensity of the latter depending on the conditions of the "bacteriofugal" process (temperature for example). Another very interesting phase of their work was the use of two capacities (100% and 50%) in the tubular centrifuge. The speed (30,000 rpm) and the acceleration (22,600 x g) remained the same. Bacteria were, in general, removed more efficiently at the lower capacity. Another of their observations was the formulation of the interdependence between the quantity of separated microorganisms and the temperatures of milk during centrifugation:

$$y = Kt + C$$

in which y is the quantity of centrifuged microorganisms (in %).

t stands for the temperature (C)

K and C are coefficients depending on the capacity.

These same authors, Surkov and Schmidt (78), using E. coli claimed that the percentage of separated microorganisms rises with the increase of the concentration.

When the content of microorganisms was changed by 10 times, the effect of bactofugation changed 4 to 5%.

Panchenko (50) utilizing an ASG-1A laboratory clarifier showed that an increase in the operating rate from 12,000 to 16,000 rpm caused the mean percentage of bacteria removed to rise from 62.4 to 92.4% (double processing). The acid value of the non-bactofuged milk (3.84×10^5 bacteriological count/ml) in 24 hr increased from 17.7°T to 22.5°T at 19 C (66.2 F). In the case of bactofuged milk, it increased from 17.2°T to 18.7°T.

Houran (27, 28) explained how the bactofuge utilized the difference in specific gravity and size between bacteria and the constituents of milk:

	Sp Gr	Size
Bacteria	1.07 - 1.13	0.5 - 8 μ
Milk (skim)	1.035	
Casein particles	1.066	500 - 800 $\mu\mu$

Actually, as mentioned by Dahlstedt (11), the difference in specific gravity between skim milk and bacteria is less than the density difference between skim milk and milk fat (0.93). This small difference made separation difficult and explains why the problem was not undertaken until specialized centrifuges and high speed centrifugation were developed.

Moreno and Kosikowski (46) described the high efficiency of the process in removing specific pathogens

(coagulase positive Staphylococcus aureus 98.5%, members of the Enterobacteriaceae 99.8%).

Surkov and Dukochaev (76), utilizing a 13-disk centrifuge (30 liters/hr) studied the influence of reducing or increasing the outer diameter of the disks. Reduction resulted in increased butterfat content of the skim milk, impaired efficiency of clarification and removal of bacteria. Increasing the diameter to a limited extent, had none of these adverse effects. Surkov et al. (77), utilizing a Volga separator and aqueous suspensions of Bacillus megaterium and S. lactis studied the separating effect of the peripheral area of the bowl of disk separators and concluded that the concentration of microorganisms in the suspension was similar to the original at the bottom of the bowl and similar to that of the clarified effluent at the top. They utilized continuous sampling by welding four hollow needles at different levels into the wall of the bowl. Peripheral separation during bactofugation required continuous removal of the sludge layer.

Bactofugation of Market Milk

Dahlstedt (11) described the first commercially operated plant located outside Brussels. Operation was started in January, 1962, after 6 months of experimental trials. The milk processing procedure was as follows: "raw milk is preheated by regeneration in a plate heat exchanger and then passes through the pasteurizing section

and on to the two Alfa-Laval high speed centrifuges connected in series. After leaving these, the milk is homogenized and led back to the heat exchanger where it passes through the regenerative section and the sections cooled by means of water and ice water respectively."

The bactofugation plant has a capacity of 6,000 liters/hr (approx. 13,000 lb./hr). The results have shown (11):

- a) High removal of microorganisms (above 99.99%).
The use of 75 C (167 F) heat assures the destruction of any remaining pathogens.
- b) The possibility of reducing the pasteurization heat treatment. The bactofugation temperature of 75 C (167 F) is a sufficient heat treatment.
- c) Improved keeping quality (approximately doubled).
- d) Absolutely natural taste and flavor which is expected with the reduction in heat treatment and the lack of significant change in chemical composition of bactofuged milk. This was even more marked when condensed bactofuged milk was compared to conventional condensed milk.

Similar plants exist in Mexico and France (39, 40).

Simonart et al. (68) reported on trials at 70 to 75 C (158 to 167 F), 6,000 liters/hr, 9,000 x g whereby double centrifugation was used. The removal of Streptococcus, Micrococcus, Microbacterium, Pseudomonas and coliforms ranged from 98.58 to 99.97% of the initial counts.

Simonart et al. (69) studied the bactofugation of summer milk and the flora changes in milk during the hot season. They described three basic operations in the industrial process:

- a) Preheating to 75 C (167 F)
- b) Bactofugation at this temperature, and
- c) A second bactofugation at the same temperature.

They studied the relative proportion of several genera of bacteria in the total count of raw milk, pasteurized, bactofuged and double bactofuged milk. They worked with milk of very low bacteriological grade (2.2 to 2.4×10^7 SPC/ml. The proportional number increased in the case of Microbacterium, Micrococcus and Lactobacillus and decreased in the case of Alcaligenes, also when Pseudomonas were mixed with coliforms. This difference reflected different proportional removal since the total number always decreased.

Simonart et al. (70) described the bactofugation of milk in a commercial plant near Brussels. They used two different stains for microscopic counts, aniline-oil-methylene blue (AOM) and periodic acid-bisulfite-toluidine (PST), as well as plate counts. The following reductions in counts were observed:

- a) AOM test (cells that did and did not stain after heating) 99.25%

b) PST test

- 1) When using milk with initial counts
 $< 2.0 \times 10^7/\text{ml}$ 98.97%
- 2) When using milk with initial counts
 $> 2.0 \times 10^7/\text{ml}$ 99.37%

Scarpari (61), in Italy, studied the inclusion of a bactofuge in the pasteurization cycle of market milk. In comparing milk that had been both bactofuged and pasteurized and milk which had been pasteurized only, the author reported no significant difference in acidity, taste and aroma, but the keeping quality of the former was "slightly superior" and the total bacterial count was lower. Reduction in total count from highly contaminated raw milk (1.62×10^7 bacteria/ml) was 94.8% when bactofuged at 40 C (104 F) and 96% when bactofuged at 70 C (158 F). The thermophilic count (117/ml in raw milk) was reduced by 88.9% at 70 C (158 F). The density of the sludge was 1.064 and 1.047 after bactofugation at 70 C (158 F) and 40 C (104 F), respectively.

Langeveld and Galesloot (35) studied the influence of bactofugation on the keeping quality of pasteurized milk as well as on the occurrence of the "bitty cream" defect. The experiments comprised both homogenized (clarifixated) and nonhomogenized milk. They were mainly concerned with the elimination of Bacillus cereus spores from the milk. On the average, bactofugation reduced the number of B. cereus spores in milk by 98%.

They reported an improvement in the keeping quality of both clarifixed and non-clarifixed milk: at 20 to 21 C (68 to 70 F), more than 15 hr for bottled milk and more than 20 hr for milk in plastic containers. The formation of "flecks" in the cream layer of nonhomogenized, pasteurized milk was reduced by bactofugation independent of the presence or absence of post pasteurization contamination.

Bactofugation of Cheese Milk

Perhaps the most studied application of bactofugation of milk is as a pretreatment process in the manufacture of cheese. Simonart and Debeer (63) in their first study suggested it as one of the most prominent possible applications of the process.

In Poland, Jakubowsky (29), studied centrifugation of cheese milk using an ordinary separator (500 liters/hr, 7,500 rpm). Kaolin added to the milk assisted the removal of bacteria. Reductions of 75 to 99% of bacteria and 99% of bacterial spores, molds and yeasts were obtained. Improved eye formation of Trappist cheese and somewhat impaired renneting capacity of cheese milk were observed.

Kosikowski and O'Sullivan (34) described the use of the process to treat low grade milk for the manufacture of Cheddar cheese. Reduction ranged from 95.8 to 99.8% for total counts (original counts 2.7 to 9.8×10^7 /ml) and 94.1 to 99.3% for coliforms (original counts 5.6×10^7 to

1.10×10^6 /ml). The composition of the cheese was not affected by bactofugation provided the sludge solids were pasteurized and reincorporated. The quality of the cheese was predominantly "atypical" in the case of the cheese made from non-bactofuged milk and always typical for the cheese made from the bactofuged milk.

Syrjänen (80, 81, 82) described the application of bactofugation in the manufacture of several specific types of cheese. In 1963 (80) he studied the effect of the process on the number of bacteria and the properties of milk for cheese making. He reported a 70% removal of bacteria at 4,400 liters/hr, 9,000 x g and 7,000 rpm and an "entire removal" of spores with double bactofugation. No changes in buffer capacity, clotting time of milk, or acid formation by starter bacteria were observed. In 1964 he studied its application in the manufacture of Edam cheese (81) and in the manufacture of Emmental cheese (82). For both types of cheese he obtained a reduction of 97 to 98% of Clostridium in the milk, a good reduction in coliforms (73.2%) and a better flavor. No changes in the manufacturing procedure were necessary. Peltola and Syrjänen (51) also investigated the application of bactofugation to milk for Emmental cheese-making. The effect was found to be insufficient to prevent butyric acid formation. The 10-fold reduction in spores of Clostridium left enough organisms to produce a "glaesler" defect in

most cheeses which suggested that the milk was highly contaminated.

Kosikowski and Fox (32, 33) studied the removal of E. coli and Aerobacter aerogenes organisms from Cheddar cheese milk by bactofugation. The milk was inoculated with E. coli and A. aerogenes and held overnight at 50 C (122 F). Populations of coliforms prior to treatment ranged from 5×10^5 to 1.5×10^7 . Both the non-bactofuged control and the milk to be bactofuged were heated to 55 C (130 F). The control milk gave Cheddar cheese with an "unclean" flavor.

In 1966 Simonart et al. (71) described the effect of bactofugation on the flora of Gouda cheese. Similarly to Syrjänen's work, the spectacular results obtained comparing cheese made with bactofuged and non-bactofuged milk were due mostly to the removal of Clostridia: 99.12% after single and 99.7% after double bactofugations. The bactofugation was after preheating at 78 C (171 F). The non-bactofuged milk was pasteurized at 80 C (176 F) prior to the cheese making. The starter, predominantly S. cremoris, was added after bactofugation or pasteurization. During the ripening of the cheese no undesirable flora changes occurred.

In Sweden, Lodin (37) and Lodin et al. (38) observed that the reduction in total counts with the use of bactofugation and pasteurization was 99.8% and 97%, respectively. The corresponding reduction in spore counts was 98% and 11%.

The reduction in the butyric acid bacteria was 96.0 to 99.1%. Texture problems in the cheese from bactofuged milk were negligible. Moller-Madsen (45) worked with a commercial bactofuge (9,400 to 10,900 x g) at 54 to 57 C (12.2 to 13.9 F) and obtained the following reductions:

Total bacteria	84%
Acid producers	86%
Micrococci	95%
Propionic acid bacteria	84%
Coliforms	88%
Lactobacilli	97%
Anaerobic sporeformers	94%

Rennet coagulation time for bactofuged, pasteurized milk were an average of 6 sec longer than for non-bactofuged milk.

Bactofugation of Milk to be Dried

Reduction of Bacillus cereus spores is of particular importance when producing certain types of milk powder used for baby foods (Made, 40). Made predicted other advantages of bactofugation in relation to the dry milk industry (40):

- a. Reduction of the total bacterial count, especially when producing low heat powder.
- b. Keeping the cell count (viable or non-viable) within tolerable limits.

Bactofugation as a Pre-sterilization Process

In early work Simonart and Debeer (63) mentioned the particular value of bactofugation for milks destined for

sterilization. Simonart et al. (66) used milk inoculated with Bacillus subtilis and studied the effect of bactofugation at 12,200 x g following preheating at 70 C (159 F). They found that the removal was approximately five times greater for the spore formers than for the non-spore formers. They used centrifugal force of 12,200 x g for 4 to 5 sec.

The Sludge

The centrifuge sludge contains a high number of microorganisms and, according to several authors, practically no fat. Kosikowski and O'Sullivan (34) and Kosikowski and Fox (32), when working with cheese milk, found that the protein content of the sludge was 8 to 12% and Moreno and Kosikowski (46) found it to be 8%. These authors agreed that the sludge constituted 3.0% of the volume processed at each centrifugation or 6% after the double process. Scarpari (61) found the density of the sludge was 1.047 to 1.064, depending on the temperature of bactofugation (40 C, 104 F or 70 C, 158 F).

In the market milk industry it is advisable to discard the sludge from the first bactofugation while the second which has <10% of the bacterial content of the first may be returned to the raw milk for reprocessing. By this method the milk solid losses are cut in half (27).

A recent patent by Alfa Laval AB (1) illustrates a system in which milk is heated to 72 C (161 F) and fed continuously into a bactofuge; the bactofuged milk is then

pasteurized while the sludge is sterilized at 130 to 140 C (266 to 284 F) for 1 to 4 sec and recombined with the milk.

In the cheese industry the reincorporation of the sludge is more critical to reduce losses and to avoid changes in the fat-casein ratio which would affect the body of the cheese. Kosikowski and O'Sullivan (34) pasteurized the sludge and incorporated it back into the bactofuged milk. The original amount of protein in the milk was closely but never completely regained, probably because of losses in the bowl and sampling. Kosikowski and Fox (32) devised a method by which, prior to reintroduction, the sludge was treated by the hydrogen peroxide-catalase method to destroy coliform and other bacteria. Another possibility (28) is "to ignore it [the sludge] completely and substitute spray dry milk powder in the cheese vat in an amount equivalent in weight to the protein carried by the sludge." This leads to "the added expense of paying for the powder but it too produces a cheese with good body" (28).

Thermoresistance and Germination

Thermoresistance and Ultra High Temperature (UHT) Treatment

The thermal destruction of microorganisms has been extensively studied. The excellent work by Pflug and Schmidt (54) thoroughly reviews the subject. The

thermoresistance of B. subtilis A₁ was studied at water boiling temperatures by Ridgeway (60) who first isolated the organism. He found a heat activation even after 30 min at 100 C. His data were plotted and the curve is shown in Figure 8. From the three B. subtilis strains isolated by Ridgeway (60) from sterilized milk, strain A was the only one that showed such an activation. Edwards et al. (15, 16) utilized this organism in their thermal inactivation (15) and heat injury studies in skim milk (16) at UHT. They used a modification of the survivor curve method in a capillary tube system and in a UHT unit. This was a steam injection system of industrial capacity. They compared two culture media, with and without sodium dipicolinate (CNA and FNA respectively). They found z values of 8.9 C (16 F) to 18 C (33 F) in the range from 113 C (235 F) to 135 C (275 F) when utilizing FNA as a recovery medium. Lower z values were observed when the CNA medium was used: 6.7 C (12 F) in the 113 C (235 F) to 127 C (260 F) range and 13 C (24 F) in the 127 C (260 F) to 135 C (275 F) range. The latter medium gave higher D values at all temperatures. The operation of large scale UHT equipment does not facilitate the use of extended holding times. Therefore the temperature-survivor curve, a plot of the number of surviving spores against temperature, best illustrated the thermal inactivation of their system (15).

Little precedent for this graphical presentation could be found in the literature (8, 18, 19). This type of curve may become more meaningful and useful with the present trend toward UHT processing of milk (7, 59, 74). Studies on evaluation of UHT processing systems are abundant (7, 8, 18, 19, 59, 74). All were based on the evaluation of the sporicidal effect on spore populations in water or milk and comprised both laboratory and plant trials.

B. subtilis strains were the most used species of microorganism for these UHT system tests.

In 1962 Arph and Hallström (4) described the vacuum therm instant sterilizer (VTIS) system as a "package plant" for the UHT treatment of milk. The direct steam injection system is used.

Lindgren and Swartling (36) studied the sterilizing efficiency of the VTIS using B. subtilis and Bacillus stearothermophilus strains. They found logarithmic reductions of >9 for B. subtilis and >7 for B. stearothermophilus obtained at temperatures of 130 to 140 C (266 to 284 F). The reduction was expressed in terms of sterilizing efficiency defined by Galesloot (21) as follows:

$$\text{sterilizing efficiency} = \log \frac{\text{initial spore count}}{\text{final spore count}}$$

Unfortunately, these authors and those following did not express heat resistance of organisms in terms of D and z values.

Thomé et al. (83) published a work that covered the engineering, bacteriological, chemical, taste, enzymatic and nutritive aspects of the VTIS. Their work was conducted on a laboratory model and on a full scale model. Modifications during their work on the latter resulted in the VTIS commercial unit. The two principal changes in flavor noted were "cooked" and "chalk" due to sterilization. No changes in color were observed.

Germination

The germination of spores has been studied by several authors. These studies have been based on changes that occur in the cell upon germination. Pulvertaft and Haynes (58) utilized changes in the microscopic properties of the cells, mainly the loss of refractability and the darkening of the spore as examined by phase contrast microscopy. Changes in the form and structure of the cell, which lead to changes in the optical density of the suspension of cells, have also been studied (22, 47). Other aspects of cell changes which have been studied include increase in stainability (55), the loss of spore components such as the release of dipicolinic acid (DPA) (85), and the reappearance of glucose oxidation (24, 41) and other metabolic activities. The loss of resistance to heat and chemical agents also has been a useful indication of the occurrence of germination.

The most used method for studying germination and the one that is easiest to apply is based on optical density (transmittance). It has been used in studying germination of spore formers related to milk (42) but because of the characteristics of milk this technique is not applicable for studies of germination in milk and similar substrates. The reduction of heat resistance has been successfully used as a spore germination index in milk (31, 43).

Many substances have been studied as possible germination agents. Glucose, L-alanine (and 18 other amino-acids), lactose, sucrose, pyruvate, succinate, fumarate, malate and phosphates, have been found to be effective germination triggers for B. subtilis (22, 23, 25, 56). No specific work on germination of B. subtilis A₁ was found.

Of the physical conditions studied, heat shock is the most effective germination trigger for most bacterial spores, among them B. subtilis (17).

Penicillin acts by principally blocking peptoglycan synthesis in the cell wall of growing cells and thus does not affect resting bacterial cells (10, 26). Davis (12) utilized this property of penicillin to isolate auxotrophic mutants.

EXPERIMENTAL PROCEDURES

Preparation of the Spore Suspension

The Organisms Used

For most experiments B. subtilis A₁, a strain provided by Dr. Z. John Ordal of the Department of Food Science, University of Illinois, Urbana, was used. This was the same as B. subtilis Type A, which was isolated from milk by Ridgeway (60). This strain was selected because it was isolated from sterilized milk and showed the highest heat resistance in comparison with the other strains (B. subtilis B and C) and the other species (Bacillus licheniformis) which he studied. Some work has been done on the thermal inactivation and heat injury of this organism at UHT in skim milk (15, 16).

For several experiments Bacillus cereus 7 was also used. This strain was isolated and provided by Dr. E. M. Mikolajcik at Ohio State University (31, 42).

For a few experiments B. stearothermophilus NCA 1518 was used. It was provided by Dr. D. H. Ashton from North Carolina State University who has studied its inhibition by milk components (5).

Growing the Spores

A general method and medium were devised that gave high yields of spores for the various strains used, including B. stearothermophilus. The medium was a modification of that described by Edwards et al. (15) and Kim and Naylor (30). As soon as the strains were received a spore crop was produced, cleaned as described below, and kept under refrigeration. After examination the first crop was labeled as the stock spore supply.

Whenever a new spore suspension was needed, a few ml of the stock suspension were heat shocked at 80 C (176 F) for 15 min for B. subtilis and B. cereus 120 C (248 F) for approximately 3 min for B. stearothermophilus. One ml of the heat shocked suspension was inoculated into a tube of dextrose tryptone starch broth* (Special Difco, Control 17.1041). Transfers (a loopful) were repeated every 4 to 6 hr into tubes of the same medium until heavy growth in 3 hr was observed. Four milliliter of this subculture were used as the inoculum into a 32 oz prescription bottle (GX-32, Armstrong) which contained a layer of Modified, Fortified Nutrient Agar (MFNA).** After >95% sporulation

*Dextrose 10 g, tryptone 5 g, starch 5 g, bromcresol purple 0.04 g per liter.

**Nutrient broth 8 g, bacto agar 20 g, yeast extract (Difco) 5 g, NaCl 8 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.089 g, dextrose 0.10 g, MnSO_4 (sol. 300 ppm) 30 ml, dist. H_2O 970 ml.

was attained (a maximum of 48 hr for B. subtilis A₁ and B. cereus 7, up to 1 week for B. stearothermophilus NCA 1518) the spores were washed from the agar by flooding the plates twice with 25 ml of chilled distilled water.

Immediately after heat shocking, aliquots of 3 ml of this suspension were used as inoculum for each of 10 to 30 bottles containing MFNA. The use of 2% agar in this medium, rather than the usual 1.5%, improved moisture retention during incubation at high temperature and facilitated washing of the spores from the agar surface (30). After approximately 48 hr >95% sporulation was attained. Incubation temperatures were 45 C (113 F) for B. subtilis A₁, 37 C (98.6 F) for B. cereus 7 and 55 C (131 F) for B. stearothermophilus NCA 1518. Excellent growth of B. cereus 7 was attained also when incubated at 45 C (113 F). Additional incubation for 24 hr and refrigeration of the culture bottles for 48 to 72 hr induced complete liberation of the spores from the remaining vegetative cell structures.

Harvesting and Cleaning the Spores

To harvest the spore crop, each bottle was flooded with 25 ml of chilled, sterile distilled water. The operation was repeated twice and the suspension thus obtained was filtered through three "Rapid Flow" (Johnson and Johnson) milk filters, collected in 250 ml polystyrene centrifuge bottles, each containing No. 10

glass beads and a magnetic stirring bar. The suspension in the different bottles was concentrated by centrifugation under refrigeration (Sorvall RC-2 centrifuge, 16,300 x g using the 5.75-inches head, for 10 min), resuspended in about 20 ml of sterile distilled water, magnetically stirred for 10 min and all bottles pooled into two bottles. These two bottles were submitted to four consecutive centrifugations (650 x g for the initial one and 1,465, 2,520 and 4,080 x g for the three subsequent ones) for 20 min each time (15). After each centrifugation the spore pellets were resuspended in distilled water by vigorous magnetic stirring. After the final washing, the spore pellets were resuspended in 20 ml of water, the content of the two bottles pooled together and the final suspension filtered (57) through two sterile 10 μ polypropylene membrane filters (Gelman Instrument Co., Ann Arbor, Mich.).

Examination of the Spores and Preparation of Suspensions

Microscopical examination (phase contrast) showed a clean spore suspension with no clumps and very few vegetative cells. A microscopic count (Petroff-Hauser chamber) utilizing a dry 100x dark phase objective, and plate counts were performed on the suspension.

The chamber counts were performed using a diluted suspension. The number of spores in 20 squares were counted and the average count per square used for the following calculation:

Average count/sq x 400 x 50 x 1,000 = spores/ml,
since

Area of square is $1/20 \times 1/20 = 1/400 \text{ mm}^2$, and

Depth of square is 0.02 mm = $1/50 \text{ mm}$

1 ml = $1,000 \text{ mm}^3$

If any dilution was necessary this result was multiplied by the factor of the dilution. The counts by the two methods differed by <10%. Sterile distilled water was added to adjust the concentration of suspension to approximately 8×10^8 spores/ml.

The Spore Counting Procedures

Agar Plate Count (APC)

The procedures described by the twelfth edition of the Standard Methods for the Examination of Dairy Products (3) for both thermoduric and thermophilic bacteria were found inadequate because of the difficulty encountered in counting spreaders and the lack of starch in the medium. This ingredient is of definite importance for the growth of spores. Olsen and Scott (48) postulated that starch inactivates inhibitory substances from the medium, and that unsaturated fatty acids are probably involved as inhibitors. This was confirmed by Wynne and Foster (86).

Several approaches were tried to reduce the difficulty of spreaders. Milk and milk dilutions were plated on a thin layer of hardened agar, and a second layer of agar

(approximately 10 ml) was added and mixed with the substrate. A third layer of agar (approximately 3 to 5 ml) was used on top to avoid spreading. The increase of agar to 2% in the medium helped to retain the moisture when high temperatures of incubation were used. The addition of 0.85% of salt decreased the spreading problem and increased the counts.

To recognize colonies in plating low dilutions of milk bromcresol purple was added. It gives a yellow color at a low pH. This dye is in Difco's m-dextrose tryptone broth, dextrose tryptone broth (special) and in Stumbo's (75) medium. These media are recommended for culturing sporeformers. The same amount used in Difco's media (0.04g/liter) was selected which is double that (0.02g/liter) recommended by Stumbo (75). The medium was a Standard Methods Agar modified for spores (SMAS).*

Membrane Filter Count (MFC)

The spreading problem, the difficulty in counting colonies when milk is plated, and several reports on the inhibition of sporeformers by milk components (5, 9) led to the trial of the Membrane Filter Technique. The procedures for this method basically were those suggested for coliform counts in milk (44) and dairy equipment (2).

*Plate count agar (Difco) 23.5 g, bacto agar 5.0 g, soluble starch 5.0 g, NaCl 8.5 g, bromcresol purple 0.04 g per liter.

Regular petri dishes instead of the special small ones, and SMAS instead of broth absorbed in pads were used. For the differentiation of colonies on the membrane after growth, several approaches were tried:

- a) Staining the membrane filter with malachite green solution as described in the Standard Methods
- b) Using prestained membrane filters (Green-6, Grid, Catalogue No. 5013, Gelman Instrument Co.)
- c) Using SMAS with pH indicator dye and placing the membrane filters upside down on the bottom layer of agar. This was the preferred procedure since it gave distinct yellow colonies on a purple background and allowed the use of white grid membrane filters (GA-6, Grid, Gelman). These filters showed better autoclavable properties than other brands tried.

Everytime that MFC was tried, identical replicate samples were plated following the regular APC procedure. Neither B. subtilis A₁ nor B. cereus 7 counts showed a significant difference when the plates contained from 1 to ~100 colonies. Only data from the agar plate counts were used for the calculations.

Samples were plated after heat shocking. The temperatures were 80 C (176 F) for B. subtilis A₁ and B. cereus 7, and 100 C (212 F) for B. stearothermophilus. The time was 15 min. Incubation conditions were as previously mentioned.

Thermoresistance and Germination

The Thermoresistance Experiments

Small scale thermoresistance experiments were conducted in the thermoresistometer designed and described by Pflug (52). For a few experiments thermal death time cans in miniature retorts were used.

The thermoresistometer cups described and studied by Pflug and Esselen (53) were utilized as a substrate holder. They concluded that 0.01 ml samples in the open cup gave a negligible lag correction factor so this volume of sample was used for the experiments. To measure the samples of a Gilmont micrometer syringe of 2.0 ml capacity (smallest division 0.002 ml) was used. The plastic model was preferred because of its relatively low price, ease of autoclaving and accuracy. For the few experiments with miniature retorts the cups were placed into special cans (10 per can); filter paper, impregnated with distilled water, was also enclosed. Miniature retorts were used when long treatments were necessary since the thermoresistometer was impractical.

In an effort to reproduce the plant conditions two different types of substrate were utilized. Difco dry skim milk which is a standardized medium and free of inhibitors was used. It was reconstituted to 10% by weight allowing for the addition of 1 ml inoculum. After reconstitution the milk was left overnight under

refrigeration to help rehydration of the particles and next day was centrifuged (10,000 rpm, 16,300 x g, RC-2 Sorvall centrifuge) to remove nonsoluble particles. Tests indicated that 0.2% of the milk solids were lost by this procedure. The milk was filtered in 100 ml amounts utilizing a Seitz filter with an S-1 (0.5 μ) asbestos pad (31). All trials utilizing membrane filters (0.45 μ) failed because the flow of milk stopped after a few milliliters. Chlorphenol-red (84) in 7.5 ppm amounts was added when the better detection of growth was desired. The second substrate was low spore count whole milk. Milk that contained <1 spore/10 ml was dispensed in 9 ml amounts in tubes, autoclaved at 10 psi for 5 min and incubated at 45 C for 48 hr. Negative tubes were used as substrate (pH 6.8).

Subculture in several milk preparations was used in some experiments and the results compared with those in DTS broth. The first one, autoclaved litmus milk, was used as aerobic and anaerobic substrate. To obtain anaerobic conditions sodium thioglycollate in amounts of 4 ppm was added to the milk. Sealing was accomplished by adding 2 ml of a mixture of paraffine-vaseline-mineral oil (1:1:4) (75). The second subculture medium consisted of UHT sterilized milk that did not spoil after 1 week at 45 C (113 F). Negative samples were distributed into sterile tubes, and rechecked for another week. The negative tubes were used as recovery substrate for thermoresistometer experiments.

The thermoresistance experiments were always performed with 10 replicates (10 cups with 0.01 inoculated substrate) prepared immediately before use as described by Eder (14) but without drying. Five cups at each time were exposed to the desired temperature-time combination in the thermoresistometer.

The initial number of spores were determined for each trial by placing random inoculated cups into dilution blanks (10 or 100 ml according to concentration), heat shocking for 15 min and shaking manually for 15 min before plating. The experiments for the survivor curves and the temperature-survivor curves also necessitated plate counts. The above procedure was followed. However in these cases no heat shocking was necessary

When "fraction-negative" (FN) tests were performed, after the heat treatment the cups were immediately placed in 8 ml of DTS broth contained in 20 ml tubes with resilient plastic foam plugs. These tubes were incubated at 45 C (113 F) for 1 week. Growth was evidenced by characteristic visual changes. The results of the fraction-negative results were processed according to the Stumbo, Murphy and Cochrane method described by Pflug and Schmidt (54). The D values obtained by this method were plotted semilogarithmically vs. temperature. From the resulting thermoresistance (TR) curves, z values were determined graphically.

The Germination Experiments

In the germination experiments only B. subtilis A₁ spores were used. Replicate bottles of 100 ml were inoculated with $>10^2$ to $>10^4$ spores of B. subtilis per ml. The initial population was determined by plating 1 ml from each of the heat shocked milk replicates. The replicates were incubated at 45 C (113 F). After time intervals of 0, 3, 6, 18, 24 and 48 hr samples were taken. After refrigeration for 24 hr or more these samples were heat shocked and plated.

The use of a penicillin-penicillinase system was tried to inhibit outgrowth of the germinated spores that could cause the formation of secondary spores. Penicillin G was added in 100 units/ml of milk. Duplicate controls containing no penicillin were tested. Refrigeration after incubation allowed the penicillin to act further on the growing cells. The action of penicillin was stopped before plating by adding a penicillinase suspension either to the bottles or to the agar. The suspension contained enough penicillinase to inactivate three times the amount of penicillin.

Plant Procedures

The Bactofuge

A Type D3187M Bactofuge provided by De Laval Separator Company was used for the experiments. It was much like a

hermetic milk clarifier but had two 0.3mm holes in the bowl for sludge outlets. The milk was fed in at the bottom of the machine, passed through the distributor and flowed into the disc stack through the holes in the discs. It passed into the center of the bowl and was discharged at the top free from foam.

The sludge containing bacteria was gathered in the bowl casing which was equipped with a special groove for the removal of the sludge and cooling air. Air and sludge were separated from each other in an attached cyclone. The contaminated air may be directed back into the hood frame for recirculation but for the experiments it was exhausted into the atmosphere. The maximum capacity of the machine was 6,000 liters/hr (approx. 13,000 lb./hr). The machine operated with a centrifugal force of about 9,000 x g. Two revolution counting devices were provided on the machine, a tachometer and a pulsing revolution counter.

The VTIS

The sterilization equipment consisted of a size A VTIS provided by the De Laval Separator Company.

A centrifugal pump fed the milk to a plate heat exchanger for preheating to 57.2 C (135 F) by means of vapors from the vacuum chamber. Then the milk flowed to a similar unit in which it was heated indirectly to 76.7 C (170 F) with steam. A timing pump controlled the flow of the milk to the steam injection head. The temperature

was raised immediately to the desired sterilization temperature within the range of 136 C (270 F) to 149 C (300 F). The time the product was in the holding tube was calculated to be 3.8 sec. The flow diversion valve was set according to the temperature of sterilization used. In forward flow the milk was flash cooled in the first vacuum chamber; its temperature dropped to approximately 136 C (270 F) in order that the same amount of water would be flashed off as was previously condensed during direct steam heating. The temperature of the milk in the vacuum chamber was controlled by the vacuum regulator in the vapor line from the vacuum chambers. The system had a diverting chamber if the temperature was below 140.6 C (285 F). A centrifugal pump removed the milk from the vacuum chamber and directed it to the aseptic homogenizer. It was then cooled to 10 to 21 C (50 to 70 F) in a specially designed aseptic plate cooler.

The Bactofugation Experiments

The general procedure used in the trials is shown in Figure 1. Approximately 780 kg (210 gal) of raw milk from the University herd were held for 1 or 2 days at 4.4 C (40 F). The milk had a fat content of 3.25 to 3.50% and a pH of 6.65 to 6.75.

Raw samples were taken and immediately inoculated with the spores while cold (approximately 4.4 C; 40 F), and agitated in a vat for not less than 30 min. The small

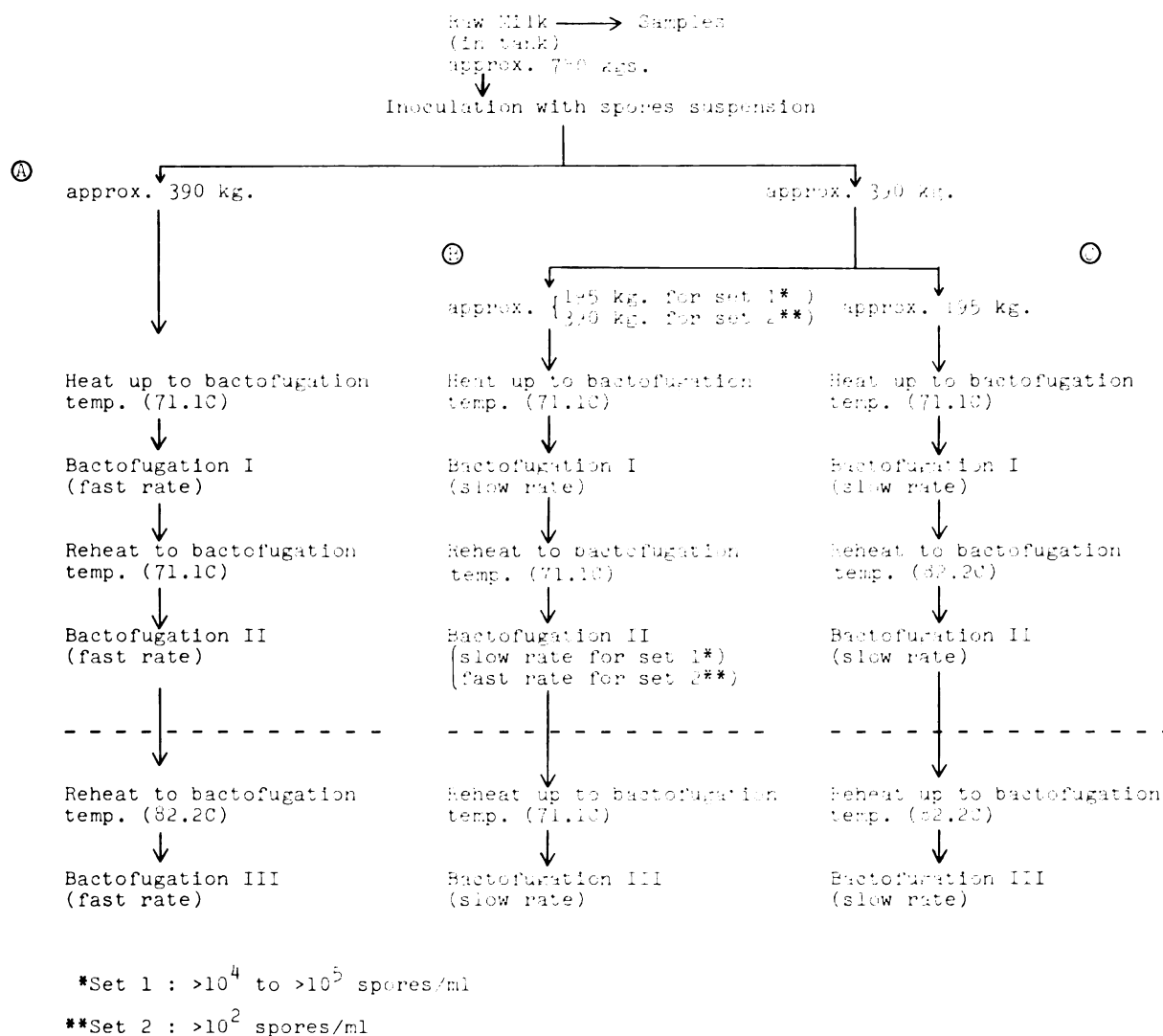


Fig. 1.--General pattern of the bactofugation experiments.

volume of inoculum prepared, as explained previously, was always placed in about 1 liter of cold raw milk and thoroughly mixed by stirring with a glass rod before being slowly poured into the bulk tank. After the mixing period, samples of the raw inoculated milk were aseptically taken. The milk to be bactofuged was then heated up to the bactofugation temperature of 71.1 C (160 F) in the double jacketed vat and pumped centrifugally into the bactofuge. The tachometer and pulsating revolution counter readings were recorded.

By weighing milk collected in a 10-gal can and recording the time with a stop watch the flow rate was calculated. From the line at the outlet of the bactofuge samples were taken aseptically during the operation. Also, samples of the sludge were taken. The total weight of the sludge at each subtrial was recorded. After the first run through the bactofuge, the milk was reheated to 71.1 or 82 C (160 or 180 F) and a second run was made through the bactofuge. Sampling, flow rate determination and revolution and pulse counts as well as sludge weights were recorded. In some trials the process was repeated a third time.

The first set of trials was conducted to compare the influence of the flow rate temperature, and a third bactofugation, on the bactofugation effect. This set comprised subtrials A, B and C in each trial. The influence of flow rate was investigated in the second set of trials. Milk

containing 1/100 less inoculated spores than on the regular trials was used. This second set had only two subtrials, A and B. Figure 1 shows the general flow scheme for these two sets of trials. Table 1 (Appendix) shows the detailed features of the operation of the bactofuge. The slow flow rate was 1,540 to 2,010 kg/hr (3,400 to 4,400 lb./hr) and the fast flow rate was 4,850 to 6,000 kg/hr (11,700 to 13,200 lb./hr). The process time was 3 to 30 min. The pressure imparted by the feeding pump was -7 to +4 psi. The tachometer readings were quite uniform, from 1,650 to 1,800 rpm and always slightly lower for the second (and third) bactofugation.

The Bactofugation-Sterilization Experiments

This group of experiments was designed to study the effect of bactofugation upon the keeping quality of UHT sterilized milk. The procedure is outlined in Figure 2, except that after sampling, the inoculated milk was divided into two equal batches, control milk and milk to be bactofuged. After bactofugation the milk was cooled to about 4.4 to 21.1 C (40 to 70 F), with the temperature depending on the time elapsed between bactofugation and VTIS treatment, usually 0.5 to 3.0 hr.

The VTIS unit was sterilized at 144.4 to 145.6 C (292 to 294 F). Bactofuged (B) milk was processed first, followed by the non-bactofuged (NB) control milk.

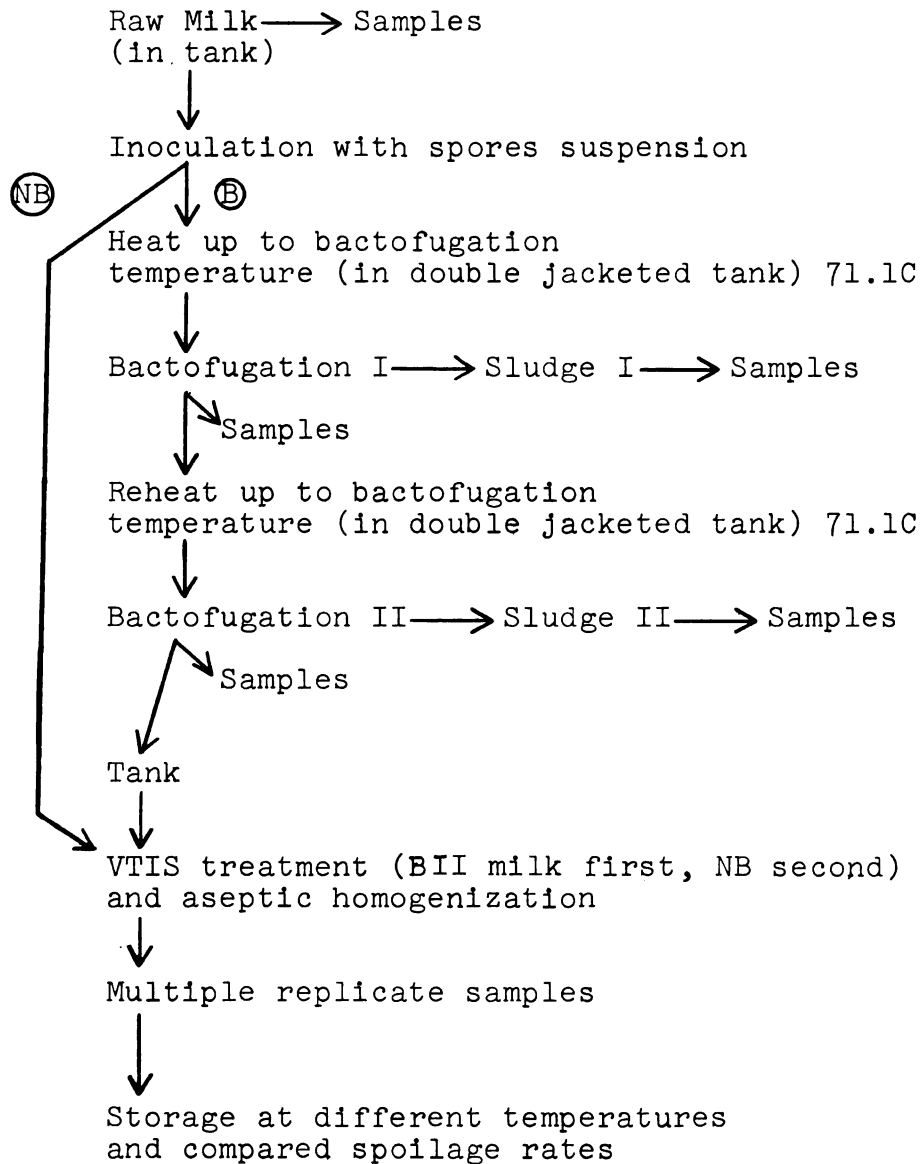


Fig. 2.--General pattern of the experiments with bacterofugation followed by sterilization.

Replicate samples were taken after sterilization. Index samples were procured by continuously sampling during sterilization of the milk. Table 1 (Appendix) shows the operating conditions of the VTIS unit during milk sterilization.

Handling the Samples

Samples of the raw (R) milk were taken before inoculation. Generally five samples of the inoculated raw milk (I) were obtained and plated in duplicate. The same applied to the bactofuged samples (BI, BII and BIII). These samples were taken by the same procedure and at about the same time during the different trials. Also two samples of each of the sludges (SI, SII, SIII) produced during each bactofugation were taken. Spore plate counts were carried out as described previously. Samples I, BI, BII, and BIII were plated in duplicate, at different dilutions. Spore count platings of each of the duplicate samples were made for R, SI, SII, and SIII at two different dilutions.

Standard plate counts of one of the replicates of each sample, selected at random, was carried out each time.

Samples from the VTIS were taken utilizing a sterilized chamber with attached rubber gloves for hands and arms. Ethylene oxide gas was the sterilization agent. The sterilizing effect of ethylene oxide was checked with filter paper strips or copper paper clips inoculated with about 10^8

spores of B. subtilis A₁ or B. subtilis var. globigii. In all cases the tests were negative, indicating complete sterilization.

Sixty samples were taken for each trial batch (30 for NB + VTIS, 30 for B + VTIS). Each sample was at 1/2 pint or approximately 200 ml of milk with a sterile cap and aluminum foil on top. Ten samples of each batch were incubated at 45 or 37 C (113 or 98.6 F), 32 C (89.6 F) and 21 C (79 F) for 8 weeks. Samples were checked visually for spoilage at 1, 2, 4 and 8 weeks and confirmed by microscopic examination, phase contrast microscopy, and by isolation of the organisms used.

The Statistical Analysis of Data and Calculations

A FORTRAN IV program was designed to make statistical analysis of data and calculate the reduction percentages in the University's CDC 3600 digital computer. The mean (AMEAN) and standard deviation (STD) of the replicate sample counts (XI) were calculated (13). The program provided for the elimination of any replicate count which deviated from the mean by more than two standard deviations. For the purified data (XAD) the means (AMEANA), standard deviation (STDAD) and 95% confidence limits (L951 and L952) were calculated. The statistical data are shown in Table 2 (Appendix). These refined data were used to calculate the per cent reduction for each bactofugation, initial and total (cumulative).

Another FORTRAN IV computer program was designed to calculate the per cent losses of sludge (PTS1, PTS2 and PTS3). This was calculated on the basis of the known initial volume (TV) converted to weight (TW), and the weight of the sludge (S1, S2 and S3) at each process. These results are shown in Table 2 (Appendix). The programs are shown in Tables 3 and 4 (Appendix).

RESULTS AND DISCUSSION

Data in Table 5 (Appendix) show the reduction in mean spore counts as well as the reduction in standard plate counts for the inoculated control (I) and the bactofuged samples (BI, BII, BIII). Data showing the spore and standard plate counts for the milk prior to inoculation (R), as well as the sludge content for the one, two and/or three bactofugations (S1, S2, S3), are also in Table 5.

The first trial (117) involved non-inoculated milk which had very low initial numbers of spores (4.25/ml); consequently it was very difficult to evaluate the remaining population after bactofugation.

Figure 3 gives the results using the slow flow rate for both BI and BII. All trials indicated a definite pattern of reduction of the spore population, an efficient removal for the first bactofugation (99.24 to 99.76%) and a much lower removal for the second bactofugation (0 to 43.21%). There were some trials where the increase in mean count after BII in relation to BI suggested an "anti-reduction" effect. However, this apparent increase in the counts after BII might also have been caused by statistical variation or experimental error in counting. These reasons may account for the negative reduction values occasionally shown in Table 5.

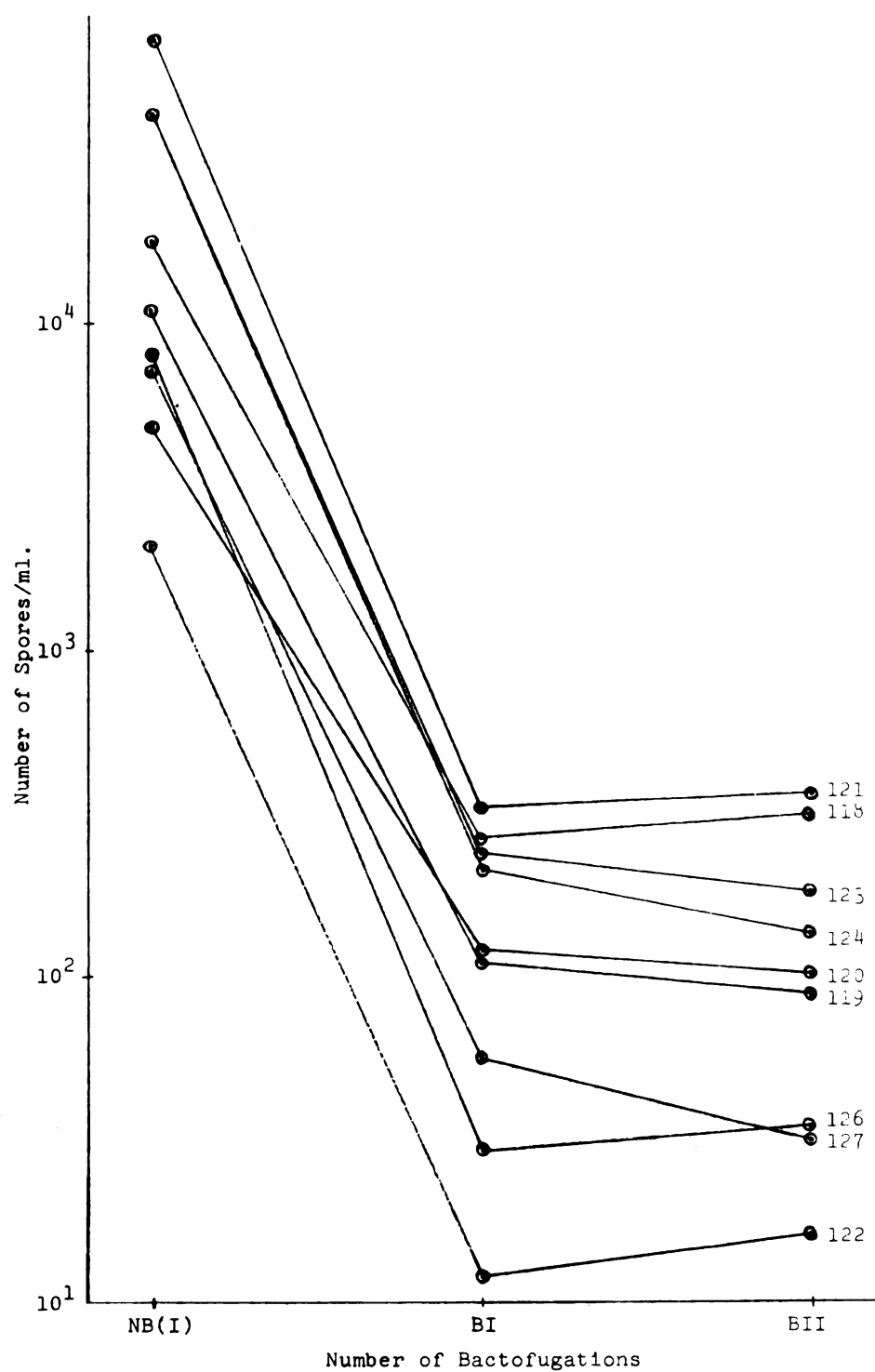


Fig. 3.--Mean counts of *B. subtilis* A₁ spores with one or two bactofugations at ~1800 kg/hr.

To eliminate the possibility that the sludge in the bowl could have been responsible for the low efficiency of removal observed during the second bactofugation, the bowl was disassembled and cleaned between BI and BII. Figure 4 shows the results of an identical procedure, except for the washing of the bowl between bactofugations. No significant increase in the removal for the second bactofugation was demonstrated by cleaning the bowl. Figure 4 also shows the reduction curves for SPC. Data in Table 5 indicate that in all trials the reduction in SPC followed the same trend as the removal of spores. However, because of the low SPC of the raw milk and the high spore inoculum, the spores contributed considerably to the SPC which was performed only as a guide.

Because of the heat resistance of the spores and the counting procedure one may assume that the spore reductions were mainly by removal while SPC reduction comprised the total bactofugation effect which consists of centrifugal force and lethality of the bactofugation temperature.

Figure 5 shows the effect of changes in the flow rate upon the removal of spores. Two different sets of trials were carried out. In the first set, besides changes in flow rate and temperature of BII, a third bactofugation took place.

The results of trials 132, 133, 152 and 154 were averaged and are illustrated in Figure 5. BI, BII and BIII

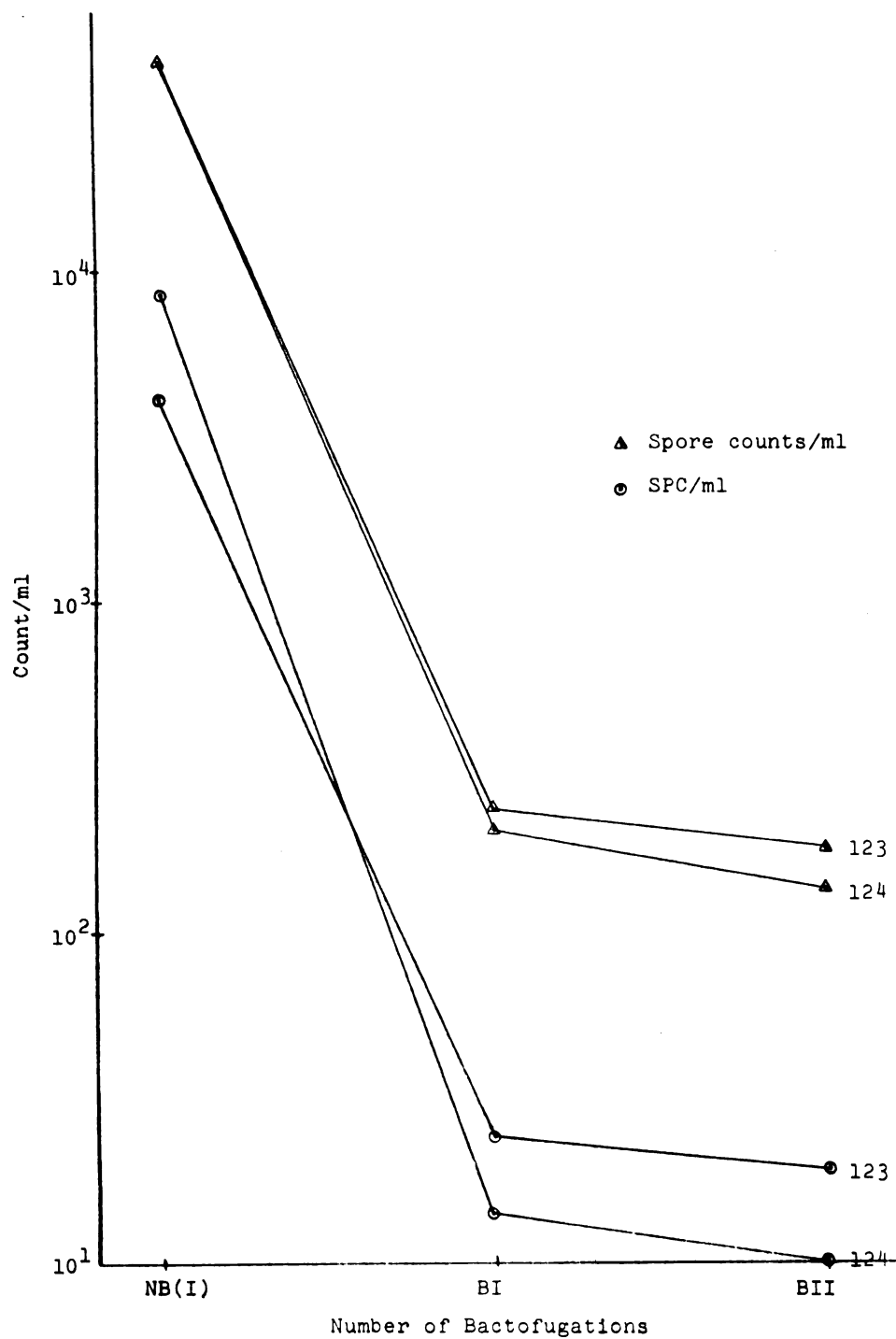


Fig. 4.--SPC and spores counted with (trial 124) and without (trial 123) cleaning the bactofuge bowl between BI and BII.

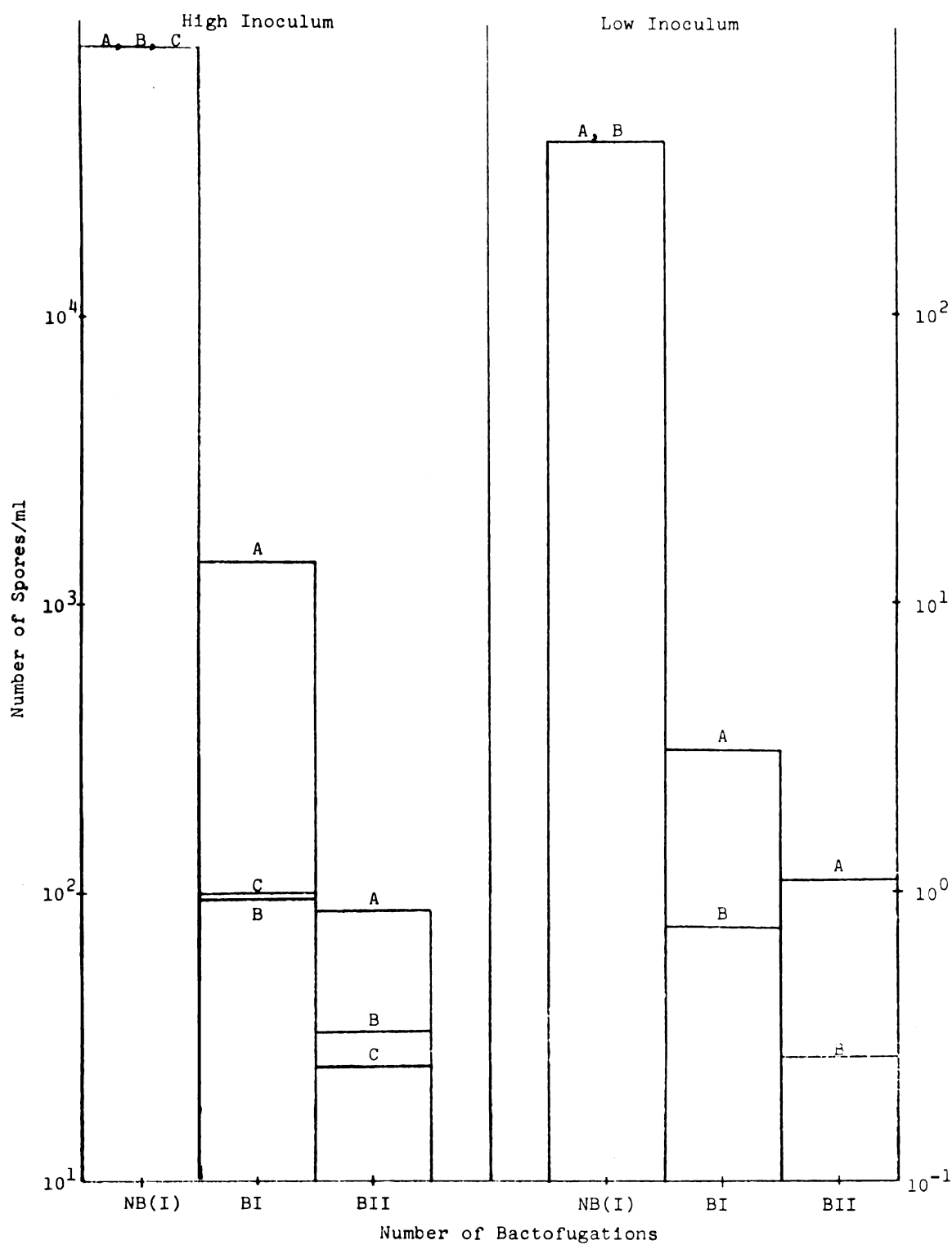


Fig. 5.--Removal of *B. subtilis* A₁ spores by bactofugation. Flow rate was ~5,400 kg/hr for subtrial A and ~1,800 kg/hr for B and C. The temperature of bactofugation was 71 C except for BII in subtrial C (82 C).

had the faster flow rate in subtrials A and the slower in subtrials B and C. In the trials with the higher spore population ($>10^4/\text{ml}$), subtrial A showed a lower per cent removal for BI (98.14 to 98.52%) than in subtrials B and C (99.87 to 99.89%). The per cent removal for BII was in contrast considerably higher in A (92.93 to 94.30%) in comparison to B and C (38.65 to 79.00%). However the percentage of spores remaining after BII in the subtrial A was approximately equal to that remaining after only BI in the B and C subtrials. Thus by single centrifugation at the slow flow rate (one-third the maximum flow rate) approximately the same effect was obtained as when double bactofugation at the fast flow rate took place. Commercially, however, there are other considerations, for example, sludge losses.

The percentage removal of spores for BII was 44.18 to 60% for subtrial A and 18.18 to 22.73% for subtrials B and C. In general BIII was found to be too inefficient and therefore it was considered unnecessary. In the trials with the lower spore population ($>10^2/\text{ml}$) the situation was similar to the trials with a higher inoculum, although in subtrial A the per cent removal for BI (98.88 to 99.30%) was not as low and the per cent removal for BII (69.33 to 71.26%) was not as high as in the trials with the higher inoculum. In subtrial B the removal was 99.79 to 99.81% for BI and 60.87 to 68.12% for BII.

Thus, these two sets of trials show that when the faster and the slower flow rates were compared during BI a higher efficiency was obtained with the slower flow rate. For the subsequent bactofugations (BII and BIII), however, although performed at different flow rates in the different subtrials, the differences in efficiency were due more to the different percentage of spores remaining after BI (for BII to act upon) than to the flow rate.

In the high inoculum trials changes in the temperature of BII from 71.1 C (160 F) to 82.2 C (180 F) as recommended by Simonart (62), did not show any significant effect on the percentage of removal of spores of B. subtilis A₁. The percentages removed were, for BII 78.65 to 78.96% at the lower temperature and 72.06 to 79.00% at the higher temperature, and for BIII 18.18% and 22.73%.

Figure 6 shows the removal of spores in 12 trials. The trials represented in this figure differ from those in Figure 5. BI took place at the slow flow rate and BII at the fast flow rate. The percentage removal was 99.72 to 99.98% for BI with the high inoculum and 99.68 to 99.82% with the low inoculum; for BII 48.19 to 80.13% with the high inoculum and 42.50 to 60.00% for the low inoculum. Again, the initial population did not seem to affect significantly the efficiency of removal, although the low inoculum gave initial populations of about the same magnitude as the population remaining after BI, when the high

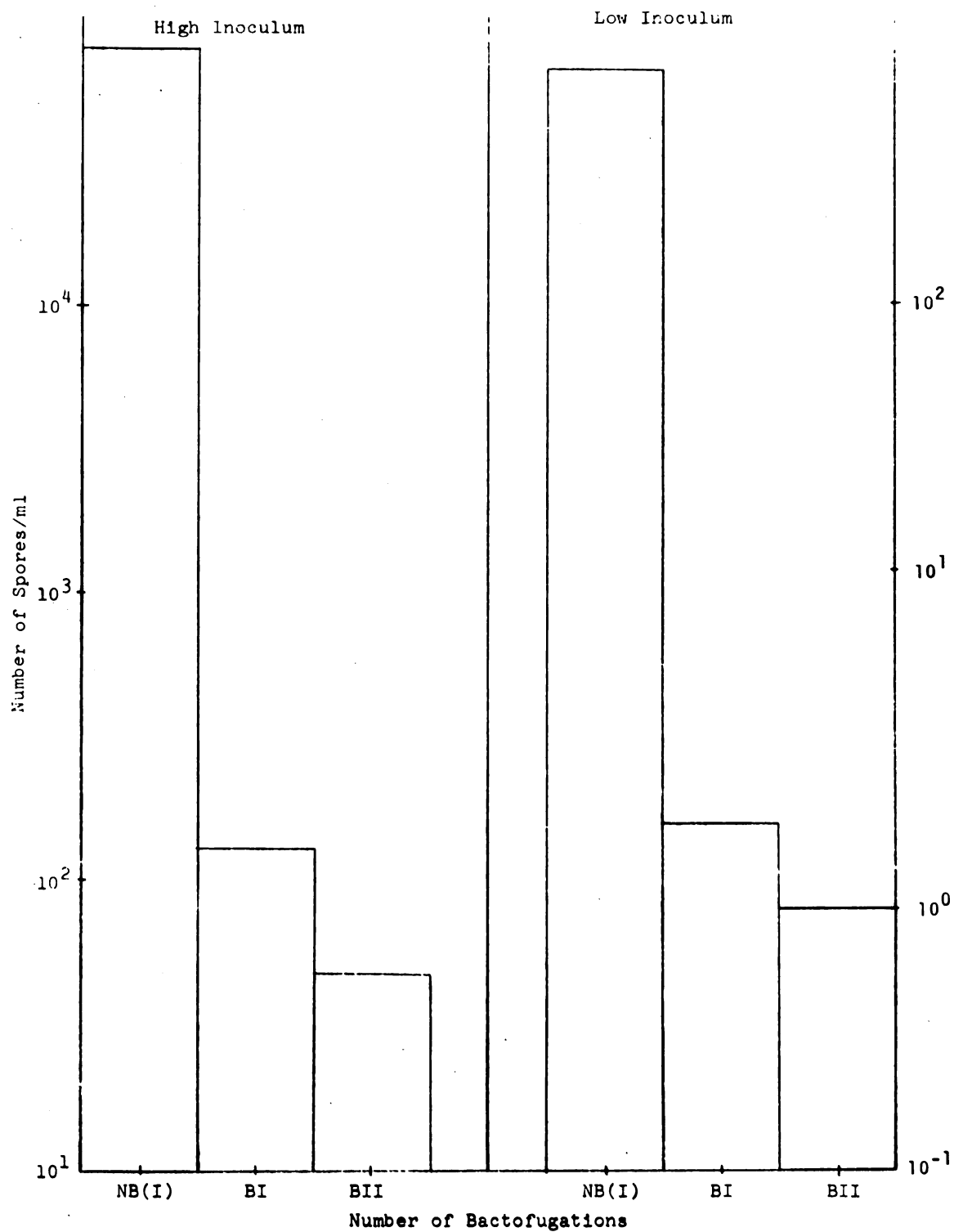


Fig. 6.--Removal of *B. subtilis* A₁ spores when BI was at ~1,800 kg/hr. and BII at ~5,400 kg/hr.

inoculum was used. Figure 6 and also Figure 5 indicate that in a particular population of spores the "removability," which is directly related to the specific gravity of the spores (Stokes law), follows a normal distribution among the cells. Thus, some of the spores were very removable, the majority were moderately removable, while some were only slightly removable, under the same conditions. A similar type of distribution is also true of the heat resistance of spores (20). BI would then have removed the highly removable and the removable spores while, no matter how many bactofugations were conducted, a percentage of the least removable spores will remain in the milk. The magnitude and importance of the number remaining depends on the initial number. This contention would explain the low efficacy of BII (and BIII) since only the less removable spores would have remained. One way to improve efficacy under these conditions would be to increase the centrifugal force (gravities) of the process.

Commercial raw milk in developing countries probably would have $\sim 10^2$ spores/ml which is similar to the counts on the samples with the low inoculum presented in Figures 5 and 6.

By carrying out the second bactofugation at the faster flow rate in the trials illustrated in Figure 6, the "anti-reduction effect" disappeared. The phenomenon of apparent or real increase had been observed in a preliminary group

of trials (Figure 3) in which BI and BII were conducted at the slower flow rate. An explanation is the use of a fast flow rate seemed to give, in some cases, a slightly better reduction in the counts of milk bactofuged twice and/or three times. This reduction might have been due to the fact that a fast flow rate accelerated the exit of the sludge and bactofuged milk from the bowl, and reduced the time of contact between the bactofuged milk and the sludge on the wall of the bowl. Such change might have prevented reincorporation of spores from sludge before they leave the bowl. Because of the small number of remaining spores, a slight variation in the counts significantly affected the percentage of removal. The reintroduction of spores might have also occurred during BI especially when slow flow rate was used but the removal effect was so great that small changes in the counts did not affect the results. Nevertheless, the phenomenon of antireduction was only of academic interest, and in practice the small difference in percentage when translated into number of spores would not be significant.

Figure 7 shows the results of four trials involving the use of B. cereus 7. The removal pattern in these trials followed the same general trend as when B. subtilis A₁ was used. BI was carried out with the slower flow rate, except for subtrial A (trial 134) shown on the left half of the graph. The percentage removal was high (98.53 to

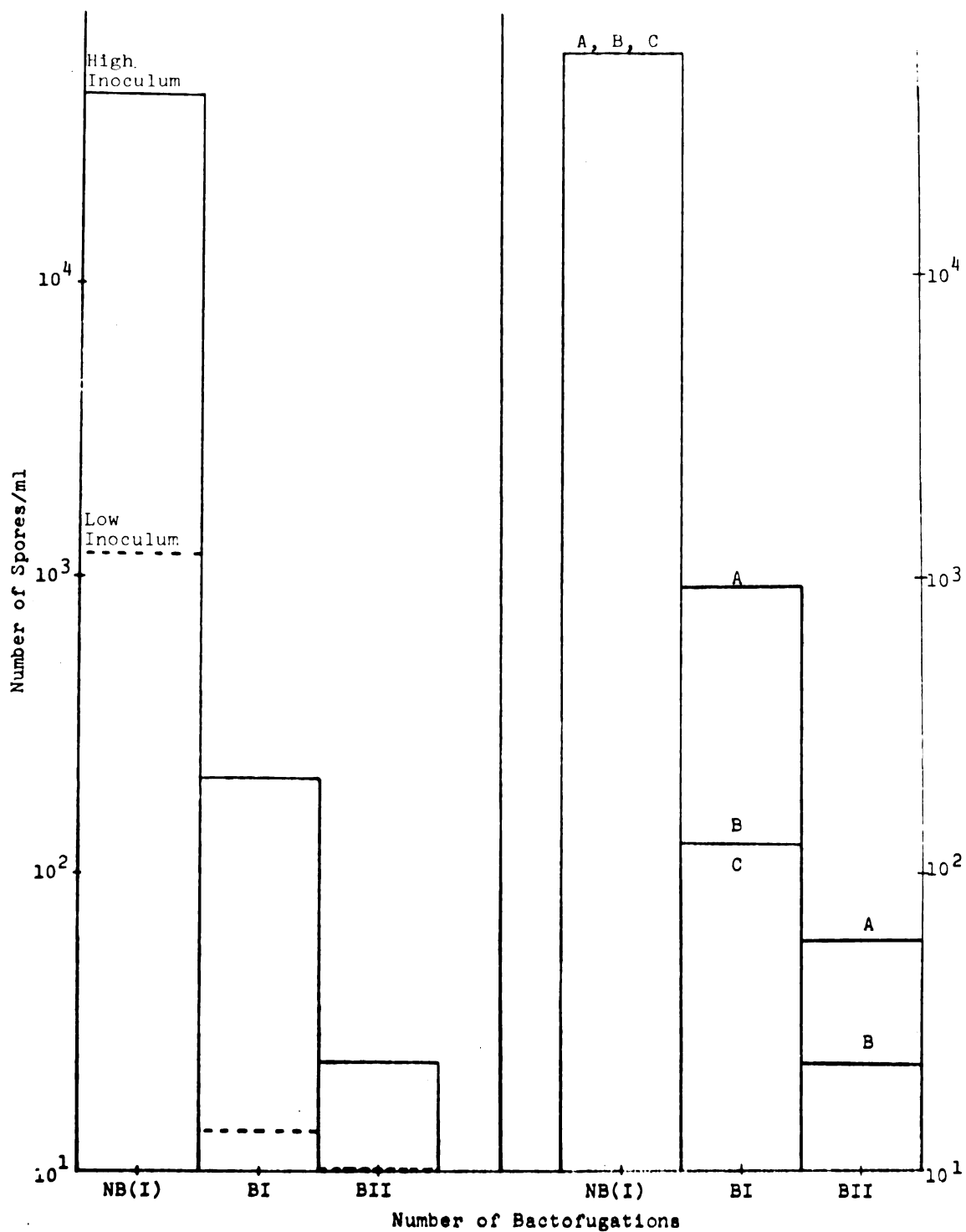


Fig. 7.--Removal of *B. cereus* 7 spores when BI flow rate was $\sim 1,800$ kg/hr and BII $\sim 5,400$ kg/hr (left graph). Subtrials A (right graph) were at the faster flow rate and B and C at the slower flow rate.

99.79%) for BI with the higher inoculum and was 98.87% for the lower inoculum but was 26.00 to 72.77% for BII for both high and low inoculum.

BII in the lower inoculum trial and in subtrial A, shown on the left half of the graph, was with the faster flow rate. BI with the faster flow rate in subtrial A gave a slightly lower per cent removal (98.65%) than with the slower flow rate (99.82%) and consequently a higher percentage of remaining spores; thus BII per cent removal (93.61%) for A was higher than normal. The per cent removal with BII was exceptionally high for B (90.29%) and C (98.14%) at 71.1 C (160 F) and 82.2 C (180 F) respectively. This suggested the possibility of lethal effect during bactofugation due to longer subjection to heat for the much less resistant species involved.

The trial with B. stearothermophilus NCA 1518 (131) was successful only for BI. The very low count after BII caused its evaluation to be difficult even with MPN techniques. However, the total removal of spores was >99.5% since the removal for BI was 99.5%.

The spore reductions were, in general, slightly higher than reported in the literature (37, 38, 39, 51, 63, 66, 80, 81, 82).

Except for the work by Simonart et al. (66) no specific spore counting techniques were described in the literature reviewed. Several authors (37, 38, 51, 80, 81, 82) referred to reductions of >90% of anaerobic (Clostridium)

spores. Syrjänen (80) reported the "entire removal" of spores which seems very improbable.

None of these authors, except Simonart et al. (66), studied aerobic spores removal specifically, and his equipment was at a semi-industrial level with a flow rate of 400 kg/hr. He utilized only one species.

Kosikowski and O'Sullivan (34) also worked with reduced flow rate due to limitations of their heating equipment. They commented on a possible increase of the efficiency of removal and mentioned similar results by Simonart in a personal communication, but provided no data.

Surkov and Schmidt (79) working with a laboratory centrifuge reported an increase in the removal of bacteria and spores by utilizing half the maximum flow rate but they did not conduct experiments on the influence of this higher removal on the efficiency of the second bactofugation.

The flow rate had a very marked influence on the amount of sludge eliminated from the milk by bactofugation. At the slower flow rate the sludge losses ranged from 5.28 to 6.98% by weight (Table 1). At the fast flow rate it constituted 1.31 to 2.59% of the milk which was approximately three to four times less than the slower flow rate. Other authors (32, 33, 34, 46), using the same type of machine at 50% normal flow rate, also found per cent losses of a higher magnitude (2.5 to 3.5%) than those reported for the normal flow rate (1.35 to 1.85%). As reported by

the same and other (80) authors, the per cent fat observed in the sludge was negligible.

Although the flow rate determined the volume of sludge, and consequently the concentration of microorganisms, there is a proportional relationship between the spore counts of sludge and their reduction in the bactofuged milk. The sludge counts, however, are two, sometimes three, log cycles greater than the count of the corresponding bactofuged milk in the majority of the trials.

The results of the fraction negative (FN) tests are shown in Tables 6 and 7 (Appendix). Collateral experiments were performed to ascertain the differences in FN results by using different subculturing substrates such as DTS broth, litmus milk and milk with anaerobic conditions. These tests were performed at 143.3 C (290 F). No significant differences were found.

Figure 8 shows the heat activation curve of B. subtilis A₁ resulting from plotting the data of Ridgeway (60).

Figure 9 shows the thermoresistance (TR) curve plotted from data in Tables 6 and 7 (Appendix). The z value for this curve is 12 C (21.5 F). Data in the two thermoresistance experiments are so compatible that the curve passes between the two sets of data, indicating almost the same slope. The z values individually calculated were 11.1 and 12.2 C (20 and 22 F). Below 121.1 C (250 F)

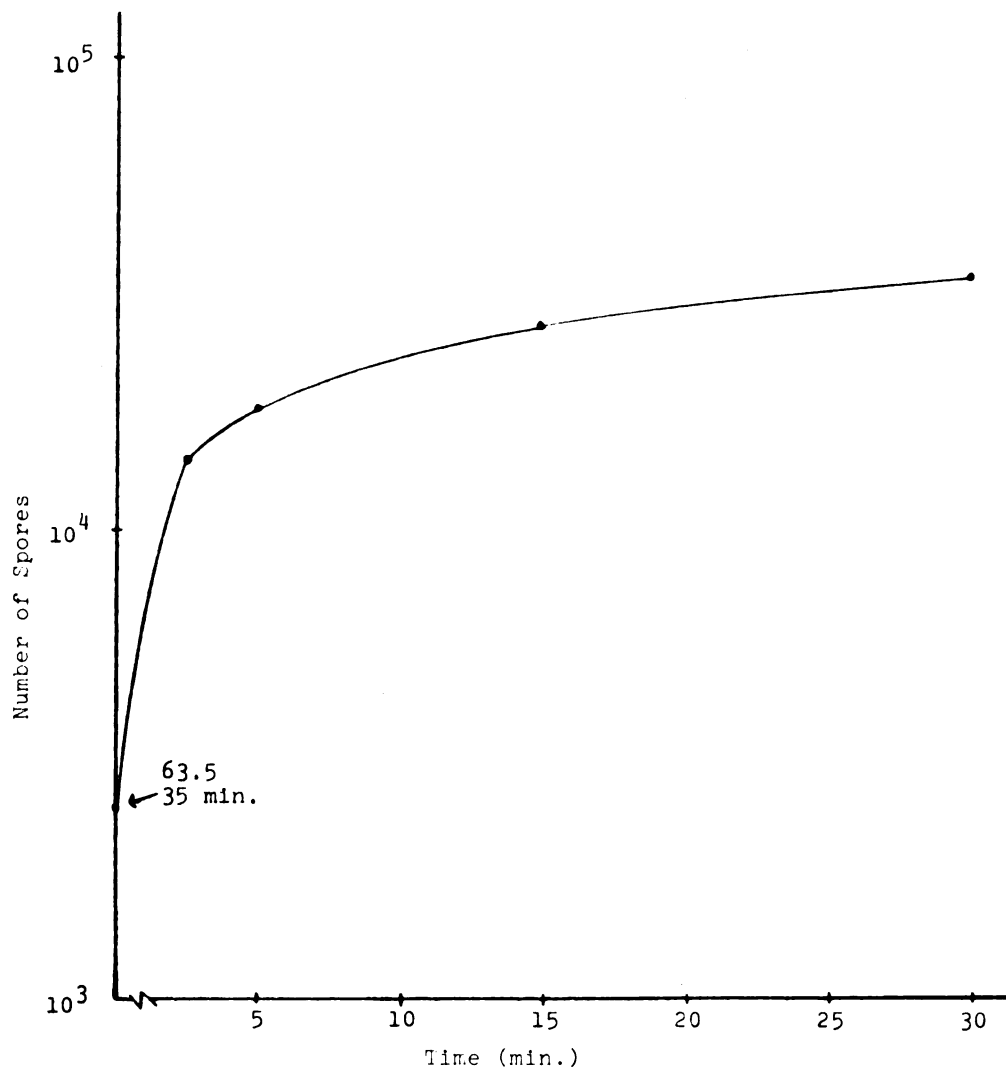


Fig.8.--Heat activation of *E. subtilis* A₁ spores at 100C (212F) according to the data by Ridgway (60).

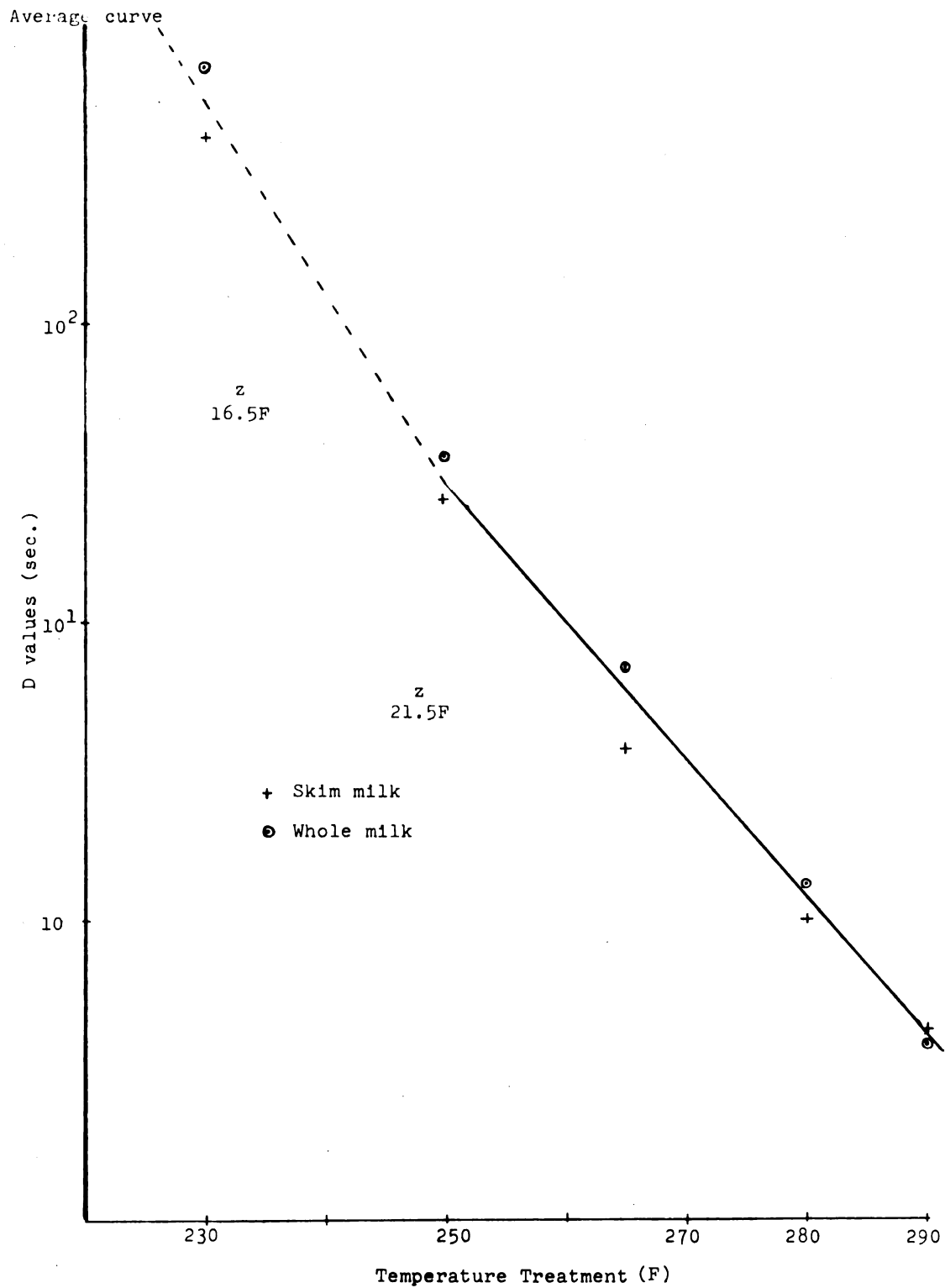


Fig. 9.--Thermoresistance curve of B. subtilis A₁ at UHT in milk.

the curve has another slope and a z value of 9.2 C (16.5 F), giving a concave TR curve.

Although the D values in skim milk are lower than in whole milk except at 143.3 C (290 F), the difference is not significant when the D values in seconds are converted into minutes. (The initial number of spores in skim milk was approximately 10 fold more than the initial number in whole milk.) This difference diminishes as the temperature increases. Skim milk and whole milk as supporting substrates thus gave approximately the same heat resistance results.

The heat resistance data and the TR curves are not significantly different from those reported by Edwards (15) for skim milk, although his technique was different. He also found concave curves with lower z values at the lower temperatures. The z values are between those found by him for FNA and CNA recovery media.

The survivor curves for 110 C (230 F) and 121.1 C (250 F) are shown in Figures 10 and 11 (data in Tables 8 and 9, Appendix). These trials were conducted only with whole milk. D_{121} is similar to the average D found by FN tests. D_{110} is considerably higher for the curve.

Temperature-survivor data for skim milk and whole milk may be seen in Tables 10 and 11 (Appendix). The temperature-survivor curves for these cannot be combined nor averaged since the counts in one are ten times greater than in the other. Thus the temperature-survivor curves

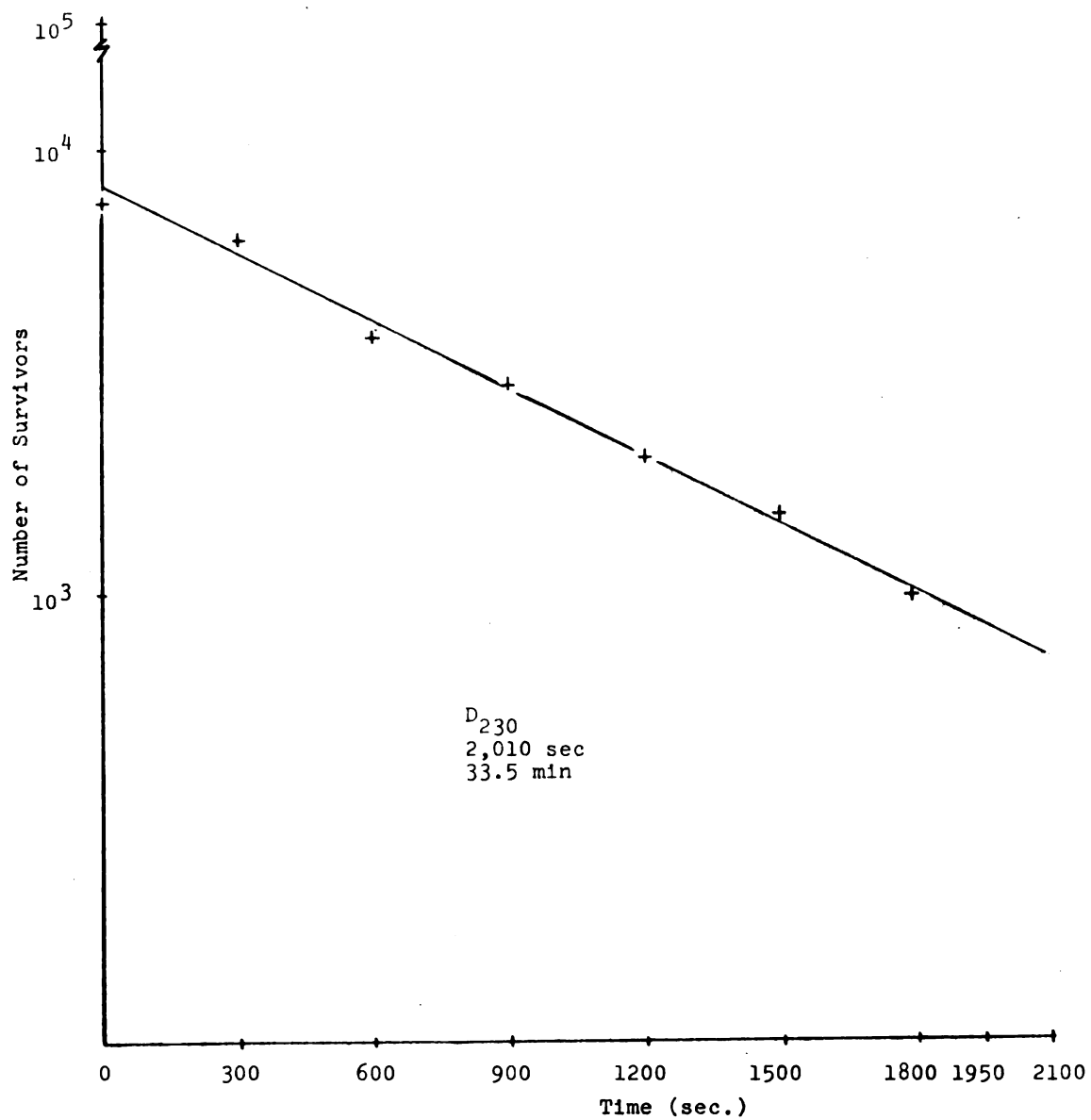


Fig. 10.--Survivor curve for B. subtilis A₁ at 110C (230F).

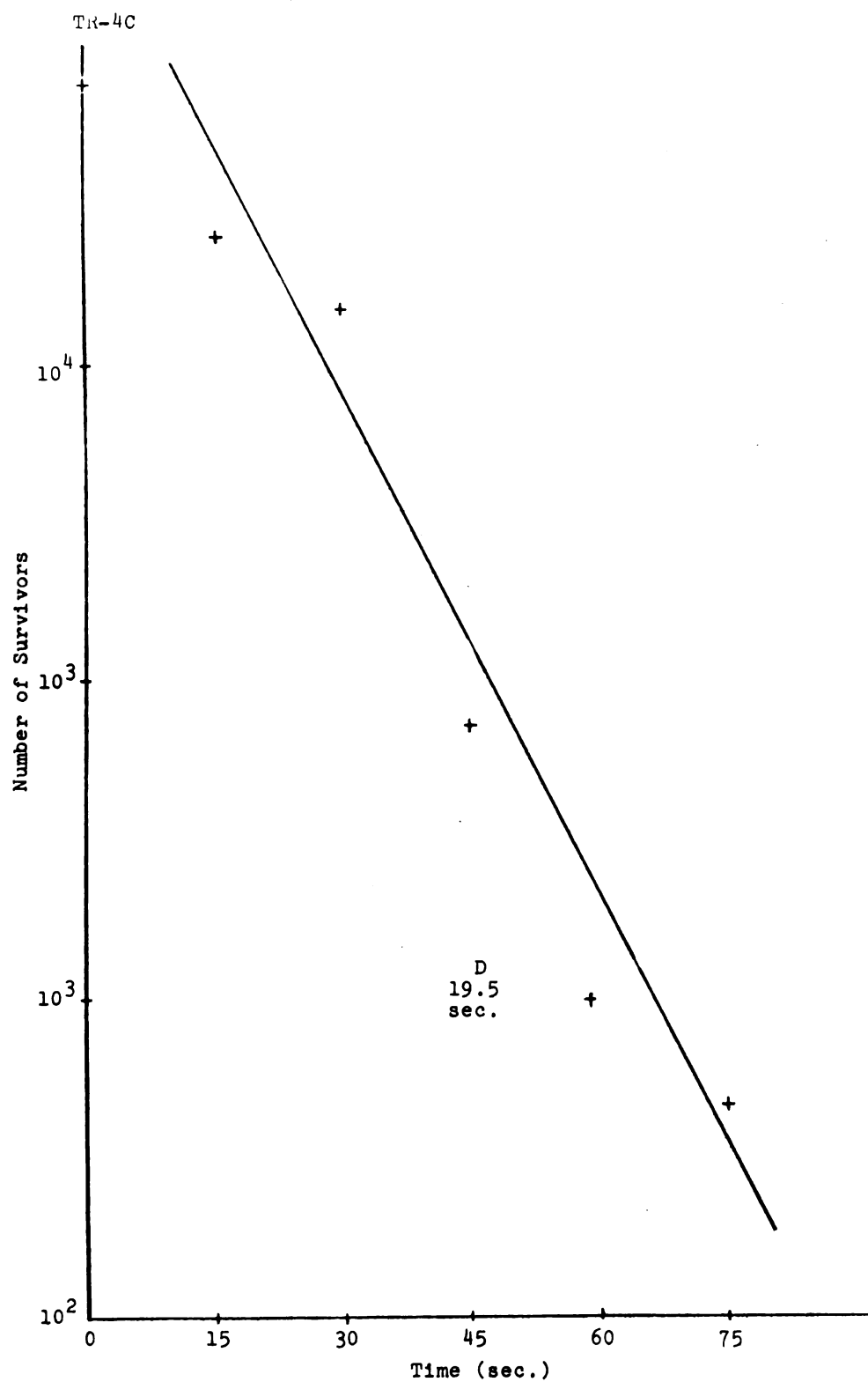


Fig. 11.--Survivor curve for B. subtilis A₁ at 121.1C (250F) in milk.

for each of the two trials were plotted (Figure 12). The two curves are similar and follow the same pattern of the curves of Edwards et al. (15).

The heat induced increase in spore count between the control and the lowest temperature treatment is worth noting. This phenomenon was checked by repeated experiments comparing counts performed after 80 C (176 F) for 15 min and counts after 110 C (230 F) for 4.0 sec. The results were similar.

The thermoresistance of B. cereus 7 was also studied. No detectable survival occurred with the minimum holding times at 132.2 C (270 F) and 143.3 C (290 F). A very low D_{121} (0.632 sec) was observed.

The germination trials were to study the possibility of reducing heat resistance of spores present in milk by stimulating their germination before sterilization. Heat shock at temperatures lower than 80 C (176 F) required a long heating time, and higher temperatures were not practical because of a cooked flavor problem. The result of these experiments was negative since no significant decrease in the spore counts occurred during incubation up to 48 hr (Table 12, Appendix). After this period of time an increase was apparent. This indicated that secondary spores were being produced from the vegetative cells germinated from primary spores. The use of a penicillin-penicillinase was tried to avoid the formation of secondary

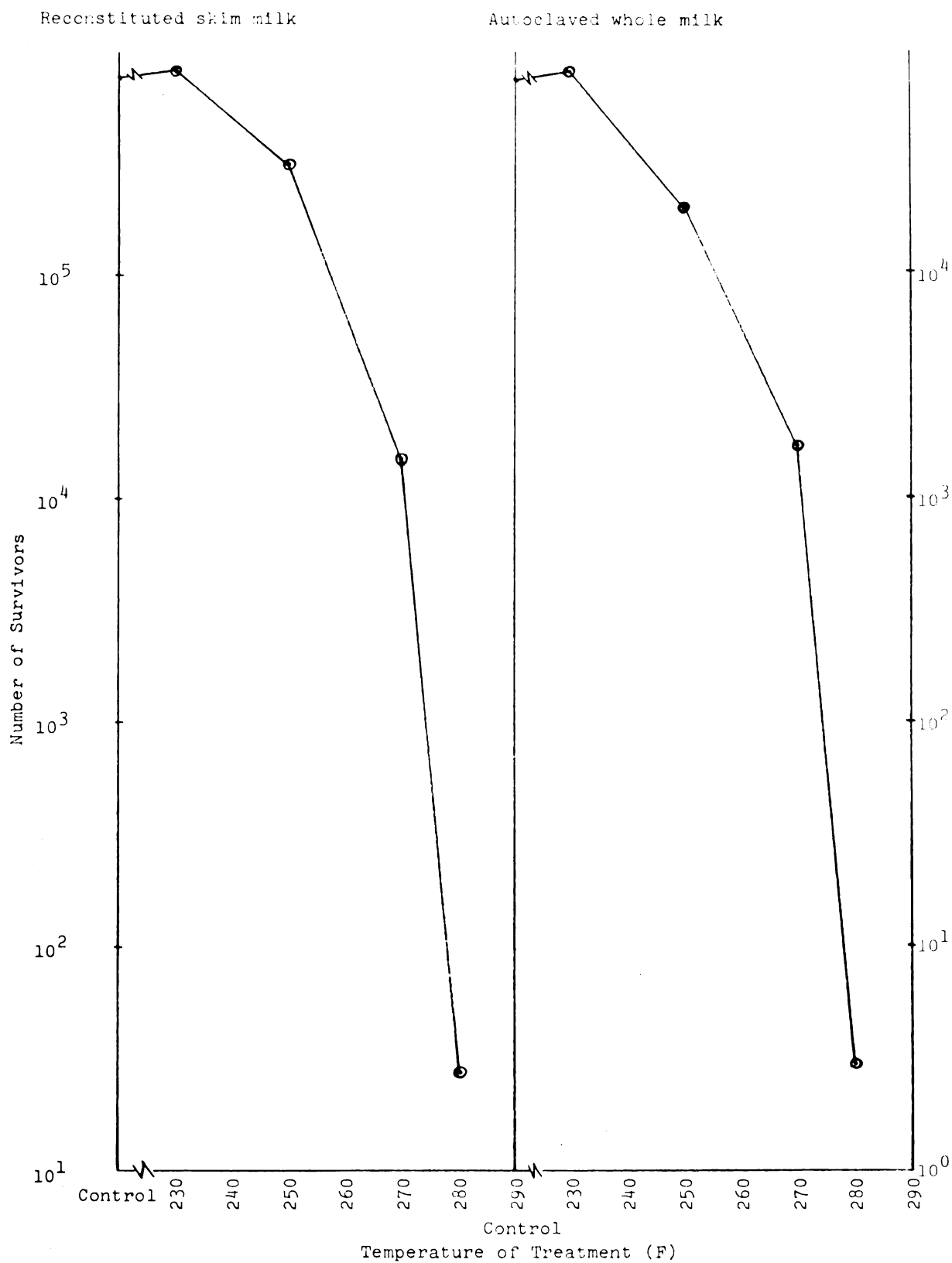


Fig. 12.--Temperature-survivor curves for *E. subtilis* A₁ in milk at UHT treatments of 4.0 sec.

spores. Several problems must be solved before the penicillin-penicillinase technique is practical. Adequate sterilization of the penicillinase solutions without changing its effectiveness is one. Precise standardization of the penicillin sensitivity to the strain used and the penicillin inactivation during the incubation also are necessary. The results of the germination experiments with whole milk incubated for 0 to 48 hr confirmed the observations made by the author (unpublished data). He worked with reconstituted skim milk and incubation periods of 0 to 3 hr.

The pH of milk after BII was 6.65 to 6.75. The acidity of the substrate has a marked influence on the lethal effect of heat, particularly on the acid side. Milk is a low acid food although it was not included in Cameron's original grouping (20). Table 13 (Appendix) shows the results of the storage trials. In many cases the samples were observed for 12 weeks but no significant spoilage was observed after 8 weeks. The most significant spoilage occurred during the first 2 weeks. The high sterilization temperatures (above 146 C, 295 F) in the first group of bactofugation-sterilization trials did not allow for the observation of very definite differences between the spoilage ratio of the NB and BII milk. The spoilage averages and their ratios are shown in Table 14. Nevertheless a slight difference in spoilage was observed at the three storage temperatures.

Table 15 (Appendix) shows the spoilage when the UHT was approximately 132 C (270 F). In the case of the high initial population the difference in the average spoilage was not significant because it was high in NB and BII milk. In the similar trials with a lower initial population of ~ 100 fold, the resulting count after BII allowed for very significant differences in the averages of spoilage. At 45 C (113 F) the difference was 100%. All NB samples spoiled but none of BII samples spoiled. Significant differences at all storage temperatures were also observed when UHT of approximately 138 C (280 F) and high population were used (Table 16, Appendix). The spoilage of NB was 18, 50 and 4 times greater than for BII milk at 21, 32 and 45 C. The spoilage ratios at 45 C for all these trials are shown in Figure 13.

The number of spores in milk prior to UHT sterilization had a marked influence on the spoilage. Bactofugation reduced by 100 to 1,000 times the initial number of spores in milk. The decrease in spore population decreased the probability of spore survival after UHT sterilization. Although changes of a few degrees in the heat treatment have a more marked influence upon the probability of survival and spoilage, as shown in the temperature-survivor curves (Figure 12), the population of spores also has influence, allowing for small reductions in the UHT treatment necessary to obtain a given per cent of spoilage.

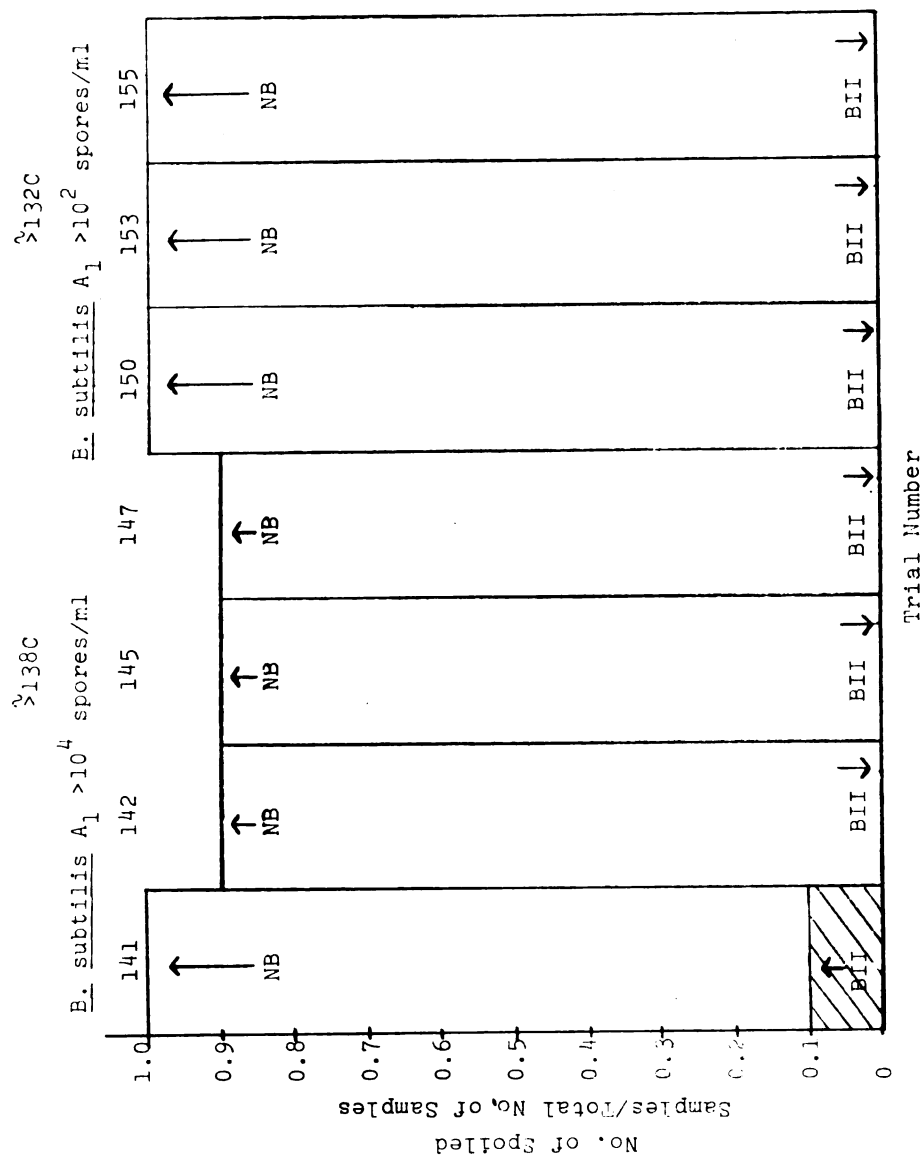


Fig. 13.--Comparison in spoilage between bacteriostatic and non-bacteriostatic UHT treated milk.

Any temperature reduction obviously will depend upon number and thermoresistance of spores as well as other conditions. Because of the characteristics of the thermal death of microorganisms, generalizations cannot be made. Also by using the regular UHT treatments the reduction in the number of spores by bactofugation will improve the efficiency of sterilization if other factors remain the same.

SUMMARY AND CONCLUSIONS

1) Bactofugation removed >99.5% bacterial spores of Bacillus subtilis, Bacillus cereus and Bacillus stearothermophilus from whole milk. The species of microorganism did not have any significant influence on the bactofugation efficiency. SPC reductions followed similar patterns.

2) Single bactofugation at ~30% of the normal flow rate of the machine gave approximately the same efficiency as double bactofugation at the normal flow rate. This reduction in flow rate gave a three to four-fold increase in sludge losses. Spore counts in the sludge were proportional but 100 to 1,000 times greater than the spore counts in the corresponding bactofuged milk.

3) More than two bactofugations were unnecessary because of the low efficiency of removal (18 to 60%) upon the low number of spores that remained after one or two bactofugations.

4) Changes in the temperature of milk for bactofugation from 71 to 82 C (160 to 180 F) did not give significant differences in the removal capacity. One trial with B. cereus 7 was an exception.

5) Cleaning of the bactofuge bowl did not improve the relative efficiency of the second bactofugation.

6) The percentage of spores removed was not significantly affected by the initial number of spores (from $>10^1$ to $>10^4$ /ml) but was affected by the percentage of spores remaining in milk after a first bactofugation.

7) Bactofugation by effectively reducing the initial number of spores in milk reduced up to 100 times the ratio of spoilage in sterilized milk when UHT treatments of approximately 132.2 C (270 F) and 134.8 C (280 F) were used for milk inoculated with $>10^2$ to $>10^4$ spores/ml.

8) D values for B. subtilis A₁ were similar when the spores were suspended in reconstituted skim milk or in autoclaved whole milk:

- a) 7.350 to 12.350 min at 110 C (230 F)
- b) 0.435 to 0.625 min at 121.1 C (250 F)
- c) 0.064 to 0.116 min at 132.2 C (270 F)
- d) 0.020 min at 137.8 C (280 F) and
- e) 0.0065 to 0.0072 min at 143.3 C (290 F)

A z value of 12 C (21.5 F) was found for the UHT range of 121.1 to 143.3 C (250 to 290 F). At 121.1 C (250 F) a D value of 0.010 min was obtained for B. cereus 7 suspended in whole milk.

9) The spores of B. subtilis A₁ did not lose heat resistance in milk after a heat shock of 80 C (170 F) for 15 min followed by incubation at 45 C (113 F) for 0 to 48 hr.

10) Temperature-survivor studies in the thermoresis-tometer showed that reduction in the initial number of spores (10^4 to 10^5 /ml) present in milk to be sterilized had an influence on the spoilage probability although numerically, changes in the UHT range of 110 to 143.3 C (230 to 290 F) had a much greater influence.

In conclusion bactofugation of milk will effectively remove spores to low levels. This process decreases the probability of spoilage when the common UHT treatments are used or may permit a small decrease in sterilization temperature by the UHT method.

APPENDIX

TABLE 1.--Bactofuge and VTIS operating conditions.

Trial No.	Total gal. Processed	Bactofugation	Bactofugation				VTIS (F)			
			Gallons	Feed Pressure	Tachom. rpm	Flow Rate $\times 10^4$ lbs/l hr.	Sludge g	Equip. Ster.	Prod. Ster.	
117	210	BI BII	100	0 0	1725 1720	0.400 0.400		292 142 min.	296-306 Av. 301	
118	200	BI BII	100	0 0	1774 1740	0.400 0.400		293 65 min	298-305 Av. 302	
119	210	BI BII	110	0 0	1765 1730	0.400 0.400		293 70 min.	294-302 Av. 294	
120	210	BI BII	110	4 0	1775 1725	0.400 0.400		292 100 min.	297-304 Av. 301	
121	210	BI BII	110	0 +2	1730 1775	0.400 0.400		293 50 min.	294-298 Av. 295	
122	210	BI BII	110	+2 +2	1775 1735	0.400 0.400		293 75 min.	291-298 Av. 295	
123	210	BI BII	110	+4 +4	1730 1730	0.400 0.400		293 65 min.	295-299 Av. 297	
124	210	BI BII	110	0 +4	1720 1775	0.400 0.400		293 50 min.	291-298 Av. 295	
126	210	BI BII	110	0 0	1730 1735	0.400 0.400		293 95 min.	292-300 Av. 297	
127	210	BI BII	110	0 0	1730 1775	0.400 0.400		293 105 min.	292-300 Av. 296	
129	210	BI BII	110	+4 +4		0.400 0.400		294 65 min.	292-299 Av. 296	
130	210	BI BII	110	+3 0	1735 1710	0.400 0.400		294 95 min.	293-300 Av. 296	
131	210	BI BII BIII	110	0 0 0	1750 1700 1680	0.440 0.440 0.440	5.03 5.74 5.37			
132	200	A BI BII BIII	100	-1 -5 -4	1800 1755 1725	1.200 1.200 1.200	1.74 1.89 2.17			

132	200	B	BI BII	50	-6 +1	1700 1690	0.440 0.440	7.44 5.28
		C	BI BII	50	+1 +1	1700 1675	0.440 0.440	5.81 6.17
133	210	A	BI BII BIII	110	-4 -2 -3	1760 1725 1700	1.170 1.170 1.170	1.59 1.50 1.31
		B	BI BII BIII	50	-1 0 0	1700 1685 1675	0.392 0.392 0.392	6.74 6.23 5.32
		C	BI BII BIII	50	0 0 0	1660 1650 1650	0.392 0.392 0.392	6.98 6.75 7.24
134	210	A		100	-4 -5 -4	1780 1775 1725	1.322 1.278 1.260	1.74 1.66 1.56
		B		50	0 0 0	1725 1700 1685	0.480 0.480 0.480	5.35 4.67 4.38
		C			0 0 1	1675 1675 1660	0.434 0.465 0.465	6.05 3.22 3.07
135	210		BI BII	110	-1 -5	1780 1725	0.423 1.190	4.33 1.99
136			BI BII	110	0 -4	1775 1725	0.440 1.335	5.07 1.89
137	230		BI BII	130	-4 -5	1750 1710	0.425 1.345	4.11 1.87
139	210		BI BII	110	0 -5	1760 1760	0.416 1.332	5.60 2.24
140	210		BI BII	110	-6 -6	1775 1725	0.468 1.296	4.44 2.32
141	210		BI BII	110	0 -6	1775 1725	0.384 1.265	6.24 2.93
142	210		BI BII	110	0 -7	1775 1710	0.424 1.332	6.13 2.59
145	320		BI BII	200	0 -7	1760 1680	0.400 1.421	6.22 1.98
							292 120 min.	268-276 Av. 272
							291 80 min.	273-280 Av. 275
							292 145 min.	282-288 Av. 280
							293 95 min.	268-273 Av. 273
							293 90 min.	270-276 Av. 273
							293 70 min.	278-284 Av. 281
							293 90 min.	278-285 Av. 282
							293 65 min.	280-284 Av. 83

TABLE 1.--Continued.

Trial No.	Total gal.	Bactofugation					VTIS (F)	
		Bactofugation	Gallons	Feed Pressure	Tachom. rpm	Flow Rate x 10 ⁴ lbs/l hr.	Sludge %	Equip. Ster. Prod. Ster.
147	320	BI BII	200	0 -4	1710 1670	0.392 1.332	6.40 2.05	293 110 min. 280-280 Av. 283
148	320	BI BII	200	0 -4	1725 1670	0.416 1.334	5.64 1.97	
150	210	BI BII	110	0 -5	1775 1700	0.432 1.490	6.24 2.25	296 90 min. 270-276 Av. 274
152	200	A BII	100	0 -6	1780 1710	0.416 1.210	5.93 2.35	
		B BII	100	-4 -6	1680 1675	1.210 1.210	1.63 1.65	
153	210	BI BII	110	-2 -5	1750 1700	0.408 1.332	6.03 2.02	293 100 min. 270-278 Av. 274
154	200	A BII	100	-3 -9	1760 1700	0.370 1.296	5.93 1.98	
		B BII	100	-6 -7	1680 1675	1.240 1.295	1.98 1.78	
155	320	BI BII	210	0 -6	1750 1675	0.340 1.380	6.04 2.00	293 90 min. 270-278 Av. 274

TABLE 2.--Statistical data on spore counts of milk for the bacterofugation experiments.

Trial No. and Sample	Counts	Adjusted Counts	Mean	Standard Deviation	C.L. 95%
117	$\times 10^0$	$\times 10^0$	$\times 10^0$	$\times 10^0$	$\times 10^0$
I	6.00 5.00 4.00 2.00	6.00 5.00 4.00 2.00	4.25	1.71	5.92 2.58
BI (MPN)	$\times 10^0$ 0.22	$\times 10^0$ 0.22	$\times 10^0$ 0.22	--	--
118	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	1.87 1.78 1.67 $\times 10^2$	1.87 1.78 1.67 $\times 10^2$	1.77 $\times 10^2$	1.00 $\times 10^0$	1.89 to 1.66 $\times 10^2$
BI	2.10 2.64 2.62 $\times 10^2$	2.70 2.64 2.62 $\times 10^2$	2.65 $\times 10^2$	4.16 $\times 10^1$	2.70 to 2.66 $\times 10^2$
BII	3.60 3.06 2.70	3.60 3.06 2.70	3.12	4.53	3.63 to 2.60
119	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	2.61 1.93 1.74	2.61 1.93 1.74	2.09	4.57	2.61 to 1.57
BI	$\times 10^2$ 1.16 1.11 1.00	$\times 10^2$ 1.16 1.11 1.00	$\times 10^2$ 1.09	$\times 10^1$ 8.19	$\times 10^2$ 1.18 to 1.00
BII	$\times 10^1$ 9.00 9.00 8.30	$\times 10^1$ 9.00 9.00 8.30	$\times 10^1$ 8.77	$\times 10^0$ 4.0	$\times 10^0$ 0.92 to 0.83
120	$\times 10^3$	$\times 10^3$	$\times 10^3$	$\times 10^2$	$\times 10^3$
I	5.85 5.30 4.80 4.55 4.05	5.85 5.30 4.80 4.55 4.05	4.91	6.92	5.51 to 4.30
BI (MPN)	$\times 10^1$ 1.23 1.19 1.15	$\times 10^1$ 1.23 1.19 1.15	$\times 10^1$ 1.19	$\times 10^0$ 0.4	$\times 10^1$ 1.24 to 1.15
BII (MPN)	$\times 10^1$ 1.20 1.00 0.80	$\times 10^1$ 1.20 1.00 0.80	$\times 10^1$ 1.00	$\times 10^0$ 2.00	$\times 10^1$ 1.23 to 0.74

TABLE 2.--Continued.

Trial No. and Sample	Counts	Adjusted Counts	Mean	Standard Deviation	C.L. 95%
121	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	9.05	9.05	7.52	9.17	8.32 to 6.72
	7.70	7.70			
	7.00	7.00			
	7.00	7.00			
	6.85	6.85			
BI	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$ 4.15 to 2.45
	4.50	4.50			
	4.00	4.00			
	3.00	3.00			
	3.00	3.00			
BII	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$ 4.07 to 3.13
	4.50	4.50			
	3.70	3.70			
	3.30	3.30			
	3.30	3.30			
122	$\times 10^3$	$\times 10^3$	$\times 10^3$	$\times 10^3$	$\times 10^3$
	3.40	3.40	2.13	1.10	3.38 to 0.89
	1.50	1.50			
	1.50	1.50			
	$\times 10^1$	$\times 10^1$			
BI	1.55	1.55	$\times 10^1$	$\times 10^0$	$\times 10^1$ 1.50 to 0.94
	1.40	1.40			
	1.25	1.25			
	1.20	1.20			
	0.70	0.70			
BII	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$ 2.38 to 0.82
	3.00	3.00			
	2.00	2.00			
	1.00	1.00			
	1.00	1.00			
123	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	4.96	4.96	4.32	4.47	4.75 to 3.88
	4.28	4.28			
	4.05	4.05			
	3.98	3.98			
BI	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$ 2.79 to 2.00
	2.99	2.99			
	2.29	2.29			
	2.17	2.17			
BII	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$ 1.90 to 1.63
	1.97	1.97			
	1.89	1.89			
	1.70	1.70			
	1.65	1.65	1.77	1.53	1.63
	1.63	1.63			

TABLE 2.--Continued.

Trial No. and Sample	Counts	Adjusted Counts	Mean	Standard Deviation	C.L. 95%
124	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	5.10	5.10	4.40	4.49	4.80
	4.59	4.59			to
	4.24	4.24			4.01
	4.17	4.12			
	3.98	3.98			
BI	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
	2.59	2.59	2.11	3.47	2.45
	2.06	2.06			to
	2.03	2.03			1.77
	1.76	1.76			
BII	$\times 10^2$	$\times 10^2$	$\times 10^3$	$\times 10^1$	$\times 10^2$
	1.82	1.82	1.35	2.91	1.60
	1.36	1.36			to
	1.31	1.31			1.09
	1.19	1.19			
	1.05	1.05			
126	$\times 10^4$	$\times 10$	$\times 10^3$	$\times 10^3$	$\times 10^4$
I	1.30	1.30	3.10	3.03	1.08
	0.86	0.86			to
	0.72	0.72			0.55
	0.67	0.67			
	0.50	0.50			
BI	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
	3.50	3.50	2.89	7.00	3.50
	3.40	3.40			to
	3.20	3.20			2.28
	2.50	2.50			
	1.85	1.85			
BII	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
	3.85	3.85	3.44	3.75	3.77
	3.75	3.75			to
	3.45	3.45			3.11
	3.20	3.20			
	2.95	2.95			
127	$\times 10^3$	$\times 10^3$	$\times 10^3$	$\times 10^2$	$\times 10^3$
I	8.30	8.30	7.40	6.63	7.98
	7.60	7.60			to
	7.50	7.50			6.82
	7.10	7.10			
	6.50	6.50			
BI	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^1$
	8.20	8.20	5.60	1.51	6.92
	5.60	5.60			to
	4.90	4.90			4.28
	4.80	4.80			
	4.50	4.50			
BII	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
	3.60	3.60	3.18	3.70	3.50
	3.40	3.40			to
	3.30	3.30			2.86
	2.90	2.90			
	2.70	2.70			

TABLE 2.--Continued

Trial No. and Sample	Counts	Adjusted Counts	Mean	Standard Deviation	C.L. 95%
129	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	3.10	3.10	2.92	2.15	3.11 to 2.74
	3.07	3.07			
	3.06	3.06			
	2.76	2.76			
	2.63	2.63			
BI	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
	1.43	1.43	1.37	9.29	1.45 to 1.29
	1.40	1.40			
	1.30	1.30			
	1.33	1.33			
	1.23	1.23			
BII	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
	4.00	4.00	3.72	2.17	3.91 to 3.53
	3.90	3.90			
	3.60	3.60			
	3.60	3.60			
	3.50	3.50			
130	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	6.30	6.30	5.74	5.60	6.23 to 5.75
	5.90	5.90			
	5.90	5.90			
	5.30	5.30			
	4.30	4.30			
BI	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
	1.39	1.39	1.20	1.34	1.32 to 1.09
	1.26	1.26			
	1.21	1.21			
	1.09	1.09			
	1.06	1.06			
BII	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$	$\times 10^1$
	1.17	1.17	3.34	2.40	1.05 to 0.62
	0.90	0.90			
	0.80	0.80			
	0.80	0.80			
	0.50	0.50			
131	$\times 10^3$	$\times 10^3$	$\times 10^3$	$\times 10^2$	$\times 10^3$
I	2.10	2.10	1.93	1.29	2.05 to 1.82
	2.00	2.00			
	1.95	1.95			
	1.83	1.83			
	1.78	1.78			
BI (MPN)	$\times 10^0$	$\times 10^0$	$\times 10^0$	--	--
	1.61	1.61	1.61		
BII (MPN)	$\times 10^0$				
	<1				
BII (MPN)	$\times 10^0$				
	<1				

TABLE 2.--Continued.

Trial No. and Sample	Counts	Adjusted Counts	Mean	Standard Deviation	C.L. 95%
132A	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	9.00 8.70 8.20	9.00 8.70 8.20	8.64	4.04	9.09 to 8.18
BI	$\times 10^3$ 1.64 1.62 1.35	$\times 10^3$ 1.54 1.62 1.35	$\times 10^3$ 1.60	$\times 10^2$ 2.45	$\times 10$ 1.88 to 1.33
BII	$\times 10^1$ 9.60 9.10 8.30	$\times 10^1$ 9.60 9.10 8.30	$\times 10^1$ 9.00	$\times 10^0$ 6.56	$\times 10^1$ 9.74 to 8.26
BIII	$\times 10^1$ 4.30 3.30 3.20	$\times 10^1$ 4.30 3.30 3.20	$\times 10^1$ 3.60	$\times 10^0$ 6.08	$\times 10^1$ 4.28 to 2.91
132B	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	9.00 8.70 8.20	9.00 8.70 8.20	8.63	4.04	9.09 to 8.81
BI	$\times 10^2$ 1.17 1.14 0.97	$\times 10^2$ 1.17 1.14 0.97	$\times 10^2$ 1.09	$\times 10^1$ 1.079	$\times 10^2$ 1.23 to 0.97
BII	$\times 10^1$ 2.50 2.30 2.10	$\times 10^1$ 2.50 2.30 2.10	$\times 10^1$ 2.30	$\times 10^0$ 2.00	$\times 10^1$ 2.53 to 2.07
132C	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^3$
I	9.00 8.70 8.20	9.00 8.70 8.20	8.63	4.04	9.09 to 8.17
BI	$\times 10^2$ 1.15 0.93 0.92	$\times 10^2$ 1.15 0.93 0.92	$\times 10^2$ 1.00	$\times 10^1$ 1.30	$\times 10^2$ 1.15 to 0.85
BII	$\times 10^1$ 2.50 2.30 1.50	$\times 10^1$ 2.50 2.30 1.50	$\times 10^1$ 2.10	$\times 10^0$ 5.29	$\times 10^1$ 2.67 to 1.50
133A	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	8.50 7.80 7.50	8.50 7.80 7.50	7.93	5.13	8.51 to 7.35
BI	$\times 10^3$ 1.30 1.21 1.01	$\times 10^3$ 1.30 1.21 1.01	$\times 10^3$ 1.17	$\times 10^2$ 1.48	$\times 10^3$ 1.34 to 1.01

TABLE 2.--Continued.

Trial No. and Sample	Counts	Adjusted Counts	Mean	Standard Deviation	C.L. 95%
BII	$\times 10^1$ 8.60 8.50 7.80	$\times 10^1$ 8.60 8.50 7.80	$\times 10^1$ 8.30	$\times 10^0$ 4.36	$\times 10^1$ 8.79 to 7.81
BIII	$\times 10^1$ 8.50 8.40 6.90	$\times 10^1$ 8.50 8.40 6.90	$\times 10^1$ 7.93	$\times 10^0$ 8.96 6.91	$\times 10^1$ 8.95 to 6.91
133B	$\times 10^4$ 8.50 7.80 7.50	$\times 10^4$ 8.50 7.80 7.50	$\times 10^4$ 7.93	$\times 10^3$ 5.13	$\times 10^4$ 8.51 to 7.35
BI	$\times 10^1$ 9.30 8.10 7.70	$\times 10^1$ 9.30 8.10 7.70	$\times 10^1$ 8.34	$\times 10^0$ 3.34	$\times 10^1$ 9.31 to 7.24
BII	$\times 10^1$ 5.70 5.30 4.40	$\times 10^1$ 5.70 5.30 4.40	$\times 10^1$ 5.13	$\times 10^0$ 6.66	$\times 10^1$ 5.88 to 4.38
BIII	$\times 10^1$ 4.60 4.50 3.50	$\times 10^1$ 4.60 4.50 3.50	$\times 10^1$ 4.20	$\times 10^0$ 6.08	$\times 10^1$ 4.89 to 3.51
133C	$\times 10^4$ 8.50 7.80 7.50	$\times 10^4$ 8.50 7.80 7.50	$\times 10^4$ 7.93	$\times 10^3$ 5.13	$\times 10^4$ 8.51 to 7.35
BI	$\times 10^2$ 1.19 1.07 0.89	$\times 10^2$ 1.19 1.07 0.89	$\times 10^2$ 1.05	$\times 10^1$ 1.50	$\times 10^2$ 1.22 to 0.89
BII	$\times 10^1$ 3.50 2.80 2.50	$\times 10^1$ 3.50 2.80 2.50	$\times 10^1$ 2.93	$\times 10^0$ 5.13	$\times 10^1$ 3.51 to 2.35
BIII	$\times 10^1$ 2.90 2.50 1.40	$\times 10^1$ 2.90 2.50 1.40	$\times 10^1$ 2.27	$\times 10^0$ 7.77	$\times 10^1$ 3.15 to 1.40
134A	$\times 10^4$ 7.30 7.10 6.40	$\times 10^4$ 7.30 7.10 6.40	$\times 10^4$ 6.93	$\times 10^3$ 4.73	$\times 10^4$ 7.47 to 6.40
BI	$\times 10^2$ 9.90 9.10 9.00	$\times 10^2$ 9.90 9.10 9.00	$\times 10^2$ 9.33	$\times 10^2$ 4.93	$\times 10^2$ 9.89 to 8.78

TABLE 2.--Continued.

Trial No. and Sample	Counts	Adjusted Counts	Mean	Standard Deviation	C.L. 95%
	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
BII	6.80	6.80	5.97	9.07	6.99
	6.10	6.10			to
	5.00	5.00			4.94
	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
BIII	3.20	3.20	2.90	2.65	3.20
	2.80	2.80			to
	2.70	2.70			2.60
134B	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	7.30	7.30	6.93	4.73	7.47
	7.10	7.10			to
	6.40	6.40			6.40
	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
BI	1.39	1.39	1.27	1.04	1.39
	1.22	1.22			to
	1.20	1.20			1.15
	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
BII	1.30	1.30	1.23	1.16	1.36
	1.30	1.30			to
	1.10	1.10			1.10
	$\times 10^0$	$\times 10^0$	$\times 10^0$	$\times 10^0$	10^0
BIII	4.00	4.00	2.67	1.16	3.97
	2.00	2.00			to
	2.00	2.00			1.36
134C	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	7.30	7.30	6.93	4.73	7.47
	7.10	7.10			to
	6.40	6.40			6.40
	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
BI	1.54	1.54	1.25	2.52	1.54
	1.15	1.15			to
	1.07	1.07			9.69
	$\times 10^0$	$\times 10^0$	$\times 10^0$	$\times 10^0$	$\times 10^0$
BII	3.00	3.00	2.33	1.16	3.64
	3.00	3.00			to
	1.00	1.00			1.03
	$\times 10^0$	$\times 10^0$	$\times 10^0$	$\times 10^0$	$\times 10^0$
BIII	1.00	1.00	1.00	0.00	1.00
	1.00	1.00			to
	1.00	1.00			1.00
135	$\times 10^5$	$\times 10^5$	$\times 10^4$	$\times 10^4$	$\times 10^5$
	1.15	1.15			
	1.06	1.06			
I	1.03	1.03	9.46	1.89	1.11
	0.75	0.75			to
	0.74	0.74			0.78

TABLE 2.--Continued.

Trial No. and Sample	Counts	Adjusted Counts	Mean	Standard Deviation	C.L. 95%
	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
BI	1.64	1.64	1.41	1.63	1.56 to 1.27
	1.53	1.53			
	1.30	1.30			
	1.30	1.30			
	1.29	1.29			
	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
BII	4.10	4.10	3.53	4.50	4.02 to 3.13
	3.80	3.80			
	3.20	3.20			
	3.20	3.20			
136	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^4$
I	3.70	3.70	6.36	1.45	8.13 to 5.59
	7.80	7.80			
	6.90	6.90			
	5.70	5.70			
	5.20	5.20			
	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^1$	$\times 10^2$
BI	1.20	1.20	9.76	1.39	1.10 to 0.85
	1.00	1.00			
	0.95	0.95			
	0.59	0.59			
	0.34	0.34			
	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
BII	5.00	5.00	4.40	5.55	4.93 to 3.95
	5.00	5.00			
	4.40	4.40			
	4.00	4.00			
	3.30	3.30			
137	$\times 10^3$	$\times 10^3$	$\times 10^3$	$\times 10^2$	$\times 10^3$
I	1.50	1.50	1.20	2.65	1.50 to 0.90
	1.10	1.10			
	1.00	1.00			
	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
BI	1.70	1.70	1.35	3.04	1.69 to 1.00
	1.20	1.20			
	1.15	1.15			
	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^1$
BII (MPN)	1.00	1.00	1.00	0.00	1.00
	1.00	1.00			
	1.00	1.00			
139	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^4$
I	8.50	8.50	7.20	1.61	8.78 to 5.62
	8.30	8.30			
	7.00	7.00			
	5.00	5.00			
	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
BI	1.40	1.40	1.20	1.95	1.42 to 0.98
	1.20	1.20			
	1.01	1.01			

TABLE 2.--Continued.

Trial No. and Sample	Counts	Adjusted Counts	Mean	Standard Deviation	C.L. 95%
	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
BII	4.60 3.90 3.60	4.60 3.90 3.60	4.03	5.13	4.61 to 3.45
140	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	5.50 4.30 4.30	5.50 4.30 4.30	4.70	6.93	5.48 to 3.92
	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^0$	$\times 10^2$
BI	1.41 1.33 1.32 1.24	1.41 1.33 1.32 1.24	1.33	6.95	1.39 to 1.26
	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
BII	2.90 2.50 2.50	2.90 2.50 2.50	2.63	2.31	2.90 to 2.37
141	$\times 10^5$	$\times 10^5$	$\times 10^5$	$\times 10^3$	$\times 10^5$
I	1.19 1.18 1.14 1.11 1.08	1.19 1.18 1.14 1.11 1.08	1.14	4.64	1.18 1.18 to 1.10
	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
BI	1.55 1.54 1.52 1.28 1.26	1.55 1.54 1.52 1.28 1.26	1.43	1.47	1.56 to 1.30
	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
BII	5.40 5.25 5.25 4.90 4.35	5.40 5.25 5.25 4.90 4.35	5.03	4.22	5.40 to 4.66
142	$\times 10^5$	$\times 10^5$	$\times 10^4$	$\times 10^4$	$\times 10^5$
I	1.13 1.04 0.99 0.90 0.86	1.13 1.04 0.99 0.90 0.86	9.84	1.08	1.08 to 0.89
	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^2$
BI	1.24 1.21 1.16 1.16 1.06	1.24 1.21 1.16 1.16 1.06	1.17	6.84	1.23 to 1.11
	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
BII	3.90 3.60 3.20 3.15 3.10	3.90 3.60 3.20 3.15 3.10	3.39	3.47	3.69 to 3.10

TABLE 2.--Continued.

Trial No. and Sample	Counts	Adjusted Counts	Mean	Standard Deviation	C.L. 95%
145	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^2$
I	9.00	9.00	7.14	1.31	3.28 to 6.00
	7.00	7.00			
	6.70	6.70			
	6.50	6.50			
	6.10	6.10			
BI	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^2$
	1.30	1.30	1.34	0.57	1.38 to 1.29
	1.30	1.30			
	1.30	1.30			
	1.30	1.30			
	1.30	1.30			
BII	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
	7.35	7.35	5.59	2.73	6.44 to 4.74
	5.55	5.55			
	5.30	5.30			
	5.10	5.10			
	5.10	5.10			
147	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	7.95	7.95	7.35	7.56	8.21 to 6.49
	7.60	7.60			
	6.50	6.50			
BI	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
	1.61	1.61	1.47	2.09	1.72 to 1.22
	1.50	1.50			
	1.21	1.21			
BII	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
	7.40	7.40	6.97	5.49	7.59 to 6.35
	7.15	7.15			
	6.35	6.35			
148	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
I	7.00	7.00	6.50	5.97	7.19 to 5.84
	6.70	6.70			
	5.65	5.65			
BI	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^0$	$\times 10^2$
	1.59	1.59	1.56	2.52	1.59 to 1.53
	1.56	1.56			
	1.54	1.54			
BII	$\times 10^1$	$\times 10^1$	$\times 10^1$	$\times 10^0$	$\times 10^1$
	8.20	8.20	8.10	1.32	8.25 to 7.95
	8.15	8.15			
	7.95	7.95			
150	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
I	7.45	7.45	6.65	7.12	7.27 to 6.03
	7.30	7.30			
	6.40	6.40			
	6.35	6.35			
	5.75	5.75			
BI	$\times 10^0$	$\times 10^0$	$\times 10^0$	$\times 10^0$	$\times 10^0$
	2.00	2.00	1.20	0.57	1.70 to 0.70
	1.50	1.50			
	1.00	1.00			
	5.00	5.00			
	1.00	1.00			
BII (MPN)	$\times 10^0$	$\times 10^0$	$\times 10^0$	--	--
	0.69	0.69	0.69	--	--

TABLE 2.--Continued.

Trial No. and Sample	Counts	Adjusted Counts	Mean	Standard Deviation	C.L. 95%
152A	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
I	5.30 4.70 4.00 3.90 3.60	5.30 4.70 4.00 3.90 3.60	4.30	6.89	4.90 to 3.70
BI (MPN)	$\times 10^0$ 5.00 3.00 3.00 2.00 2.00	$\times 10^1$ 5.00 3.00 3.00 2.00 2.00	$\times 10^1$ 3.00	$\times 10^0$ 1.23	10^0 4.07 to 1.93
BII (MPN)	$\times 10^0$ 0.92	$\times 10^0$ 0.92	$\times 10^0$ 0.92	--	--
152B	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
I	5.30 4.70 4.00 3.90 3.60	5.30 4.70 4.00 3.90 3.60	4.30	6.98	4.90 to 3.70
BI	$\times 10^0$ 0.30	$\times 10^0$ 0.30	$\times 10^0$ 0.30	--	--
BII (MPN)	$\times 10^0$ 0.92	$\times 10^0$ 0.92	$\times 10^0$ 0.92	--	--
153	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
I	8.40 7.90 7.80 7.70 7.10	8.40 7.90 7.80 7.70 7.10	7.78	4.66	8.19 to 7.37
BI	$\times 10^0$ 3.00 3.00 2.00 2.00 1.00	$\times 10^0$ 3.00 3.00 2.00 2.00 1.00	$\times 10^0$ 2.20	$\times 10^0$ 0.84	$\times 10^0$ 2.93 1.47
BII (MPN)	$\times 10^0$ 0.92	$\times 10^0$ 0.92	$\times 10^0$ 0.92	--	--
154A	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
I	4.20 4.00 3.80 3.40 3.20	4.20 4.00 3.80 3.40 3.20	3.72	4.15	4.08 to 3.36
BI (MPN)	$\times 10^0$ 4.90 4.10 3.90 3.80	$\times 10^0$ 4.90 4.10 3.90 3.80	$\times 10^0$ 4.18	$\times 10^0$ 0.05	$\times 10^0$ 4.66 to 3.70

TABLE 2.--Continued.

Trial No. and Sample	Counts	Adjusted Counts	Mean	Standard Deviation	C.L. 95%
	$\times 10^0$	$\times 10^0$	$\times 10^0$	$\times 10^0$	$\times 10^0$
BII (MPN)	1.40	1.40	1.20	0.16	1.34 to 1.06
	1.30				
	1.20	1.20			
	1.10	1.10			
	1.00	1.00			
154B	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
I	4.20	4.20	3.72	4.15	4.08 to 3.36
	4.00	4.00			
	3.80	3.80			
	3.40	3.40			
	3.20	3.20			
BI (MPN)	$\times 10^0$	$\times 10^0$	$\times 10^0$	--	--
	0.69	0.69	0.69		
BII (MPN)	$\times 10^0$	$\times 10^0$	$\times 10^0$	--	--
	0.22	0.22	0.22		
155	$\times 10^2$	$\times 10^2$	$\times 10^2$	$\times 10^1$	$\times 10^2$
I	7.60	7.60	7.10	4.53	7.50 to 6.70
	7.50	7.50			
	7.00	7.00			
	6.90	6.90			
	6.50	6.50			
BI (MPN)	$\times 10^0$	$\times 10^0$	$\times 10^0$	--	--
	2.30	2.30	2.30		
BII (MPN)	$\times 10^0$	$\times 10^0$	$\times 10^0$	--	--
	0.92	0.92	0.92		

TABLE 3.--FORTRAN IV program for the statistical and reduction-per cent calculations.

```

JOB,133652,456483,.45,          1          TORRES,MANUEL          FSC 699
FOR,X,L
PROGRAM BACTOF

REAL I,BI,BII,BIII,L951,L952
DIMENSION XI (5,4,3)
NEX = 39
READ (60,700) XI
FORMAT (5E10.3)
DO 500 L = 1,NEX
  PRINT 50
50  FORMAT (1H1)
  PRINT 115, L
115  FORMAT ( 19H1EXPERIMENT NUMBER , 12)
DO 400 K = 1,4
  KLX = 0
  SUM = 0.0
  DIF = 0.0
  JSTD = 0
  SUMI = 0.0
  DIFI = 0.0
  WRITE (61,300)
300  FORMAT ( / 5X, 2H1)
  PRINT 351, (XI(J,K,L), J = 1,5)
351  FORMAT (10X,2H1,3,FX)
  DO 300 J = 1,5
    SUM = SUM + XI(J,K,L)
    IF (XI(J,K,L).LE.1.E-10) KLX=KLX+1
    JNEW = 5 - KLX
    CONTINUE
    IF (JNEW.EQ.0) GO TO 501
    AMEAN = SUM / JNEW
    DO 300 I = 1, JNEW
      DIF = DIF + (XI(J,K,L) - AMEAN)**2
    CONTINUE
    IF (JNEW.EQ.1) JNEW=E4.0 GO TO 402
    STD = SQRT(DIF/(JNEW-1))
    GO TO 803
  STD = 0.
802  CONTINUE
803  PRINT 201
201  FORMAT ( / 5X, 3HXAD)
  DO 360 J=1, JNEW
    IF ( ABS(XI(J,K,L) - AMEAN). GT. (2.*STD)) GO TO 360
    XAD = XI (J,K,L)
    JSTD = JSTD+1
    SUMI = SUMI + XAD
  PRINT 200, XAD
200  FORMAT (10X, E10,3)

```

```

360 CONTINUE
   AMEANA = SUMI / JSTD
   IF(JSTD.EQ.1.OR.JSTD.EQ.0) GO TO 804
   DO 370 J=1, JNEW
   IF(ABS(XI(J,K,L) - AMEAN). GT. (2.*STD)) GO TO 370
   DIFI = DIFI + XI(J,K,L) - AMEANA
   CONTINUE
   STDAD = SQRT(DIFI/(JSTD-1))
   GO TO 805
804 STDAD = 0.
   PQ = 0.
   GO TO 806
805 PLUPE = JSTD
   PQ = 1.96*STDAD/SQRT(PLUPE)
806 L951 = AMEANA + PQ
   L952 = AMEANA - PQ
   IF (K-1) 20,25,20
25 I = AMEANA
   PRINT 202, I, STDAD, L951, L952
202 FORMAT (/ 10X, 15H I, 15H STDAD, 15H L951, 15H L952)
1, 15H L952 // 10X, 4(E10.3,5X))
   GO TO 400
20 IF (K - 2) 30,35,30
35 BI = AMEANA
   PRINT 203, BI, STDAD, L951, L952
203 FORMAT (/ 10X, 15H BI, 15H STDAD, 15H L951, 15H L952)
1, 15H L952 // 10X, 4(E10.3,5X))
   GO TO 400
30 IF (K - 3) 40,45,40
45 BII = AMEANA
   PRINT 204, BII, STDAD, L951, L952
204 FORMAT (/ 10X, 15H BII, 15H STDAD, 15H L951, 15H L952)
1, 15H L952 // 10X, 4(E10.3,5X))
   GO TO 400
40 IF (K - 4) 400, 55, 400
55 BIII = AMEANA
   IF (BIII.EQ.1.E-10) GO TO 400
   PRINT 205, BIII, STDAD, L951, L952
205 FORMAT (/ 10X, 15H BIII, 15H STDAD, 15H L951, 15H L952)
1, 15H L952 // 10X, 4(E10.3,5X))
   GO TO 400
400 CONTINUE
   IF (I.EQ.1.E-10) GO TO 562
   RED1 = 100. - BI *100./I
562 IF (BI.EQ.1.E-10) GO TO 563
   RED2 = 100. - BII *100./BI
563 IF (BII.EQ.1.E-10) GO TO 564
   RED3 = 100. - BIII*100./BIII
564 IF (I.EQ.1.E-10) GO TO 500
   IF BIII-1.E-10)551,551,21
21 TRED1 = 100. - BIII*100./I
551 TRED = 100. - BII*100./I
500 WRITE (61,550) RED1,RED2,RED3, TRED, TRED1
550 FORMAT (10X,15H RED1,15H RED2,15H RED3,15H TRED,15H TRED1,15H //5(E10.3,5X))
   END
*RUN, .45,2600

```

105 CARDS

TABLE 4.--FORTRAN IV program for the calculation of the sludge--losses per cent.

	'JOB,133652,45683,.45, 'FOR. X,L PROGRAM SLUDGE	2	TORRES, MANUEL	FSC 899
10	DIMENSION TV(27),S1(27),S2(27),S3(27),TQ(27),PTS1(27),PTS2(27), PT 153(27)			
11	DO 10 I = 1,27			
12	READ (60,11) TV(I), S1(I),S2(I),S3(I)			
13	FORMAT (4F10.1)			
14	DO 12 I = 1,27			
15	TW (I) = 8.6*TV*I			
16	DO 13 I = 1,27			
17	PTS1 (I) = (S1(I)/TW(I))*100.			
18	DO 14 I = 1,27			
19	PTS2 (I) = (S2(I)/(TW(I) - S1(I)))*100.			
20	DO 15 I = 1,27			
21	PTS3(I) = (S3(I)/(TW(I) - S1*I) - S2(I))*100.			
22	DO 16 I = 1,27			
23	N = 12 + I			
24	WRITE (61,17) N, TW(I), PTS1(I), PTS2(I), PTS3(I)			
25	FORMAT (13,4F10.2)			
26	STOP			
27	END			
28	'RUN,.45, 2100			

23 CARDS

TABLE 5.--Results of the trials performed on bactofugation and bactofugation-sterilization.

Trial No.*	Sample	SPC/ml**	Reduction SPC% initial	SPC% total	Spores, count/ml***	Reduction spores % initial	spores total
117 No. inoc.	R BI BII	1.23 x 10 ⁴ 9.00 x 10 ⁰ 1.60 x 10 ⁰	99.93 82.00	99.98	4.25 x 10 ⁰ 0.22 x 10 ⁰ <1	94.82 --	>94.82
118	R I BI BII	9.70 x 10 ³ 4.40 x 10 ³ 3.60 x 10 ¹ 2.90 x 10 ¹	99.18 19.44	99.34	5.00 x 10 ⁴ 1.77 x 10 ⁴ 2.65 x 10 ² 3.12 x 10 ²	98.50 -17.59	98.24
119	R I BI SI BII SII	1.60 x 10 ⁶ 1.19 x 10 ¹ 3.30 x 10 ¹ 4.40 x 10 ¹	99.72 -33.33	99.63	5.00 x 10 ⁴ 2.09 x 10 ² 1.09 x 10 ² 8.77 x 10 ¹	99.48 19.57	99.58
120	R I BI SI BII SII	4.70 x 10 ³ 3.10 x 10 ³ 1.20 x 10 ¹ 3.10 x 10 ³ 1.10 x 10 ⁰	00.61 90.83	99.96	7.00 x 10 ³ 4.91 x 10 ¹ 1.19 x 10 ⁴ 4.00 x 10 ¹ 1.00 x 10 ¹ 5.00 x 10 ²	99.76 15.97	99.80
121	R I BI SI BII SII	1.50 x 10 ³ 2.03 x 10 ⁰ 9.00 x 10 ³ 7.20 x 10 ¹ 1.50 x 10 ¹ 9.80 x 10 ¹	99.56 neg.	>99.0	5.00 x 10 ⁴ 7.52 x 10 ² 3.30 x 10 ² 3.60 x 10 ²	99.56 - 9.00	99.52
122	R I BI SI BII SII	6.30 x 10 ³ 4.70 x 10 ¹ 1.50 x 10 ³ 9.80 x 10 ¹ 1.00 x 10 ² 7.80 x 10 ²	99.68 33.33	99.79	6.00 x 10 ³ 2.13 x 10 ¹ 1.22 x 10 ¹ 4.70 x 10 ¹ 1.60 x 10 ² 2.00 x 10 ²	99.43 -31.15	99.25
123	R I BI SI BII SII	2.02 x 10 ³ 4.16 x 10 ¹ 2.40 x 10 ³ 4.96 x 10 ¹ 1.90 x 10 ² 1.80 x 10 ²	99.42 20.83	99.54	4.50 x 10 ⁴ 4.32 x 10 ² 2.39 x 10 ⁴ 2.42 x 10 ⁴ 1.77 x 10 ² 5.00 x 10 ²	99.45 26.10	99.59

TABLE 5.--Continued.

Trial No.*	Sample	SPC/ml**	Reduction SPC% initial total	Spores, count/ml***	Reduction spores % initial total
132 B	R	2.42 x 10 ⁴		4.0 x 10 ⁴	
	I	4.64 x 10 ¹		8.63 x 10 ²	
	BI	7.80 x 10 ⁵	99.83	1.09 x 10 ⁵	99.87
	SI	1.64 x 10 ¹		5.10 x 10 ¹	
	BII	2.50 x 10 ¹	67.95	2.30 x 10 ¹	78.96
C	SII	3.44 x 10 ³	99.95	3.20 x 10 ³	99.97
	R	2.42 x 10 ³		4.00 x 10 ⁴	
	I	4.64 x 10 ²		8.63 x 10 ²	
	BI	1.03 x 10 ⁵	99.79	1.00 x 10 ⁵	99.88
	SI	1.10 x 10 ¹		6.90 x 10 ¹	
33 A	BII	2.50 x 10 ²	75.73	2.10 x 10 ³	79.00
	SII	5.60 x 10 ²	99.95	1.80 x 10 ³	99.90
	R	4.50 x 10 ³		6.00 x 10 ⁴	
	I	3.28 x 10 ²		7.93 x 10 ³	
	BI	7.84 x 10 ⁵	97.61	1.17 x 10 ⁵	98.52
B	SI	5.44 x 10 ¹		6.50 x 10 ¹	
	BII	6.00 x 10 ³	89.80	8.30 x 10 ³	92.93
	SII	7.90 x 10 ¹	99.76	6.30 x 10 ³	99.90
	BIII	5.60 x 10 ¹	30.00	7.93 x 10 ²	44.13
	SIII	5.53 x 10 ³	99.53	5.50 x 10 ²	99.90
C	R	4.50 x 10 ³		7.93 x 10 ⁴	
	I	3.28 x 10 ¹		5.34 x 10 ¹	
	BI	6.00 x 10 ⁵	99.92	5.10 x 10 ⁵	99.93
	SI	1.26 x 10 ¹		5.10 x 10 ¹	
	BII	3.50 x 10 ³	41.67	5.13 x 10 ³	38.25
B	SII	3.36 x 10 ¹	99.59	5.10 x 10 ¹	99.94
	BIII	2.50 x 10 ¹	28.57	4.20 x 10 ¹	18.18
	SIII	2.32 x 10 ²	99.92	4.70 x 10 ²	99.95
C	R	4.50 x 10 ³		7.93 x 10 ⁴	
	I	3.28 x 10 ²		1.05 x 10 ²	
	BI	1.54 x 10 ⁵	99.53	6.70 x 10 ⁴	99.87
	SI	5.68 x 10 ¹		2.93 x 10 ¹	
	BII	3.50 x 10 ³	77.27	1.58 x 10 ⁴	72.06
B	SII	1.43 x 10 ¹	99.89	2.27 x 10 ¹	99.96
	BIII	3.20 x 10 ²	85.71	2.04 x 10 ³	22.73
	SIII	7.36 x 10 ²	99.90		99.97

TABLE 5.--Continued.

Trial No.	Sample	SPC/ml*	Reduction SPC% initial total	Spores, count/ml**	Reduction spores % initial total
139	R	5.20 x 10 ⁴		3.0 x 10 ⁴	
	I	1.30 x 10 ⁰		7.20 x 10 ²	
	BI	2.00 x 10 ⁴	99.98	1.20 x 10 ⁵	99.83
	SI	1.13 x 10 ⁴		6.40 x 10 ⁵	
	BI	1.72 x 10 ²	98.68	4.03 x 10 ¹	99.94
140	SI	7.80 x 10 ³		6.30 x 10 ³	
	R	8.00 x 10 ²		2.00 x 10 ⁴	
	I	6.80 x 10 ³		4.70 x 10 ²	
	BI	9.00 x 10 ⁴	99.87	1.33 x 10 ⁴	99.72
	SI	6.50 x 10 ⁰		2.60 x 10 ⁴	
141	BI	7.00 x 10 ³	22.22	2.63 x 10 ¹	80.13
	SI	4.48 x 10 ³	99.90	7.00 x 10 ²	99.94
	R	2.60 x 10 ³			
	I	5.96 x 10 ³		1.14 x 10 ⁵	
	BI	8.00 x 10 ³	93.87	1.43 x 10 ⁵	99.87
142	SI	4.12 x 10 ³		1.09 x 10 ⁵	
	BI	4.00 x 10 ³	50.00	9.03 x 10 ¹	64.83
	SI	1.20 x 10 ³	99.93	3.72 x 10 ³	99.96
	R	8.36 x 10 ³		1.40 x 10 ¹	
	I	1.67 x 10 ⁰		9.34 x 10 ²	
145	BI	1.00 x 10 ⁰	93.99	1.17 x 10 ⁵	99.88
	SI			6.0 x 10 ⁵	
	BI	1.50 x 10 ³	-50.00	3.47 x 10 ¹	70.93
	SI	1.56 x 10 ³	99.99	4.74 x 10 ³	99.97
	R	5.04 x 10 ³		2.0 x 10 ⁴	
147	I	6.68 x 10 ³		7.14 x 10 ²	
	BI	6.00 x 10 ⁴	99.91	1.34 x 10 ³	99.51
	SI	1.76 x 10 ⁰		6.65 x 10 ³	
	BI	4.00 x 10 ³	53.33	5.54 x 10 ¹	58.21
	SI	1.45 x 10 ³	99.94	6.00 x 10 ²	99.99
148	R	7.00 x 10 ³		3.00 x 10 ⁴	
	I	8.96 x 10 ³		7.35 x 10 ⁴	
	BI	7.06 x 10 ⁴	99.40	1.47 x 10 ⁵	99.50
	SI	7.30 x 10 ⁰		3.8 x 10 ⁵	
	BI	4.00 x 10 ³	45.45	6.97 x 10 ⁴	99.50
149	SI	1.45 x 10 ³	99.96	9.23 x 10 ³	99.96
	R	1.94 x 10 ⁴		6.00 x 10 ⁴	
	I	1.33 x 10 ³		6.52 x 10 ²	
	BI	3.00 x 10 ³	>99.99	1.56 x 10 ⁵	99.76
	SI	8.40 x 10 ³		4.96 x 10 ³	
150	BI	3.00 x 10 ³	0.00	8.10 x 10 ¹	48.19
	SI	1.40 x 10 ³	>99.99	8.26 x 10 ³	99.84

TABLE 6.--Results of the fraction negative thermoresistance tests for *B. subtilis* A₁ suspended in reconstituted skim milk.

T	t	+	-	r/q	Nu=ln $\frac{r}{q}$	$\log_{10} \frac{N_0}{Nu}$	$\log_{10} \frac{N_0}{Nu}$	D _T (sec.)
110C (230F)	120	10	0	0	--			
	240	10	0	0	--			
	360	10	0	0	--			
	480	10	0	0	--			
	900	10	0	0	--			
	1,800	10	0	0	--			
	2,700	1	9	1.11	0.105	-0.9788	6.908	390
	2,400	9	1	10.00	2.303	0.3623	4.5927	450
	3,600	1	9	1.11	0.105	-0.9788	6.908	520
	4,800	0	10	1.00	0.000	--		440
121.1C (250F)	30	10	0	0	--			
	90	10	0	0	--			
	150	3	7	1.43	0.358	-0.4477	6.3477	23.30
	150	3	7	1.43	0.358	-0.4477	6.3477	23.30
	180	2	8	1.25	0.223	-0.6517	6.5517	27.50
	210	1	9	1.11	0.105	-0.9788	6.8788	30.50
	240	0	10	1.00	0.000	--		
							Av.	26.15
132.2C (270F)	10	3	7	1.43	0.358	-0.4477	6.3477	1.57
	20	2	8	1.25	0.223	-0.6517	6.5517	3.30
	30	2	8	1.25	0.223	-0.6517	6.5517	4.58
	40	1	9	1.11	0.105	-0.9788	6.8788	5.80
							Av.	3.81
143.3C (290F)	1.5	2	8	1.25	0.223	-0.6517	6.5517	0.229
	2.5	1	9	1.11	0.105	-0.9788	6.8788	0.362
	3.5	1	9	1.11	0.105	-0.9788	6.8788	0.507
	4.5	0	10	1.00	0.00	--	--	
							Av.	0.428
143.3C (290F) Aer. litmus milk	1.5*	8	2	5.0	1.609	+0.2066	5.0290	0.298
	2.5*	1	9	1.11	0.105	-0.9788	6.2148	0.322
	3.5*	1	9	1.11	0.105	-0.9788	6.2148	0.485
	4.5*	0	10	1.00	0.00	--	--	
							Av.	0.368
143.3C (290F) Anaer. litmus milk	1.5*	8	2	5.0	1.609	+0.2066	5.0290	0.290
	2.5*	2	8	1.25	0.223	-0.6517	5.8877	0.338
	3.5*	0	10					
	4.5*	0	10	1.00	0.00	--	--	
							Av.	0.318

*Tests where substrates other than DTS broth was used.

**log N₀ was: 5.9300 for 110C
 5.9000 for 121.1, 132.2 and 143.3C
 5.2400 for 143.3C when other than DTS subcultures were used.

TABLE 7.--Results of the fraction negative tests for *B. subtilis* A₁ suspended in autoclaved whole milk.

T	t	+	-	r/q	Nu=ln ^r /q	log ₁₀ Nu	log ₁₀ ^{No**} /Nu	D _T (sec)
110C (230F)	1,200	10	0	0	--			
	1,800	10	0	0	--			
	2,400	10	0	0	--			
	3,000	10	0	0	--			
	3,600	6	4	2.50	0.916	-0.0381	4.9831	720
	4,500	1	9	1.11	0.105	-0.9788	5.9340	760
	5,100	0	10	1.00	0.000	--	--	
								Av. 740
121.1C (250F)	30	10	0	0	--			
	40	10	0	0	--			
	50	10	0	0	--			
	60	10	0	0	--			
	90	10	0	0	--			
	120	10	0	0	--			
	150	9	1	10.0	2.303	0.3623	4.6830	32.0
	180	6	4	2.5	0.916	-0.0381	5.0840	35.0
	210	5	5	2.0	0.693	-0.1593	5.2046	40.0
	240	2	8	1.25	0.223	-0.6517	5.6970	42.0
								Av. 37.4
132.2C (270F)	5	10	0	0	--			
	10	10	0	0	--			
	15	10	0	0	--			
	20	4	6*	1.67	0.513	-0.2899	5.2452	3.68
	30	3	7*	1.43	0.358	-0.4461	5.4016	5.55
	40	2	8	1.25	0.223	-0.6517	5.6069	7.15
	50	1	9	1.11	0.105	-0.9788	5.9340	8.42
	60	1	9	1.11	0.105	-0.9788	5.9340	10.12
								Av. 6.98
137.8C (280F)	4	10	0	∞				
	6	6	4	2.50	0.916	-0.0381	4.9933	1.20
	8	2	8	1.25	0.223	-0.6517	5.6069	1.43
	10	0	10	1.00	0.000	--	--	
								Av. 1.32
143.3C (290F)	1.5	4	6	1.67	0.513	-0.2898	5.3350	0.28
	2.0	2	8	1.25	0.223	-0.6517	5.6980	0.35
	2.5	1	9	1.11	0.105	-0.9788	6.0230	0.42
	3.0	1	9	1.11	0.105	-0.9788	6.0230	0.50
	3.5	0	10	1.00	0.000	--	--	
								Av. 0.39

*When others substrates were used results were:

t	+	-
20	4	6
30	1	9

**log N₀ was: 4.9552 for 110C, 132.2C, and 137.8C.
5.0453 for 121.1C and 143.3C.

TABLE 8.--Counts of *B. subtilis* A₁ spores after treatment at 110 C (230 F) for different intervals of time.

Time (sec)	Counts	Adjusted Counts	Adjusted Mean	Standard Deviation	C.L. 95%
N ₀	x10 ⁴	x10 ⁴	x10 ⁴	x10 ³	x10 ⁴
	8.20	8.20			8.24
(after 80C	7.90	7.90	7.62	7.01	to
15 min)	7.80	7.80			7.01
	7.80	7.80			
	6.40	6.40			
300	x10 ⁴	x10 ⁴	x10 ⁴	x10 ³	10 ³
	7.50	7.50			7.58
	6.40	6.40	6.50	9.54	to
	5.60	5.60			5.42
600	x10 ⁴	x10 ⁴	x10 ⁴	x10 ⁴	x10 ⁴
	3.90	3.90			3.90
	3.70	3.70	3.73	1.53	to
	3.60	3.60			3.56
900	x10 ⁴	x10 ⁴	x10 ⁴	x10 ³	x10 ⁴
	3.10	3.10			3.13
	2.90	2.90	2.90	2.00	to
	2.70	2.70			2.67
1,500	x10 ⁴	x10 ⁴	x10 ⁴	x10 ³	x10 ⁴
	2.00	2.00			2.07
	1.50	1.50	1.50	5.00	to
	1.00	1.00			0.93
1,800	x10 ⁴	x10 ⁴	x10 ⁴		
	1.00	1.00			
	1.00	1.00	1.00		
	1.00	1.00			

TABLE 9.--Counts of *B. subtilis* A₁ spores, suspended in autoclaved whole milk, after treatment at 121.1 C (250 F) for different intervals of time.

Time (sec)	Counts	Adjusted Counts	Adjusted Means	Standard Deviation	C.L. 95%
N ₀	x10 ⁴	x10 ⁴	x10 ⁴	x10 ³	x10 ⁴
(after 80C, 15 min).	8.20	8.20			8.24
	7.90	7.90			to
	7.80	7.80	7.62	7.01	7.01
	7.80	7.80			
	6.40	6.40			
15	x10 ⁴	x10 ⁴	x10 ⁴	x10 ³	x10 ⁴
	2.80	2.80			2.80
	2.40	2.40	2.53	2.31	to
	2.40	2.40			2.27
30	x10 ⁴	x10 ⁴	x10 ⁴	x10 ³	x10 ⁴
	1.93	1.93			1.97
	1.51	1.51	1.53	3.86	to
	1.16	1.16			1.10
45	x10 ³	x10 ³	x10 ³	x10 ²	x10 ³
	7.50	7.50			7.60
	7.40	7.40	7.30	2.65	to
	7.00	7.00			7.00
60	x10 ³	x10 ³	x10 ³	x10 ²	x10 ³
	1.23	1.23			1.23
	1.00	1.00	1.05	1.65	to
	0.91	0.91			8.60
75	x10 ²	x10 ²	x10 ²	x10 ²	x10 ²
	7.40	7.40			7.74
	6.90	6.90	6.57	1.04	to
	5.40	5.40			5.39

TABLE 10.--Counts of B. subtilis A₁ spores, suspended in reconstituted skim milk, after different temperatures for 4.0 sec.

Heat Treat.	Counts	Adjusted Counts	Adjusted Mean	Standard Deviation	C.L. 95%
N _o	x10 ⁵	x10 ⁵	x10 ⁵	x10 ⁵	x10 ⁵
(after 80C, 15 min.)	8.90	8.90			8.84
	8.50	8.50	7.85	1.00	to
	7.20	7.20			6.84
	6.70	6.80			
110C (230F)	x10 ⁶	x10 ⁶	x10 ⁵	x10 ⁵	95%
	1.02	1.02			
	0.89	0.89			9.55
	0.83	0.83	8.40	1.31	to
	0.80	0.80			7.25
	0.66	0.66			
121.1C (250F)	x10 ⁵	x10 ⁵	x10 ⁵	x10 ⁴	x10 ⁵
	1.55	1.55			
	1.47	1.47			1.50
	1.36	1.35	1.35	1.67	to
	1.22	1.22			1.20
	1.15	1.15			
132.2C (270F)	x10 ⁴	x10 ⁴	x10 ⁴	x10 ²	10 ⁴
	1.60	1.60			1.58
	1.58	1.58			to
	1.48	1.48	1.50	8.60	1.43
	1.42	1.42			
	1.42	1.42			
137.8C (280F)	1/5 x10 ²	x10 ²	x10 ²	x10 ¹	x10 ²
	1.90	1.90			1.67
	1.00	1.00	1.13	5.32	to
	0.90	0.90			0.60
	0.70	0.70			

TABLE 11.--Counts of B. subtilis A₁ spores, suspended in autoclaved whole milk, after different temperatures for 4.0 sec..

Heat treat.	Counts	Adjusted Counts	Adjusted Mean	Standard Deviation	C.L. 95%
	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
N _o (after 80C, 15 min.)	8.20	8.20			
	7.90	7.90			8.24
	7.80	7.80	7.62	7.01	to
	7.80	7.80			7.00
	6.40	6.40			
110C (230F)	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^3$	$\times 10^4$
	8.90	8.90			
	8.50	8.50			8.82
	8.50	8.50	8.26	6.43	to
	8.20	8.20			7.70
	7.20	7.20			
121.1C (250F)	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^2$	$\times 10^4$
	2.06	2.06			2.06
	1.97	1.97	1.98	7.10	to
	1.92	1.92			1.90
132.2C (270F)	$\times 10^3$	$\times 10^3$	$\times 10^3$	$\times 10^2$	$\times 10^3$
	2.08	2.08			2.09
	1.70	1.70	1.77	2.77	to
	1.54	1.54			1.46
137.8C (270F)	$\times 10^0$	$\times 10^0$	$\times 10^0$	$\times 10^0$	10^0
	4.00	4.00			4.40
	3.00	3.00	2.67	1.53	to
	1.00	1.00			0.94
143.3C (290F) (MPN)	$\times 10^0$ <1				

TABLE 12.--Counts of *B. subtilis* A₁ spores after heat shock at 80 C for 15 min, and incubated for different times.

Time (hr)	Counts	Adjusted Counts	Mean	Standard Deviation	95% C.L.
0	x10 ²	x10 ²	x10 ²	x10 ¹	x10 ²
	3.90	3.90	3.43	4.03	3.82
	3.60	3.60			to
	3.20	3.20			3.03
	3.00	3.00			
3	x10 ²	x10 ²	x10 ²	x10 ¹	x10 ²
	3.70	3.70	3.20	3.46	3.54
	3.10	3.10			to
	3.10	3.10			2.86
	2.90	2.90			
6	x10 ²	x10 ²	x10 ²	x10 ¹	x10 ²
	3.20	3.20	2.68	3.83	3.06
	2.70	2.70			to
	2.50	2.50			2.30
	2.30	2.30			
18	x10 ²	x10 ²	x10 ²	x10 ¹	x10 ²
	4.90	4.90	6.65	1.92	4.84
	4.70	4.70			to
	4.50	4.50			4.46
	4.50	4.50			
24	x10 ²	x10 ²	x10 ²	x10 ¹	x10 ²
	6.10	6.10	6.00	1.41	6.14
	6.10	6.10			to
	6.00	6.00			5.86
	5.80	5.80			
48	x10 ²	x10 ²	x10 ²	x10 ¹	x10 ²
	9.90	9.90	9.63	2.31	9.90
	9.50	9.50			to
	9.50	9.50			9.37

TABLE 13.--Spoilage of non-bactofuged and bactofuged, UHT treated milk after 8 weeks storage.

Trial No.	Inoculation	Treatment	Storage					
			37 or 45C		32C		21C	
			+	-	+	-	+	-
UHT > 146C								
117	None	NB BII	5 2	1 4	6 4	0 2	6 1	0 5
118	<u>B. subtilis</u> A ₁	NB BII	Mold contamination					
119	<u>B. subtilis</u> A ₁	NB BII	5 6	5 4	4 2	6 8	5 1	5 9
120	<u>B. subtilis</u> A ₁	NB BII	0 0	10 10	0 0	10 10	0 0	10 10
121	<u>B. subtilis</u> A ₁	NB BII	4 3	6 7	2 1	8 9	1 4	9 6
122	<u>B. subtilis</u> A ₁	NB BII	0 0	10 10	0 0	10 10	0 0	10 10
123	<u>B. subtilis</u> A ₁	NB BII	0 0	10 10	0 0	10 10	0 0	10 10
124	<u>B. subtilis</u> A ₁	NB BII	3 0	7 10	0 1	10 9	2 0	8 10
126	<u>B. subtilis</u> A ₁	NB BII	0 0	10 10	0 0	10 10	2 0	8 10
127	<u>B. subtilis</u> A ₁	NB BII	0 0	10 10	0 0	10 10	0 0	10 10
129	<u>B. cereus</u> 7	NB BII	1 0	9 10	0 0	10 10	0 0	10 10
130	<u>B. cereus</u> 7	NB BII	0 0	10 10	0 0	10 10	0 0	10 10
UHT > 134C								
135	<u>B. subtilis</u> A ₁	NB BII	4 5	6 5	4 0	7 0	Mold surf. cont.	
136	<u>B. subtilis</u> A ₁	NB BII	0 1	10 9	1 5	7 5	Mold surf. cont.	
137	<u>B. cereus</u> 7	NB BII	0 0	10 10	0 0	10 10	0 0	10 10
139	<u>B. subtilis</u> A ₁	NB BII	10 0	0 4	10 10	0 0	10 10	0 0
140	<u>B. subtilis</u> A ₁	NB BII	10 10	0 0	10 2	0 8	1 0	9 10
UHT > 132C								
141	<u>B. subtilis</u> A ₁	NB BII	10 1	0 0	10 0	0 10	0 2	10 8
142	<u>B. subtilis</u> A ₁	NB BII	9 0	1 10	10 0	0 10	7 0	3 10
145	<u>B. subtilis</u> A ₁	NB BII	9 0	1 10	0 0	10 10	0 0	10 10
147	<u>B. subtilis</u> A ₁	NB BII	9 0	1 10	0 0	10 10	1 0	9 10
UHT > 132C								
150	<u>B. subtilis</u> A ₁	NB BII	10 0	0 10	10 2	0 8	5 0	5 10
153	<u>B. subtilis</u> A ₁	NB BII	10 0	0 10	10 0	0 10	6 0	4 10
155	<u>B. subtilis</u> A ₁	NB BII	10 0	0 10	3 0	7 10	10 1	0 9

TABLE 14.--Spoilage ratio of milk sterilized by UHT at ~146 C. Initial population of spores >100 to >10⁴/ml.

Trial No.	Storage					
	37 of 45 C			32 C		
	NB	BII	NB	BII	NB	BII
117(1)**	0.83	0.33	1.00	0.67	1.0	0.17
119(3)	0.50	0.60	0.40	0.20	0.5	0.10
121(5)	0.40	0.30	0.20	0.10	0.10	0.40
124(8)	0.30	0.00	0.00	0.10	0.50	0.00
AVERAGE	0.40	0.30	0.40	0.27	0.53	0.17

* Number of spoiled samples/number of tested samples

** For this trial only six samples at each temperature were tested.
In all other trials 10 samples at each temperature were tested.

TABLE 15.--Spoilage ratio of milk sterilized by UHT at $\sim 132^\circ\text{C}$.

Trial No.	45C			Storage 32C			21C		
	Spores initial pop. $>10^4/\text{ml}$			Spores initial pop. $>10^4/\text{ml}$			Spores initial pop. $>10^4/\text{ml}$		
	NB	BII	NB	BII	NB	BII	NB	BII	NB
135	0.40	0.50	0.40	0.00					
136	0.00	0.10	0.20	0.50					
139	1.00	0.60	1.00	1.00	1.00	1.00	1.00	1.00	
140	1.00	1.00	1.00	0.20	0.10	0.00			
AVERAGE	0.60	0.55	0.65	0.43	0.55	0.50			
Spores initial pop. $>10^2/\text{ml}$									
150	1.00	0.00	1.00	0.20	0.50	0.00			
153	1.00	0.00	1.00	0.00	0.50	0.40			
155	1.00	0.00	1.00	0.30	1.00	0.10			
AVERAGE	1.00	0.00	1.00	0.17	0.67	0.17			

TABLE 16.--Spoilage of milk sterilized by UHT at 138 C with initial spore population of $>10^4$ /ml.

Trial No.	Storage					
	45C			32C		
	NB	BII		NB	BII	
141	1.00	0.10		1.00	0.00	0.20
142	0.90	0.00		1.00	0.00	0.00
145	0.90	0.10		0.00	0.00	0.00
147	0.90	0.00		0.00	0.00	0.00
AVERAGE	0.93	0.05		0.50	0.00	0.05

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