THE RECOVERY AND ISOLATION OF HEAT-INJURED BACTERIA BY A MEMBRANE FILTER TECHNIQUE

by

JAMES HAROLD GOFF

B. S., Kansas State University, 1969

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

in

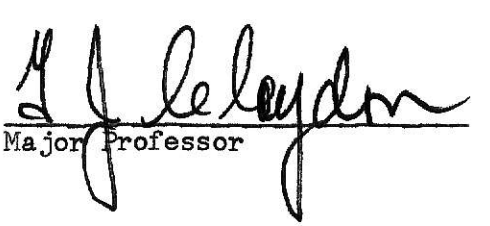
Food Science

Department of Dairy and Poultry Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1971

Approved by:



Major Professor

THIS BOOK CONTAINS NUMEROUS PAGES WITH THE ORIGINAL PRINTING BEING SKEWED DIFFERENTLY FROM THE TOP OF THE PAGE TO THE BOTTOM.

THIS IS AS RECEIVED FROM THE CUSTOMER.

TABLE OF CONTENTS

INTRODUCTION

In the dairy industry various bacteriological tests have been used in attempts to predict the keeping quality of pasteurized milk. In general, these have given helpful information. However, in many cases the correlation between predicted keeping quality and actual keeping quality has been low. The original objective of the present investigation was to determine if the low correlation might arise from subsequent recovery of bacteria that were sub-lethally injured in the pasteurization process. For such a study it seemed desirable to apply a technique that could segregate the injured from non-injured cells. Previous reported studies of heat-injured organisms utilize indirect methods of detection and enumeration. Some questions have been raised as to whether designated organisms were non-injured survivors or recovered injured cells.

As a new approach to the problem it was speculated that the membrane filter technique with modifications could be used to detect both injured and non-injured cells.

Work of others has previously shown that while heat-injured cells will usually grow in complex media containing a variety of supplemental nutrients, they are suppressed in media containing a selective or inhibitory agent that does not effect uninjured cells.

In this study it was postulated that with the membrane filter procedure and a selective medium, the growth detected would reflect the uninjured population. Subsequent transfer of the membrane to a complex, supplemented medium would allow growth of injured cells to form additional colonies. These then could be differentiated from the other colonies and could be enumerated, isolated and studied further.

The objective of the investigation was to evaluate the proposed procedure as a technique for segregating heat-injured and uninjured bacteria.

LITERATURE REVIEW

In recent years, increasing attention has been given by microbiologists to the concept of sub-lethal injury and recovery of bacterial cells. Early observations (4, 8, 9, 23, 28, 31, and 35) that bacteria in milk after pasteurizing grew better on complex, supplemented media than on the usual nutrient agar suggested that the heat treatment had impaired cell development. Subsequently, various workers utilized different approaches to demonstrate that certain treatments resulted in sub-lethal injury, but not death of some cells. Under suitable conditions the injured cells were able to recover and produce detectable growth.

Effect of Plating Media on Counts of Heated Cells

Prior to development of the concept of cell injury, Sherman (31) found increases of 141 to 1050% in the count of pasteurized milk when lactose was included in the plating medium. Ayers and Mudge (4) used added milk, peptone, yeast extract and meat extract in the plating medium and obtained significantly higher plate counts than those on standard agar when plating pasteurized milk.

Bowers and Hucker (8) developed tryptone glucose skimmilk agar and obtained higher plate counts with both pasteurized and raw milk compared to plate count agar. Phelum (28) found results similar to those of Bowers and Hucker. Bradfield (9) and Yale (35) used tryptone glucose skimmilk agar to evaluate pasteurized and raw milk. With this medium they found large increases in counts of pasteurized milk compared to nutrient agar. Raw milk did not show the increases found with pasteurized milk. Nelson (23) also noted increased counts with heated milk but no increases in count when examining raw milk using improved media and plan nutrient agar. Nelson (24, 25) and Hansen and Riemann (16) found that heated organisms

required a more complex growth medium than unheated organisms in order to produce colonies on agar plates. The ability to grow on ordinary media was regained after incubation in the complex medium.

Baird-Parker and Davenport (5) heated Staphylococcus aureus at 63 C for 2.5 min. and enumerated the bacterial content on various media using a surface plating technique. Blood agar and sodium pyruvate added to various plating media produced colonies more rapidly than less nutritious media.

Iandolo and Ordal (19) determined that a complete medium containing amino acids, glucose and phosphate was necessary for growth of heated S. aureus MF31.

Trypticase soy agar (TSA) and trypticase soy broth (TSB) have been used by several workers (12, 19, 26, 27 and 34) as enriched media for incubation and growth of organisms in work with heat treatments of bacteria. TSA and TSB are excellent sources of amino acids, glucose and phosphate.

"Chemical Reactivation" of Heated Cells

An early indication that cells may recover from heat-induced injury was provided by Heinmets et al (17). They developed the term "chemical reactivation" to describe the apparent increase in viable organisms following the treatment of heated cells with various Kreb's cycle metabolites. Lactate, pyruvate, fumarate, acetate, succinate and a-ketoglutarate were used in their study. Garvie (15) suspected that the metabolites used were not sufficiently free from nitrogen sources to prevent growth of the uninjured organisms which survived heating. Increases in count would appear to be the result of injured cells being reactivated, when the actual increase was due to the growth of uninjured cells.

Hurwitz et al (18) duplicated Heinmets's experiment and observed increases in count. Then, to test Garvie's contentions, a dilution

method was used to show that the increases in count were actually survivor growth. The dilution method was based on the hypothesis that the very small number of non-injured survivors could be diluted out and therefore survivor growth would not be significant. No growth on surface plating on nutrient agar was observed after dilution. They concluded that Heinmets had observed survivor growth. Stiles and Witter (34) found that metabolites alone would not promote the recovery of S. aureus MF31 following heating at 130 F for varying periods.

Nature of Heat Injury

Indications that heating of bacteria increased the nutritional requirements for growth do not offer conclusive evidence of the phenomenon of sub-lethal injury. Several workers have observed actual biochemical alteration to bacterial cells.

Beuchat and Lechowich (7) found that cellular ribonucleic acid (RNA) in heated cells was lowered significantly when compared to unheated cells. Rosenthal and Iandolo (29) found intracellular degradation of ribosomes after heating S. aureus MF31 at 55 C. A decrease in RNA was also noted. Iandolo and Ordal (19) found cell membrane damage after heating S. aureus MF31. Russell and Harries (30) determined that pentose materials were leaked into the suspending medium from E. coli cells following heating at 50 to 60 C for 30 min. The leakage was found to be due to damage to the cytoplasmic membrane. Cell wall damage, RNA degradation and cytoplasmic protein alteration were also listed as causes of heat injury. Clark et al (11) found that RNA synthesis by Streptococcus faecalis R57 was impaired by heating at 60 C for 15 min.

Effects of Heat Injury

While some workers observed the actual physical injury of cells, others observed the effects of heating on the growth patterns of bacteria.

Allen (1) considered that bacterial cells entered a period of attenuation following heating. Dahlberg (13) attributed delayed growth of cells to heat-induced dormancy.

Jackson and Woodbine (20) determined that sub-lethal heating of S. aureus caused an immediate decrease in the viability of organisms and an increased lag phase prior to growth. The viability returned following incubation and no permanent loss of viability occurred. They concluded that delayed growth is the only effect of sub-lethal heating.

Kaufmann et al (21) also observed an increased lag phase in heated Micrococcus strain MS 102 cultures. Youssef et al (36) found that laboratory pasteurization of Pseudomonas fragi and Ps. fluorescens at 63 C for 30 min. caused a 7 day lag in growth of colonies on standard plate count agar (SPC) at 5 C. After 7 days psychrophillic growth was observed.

Another effect of heat injury is the loss of tolerance for certain chemicals. Research indicating that chemical compounds may suppress growth of injured cells at levels not affecting uninjured cells has been conducted by several workers.

Maxcy (22) found that heat-injured coliform organisms would not produce colonies on violet red bile agar (VRB) as readily as on SPC agar. Heated cultures were plated on both media and 78% of the population surviving laboratory pasteurization and growing on SPC could not produce colonies on VRB. The selective medium suppressed the growth of injured organisms. The surface active agents in the VRB were found to be responsible for the lack of growth. The amount of heat injury was quantified by the difference between SPC and VRB counts. Desoxycholate lactose

agar and brilliant green bile broth also were inhibitory to injured cells but had no effect on uninjured cells. Surface active agents were again the cause of inhibition.

Other work with suppressants utilized NaCl. Busta and Jeseski (10) found that the Na Cl content of Staphylococcus Medium No. 110 suppressed the growth of the injured population and decreased the total colony count of heat-shocked S. aureus 196E compared to plate count agar. S-110 medium with reduced NaCl content caused less suppression. Subsequent incubation of cells in skimmilk resulted in restoration of equal numbers of viable cells in both SPC and S-110 media.

Methods of Detecting Injured Organisms

Following the discovery by Busta and Jeseski that NaCl tolerance was reduced by heat injury, several methods were devised for determining the proportion of injured cells present in a culture.

Iandolo and Ordal (19) used TSA and TSA containing 7.5% NaCl (TSAS) as differential media for determining the number of injured cells of S. aureus MF31. Heat injury caused the cells to lose their ability to grow in TSAS. Cells in duplicate aliquots of heated cell suspension would recover and grow on TSA. Incubation in TSB allowed the injured cells to recover salt tolerance and grow on TSAS.

Clark et al (11) used 6% TSAS to evaluate heat injury in S. faecalis R57. Heat-injured cells would not grow in TSAS but recovered salt tolerance when incubated in TSB. TSAS had no effect on the unheated culture.

In the previous cases, approximately five log cycles of cells were injured giving 99.999% injury. The cells which were not injured accounted for only .001% of the total population. The growth of this small number of survivors would not make a significant contribution to the amount of cells counted as recovered.

The Membrane Filter

With small numbers of injured cells surviving, the percentage of uninjured survivors is proportionally larger and growth of survivors is a distinct possibility when attempting to distinguish the two types. Some method of fixing the position of cells is needed in order to produce colonies which can be detected and identified after incubation. Hurwitz et al (18) noted that the membrane filter could be used to fix the positions of cells by filtering bacterial suspensions onto the membrane after heat treatment.

The membrane filter was developed in Germany during World War II and was introduced into the United States in 1947. The membranes with 0.45 micron pores will filter bacteria from fluids. In making bacterial counts, the membranes, after such filtration, are incubated on absorbent pads saturated with the culture medium. Visible colonies are produced. The filtering process is limited by the amount of organic material in the solution. Large particles will clog the pores and filtration will cease. In making counts on whole milk the Millipore Corporation (3) recommends the use of a surfactant, Triton-X (Rohm and Haas), to aid in filtration; however, Maxcy (22) demonstrated that surfactants suppressed the growth of heat-injured cells.

Implications of Heat Injury

Maxcy (22) noted that coliform counts in milk are generally reported as <1 per ml following pasteurization. Problems develop later when regulatory laboratories find higher numbers of organisms. Maxcy indicated that recovery of organisms from heat injury may be a factor in the subsequent higher counts.

Speak (33) suggested that food spoilage organisms which are thought to be killed may be only injured. Subsequent recovery and growth may

cause rapid spoilage of foods which were thought to be satisfactorily pasteurized or sterilized.

Pariza and Iandolo (27) noted that S. aureus MF31, which was incapable of growing on TSAS could still produce coagulase. Such injured cells would still be active as pathogens.

Summary

The full significance of sub-lethal heat injury is not clearly understood. Increasing attention is being given to this phenomenon in order to appreciate more fully its impact on the food industry. Improved methods for direct isolation of heat-injured cells would facilitate study and contribute to the understanding of problems associate with sub-lethal heat injury.

EXPERIMENTAL PROCEDURES

The original intention in this research was to determine the effect of heat-injured bacterial cells on tests that have been used to predict the keeping quality of pasteurized milk. After initial exploration, the principal objective became the evaluation of the membrane filter technique as a possible adjuvant in facilitating such investigation. Since membrane filtration of whole milk presented problems even with surfactant filtering aids, it was necessary to conduct the studies with broth media. In order to maintain a flora somewhat representative of milk, raw milk was used as the inoculum. Following incubation, broth cultures were heat treated and subsequently cultured on membrane filters in a technique that distinguished colonies of recovered cells from those of non-injured organisms.

Sources of Milk

The raw milk used in this work was obtained from three sources. One was from a local grade A producer. Milk samples from the bulk tank were taken aseptically after thorough agitation, placed in sterile tubes and transported to the laboratory. The other sources were the herd of the University and bulk milk purchased by the University from Mid-America Dairymen, Sabetha, Kansas. Samples were taken from raw milk storage tanks in the University Dairy. Milk samples were refrigerated until used, always within 2 hrs.

Preparation of Culture Media

Bacteriological media were made according to the recommendations of the American Public Health Association (2).

TSB for use as a culture and recovery medium was formulated using 17.0 g pancreatic digest of casein USP (Casitone, Difco Laboratories), 3.0 g pancreatic digest of soya meal (Phytone, Baltimore Biological Laboratories), 5.0 g NaCl, 2.5 g HK_2PO_4, 2.5 g dextrose (Difco) and 1.0 liter distilled water.

TSB containing NaCl (TSBS) was used to determine the highest level at which no inhibition of unheated cells occurred. TSBS was made from the same formula as TSB except that the amount of NaCl was increased to obtain the desired percentage of salt in the final media. Media containing 1, 2, 3, 4 and 5 percent NaCl (TSBS1%,...,TSBS5%) were prepared and dispensed into tubes. After determination of the inhibitory level of NaCl as described below, TSBS of that NaCl content was used as a differential medium to determine the amount of heat injury.

Water blanks for dilution and suspension of cultures to aid in filtering were made by dispensing 9 ml of distilled water in screw cap tubes.

Litmus milk for growth and identification of organisms was made by dissolving 110 g nonfat dry milk powder in 1 liter of distilled water, adding litmus solution and dispensing into tubes with Morton closures.

All liquids except litmus milk were sterilized in an autoclave for 15 min. at 15 psi. Litmus milk was sterilized at 25 psi for 12 min. to prevent excessive browning.

Preparation of Dry Materials

Dabbah, et al (12) stated that the use of ampules in the heating of bacterial suspensions eliminated the possibility of contamination of unheated cells. Ampules were used as containers for cultures in all heating experiments. Ampules, syringes for filling ampules, petri plates

and filter equipment were wrapped in foil or kraft paper. Membrane filters (47 mm, Type HAWG, 0.45 micron pore size, Millipore Corporation, Bedford, Mass.) and absorbent pads were purchased in autoclave packaging ready for sterilization. All dry materials were sterilized in an autoclave at 15 psi for 30 min. and dried under negative pressure for 15 min.

Inoculation and Incubation of Cultures

The difficulty encountered in membrane filtration of whole milk made it necessary to use another medium in this work. A small amount of raw milk inoculated into TSB and incubated, produced a milk-type flora and gave no filtration difficulties. TSB was inoculated with raw milk at the rate of 0.1 ml per 9 ml TSB. The inoculated tubes were shaken on a Vortex mixer for 20 sec. to disperse the milk uniformly throughout the TSB. The tubes were incubated at 32 C for 24 hr. Duplicate tubes were combined after incubation and remixed to break up clumps of cells and to give a uniform culture before sampling.

Determination of Salt Sensitivity

It was necessary to determine the highest level of NaCl in TSB which would not be inhibitory to unheated cells in order to determine if NaCl inhibited heated cells. Preliminary experiments were performed to determine the dilution factor of the TSB culture needed to obtain the recommended (3) 20 to 200 colonies per membrane filter after 48 hr. incubation of the latter at 32 C with unheated cultures. Under the conditions of this study a dilution of 10^{-8} was found to be most suitable and was used in all work with unheated cultures. Petri plates (48 mm) containing absorbent pads were charged with 2.0, 1 of media. Media used were TSB, TSBS1%, TSBS2%, TSBS3%, TSBS4%, and TSBS5%. Incubated cultures were diluted and filtered in sextuplicate. Filters were placed on each of the six prepared absorbent pads in the plates and incubated at 32 C

for 48 hrs. A tray of water was kept in the incubator to maintain a high level of humidity to prevent excessive drying of the plates.

After growth most colonies were small, and some were difficult to see under normal room light. These colonies were counted by illuminating the filter with a microscope illuminator placed at an angle of about 15 degrees to the surface. The colonies then were observed as shadows behind the raised colonies. This procedure was followed throughout the study.

Data were analyzed for differences in counts caused by increasing NaCl concentration. Snedecor and Cochran's (32) two-way analysis of variance was used to determine the level at which salt-induced inhibition occurred. Significant differences in means were identified with the New Multiple Range Test of Duncan, described by Fryer (14). The NaCl level used in subsequent trials for determining sub-lethal injury was the highest concentration at which no significant reduction in the plate count of the non-heated culture occurred. This experiment showed that TSBS3% was the highest NaCl level at which no inhibition of cell growth and colony formation occurred. This level was used in subsequent trials.

Determination of Heat Injury

Heat Treatment. Cultures were placed into 3 ml ampules in 2.6 ml lots with a 5 cc syringe. Ampules were sealed by heating the neck and drawing the hot glass with forceps. Charged ampules were allowed to equilibrate to ambient temperature, (22.7 C) then immersed in a water bath held at 62.8 C. The time required for the internal temperature of the ampule to reach 62.8 C was determined with a thermometer inserted into an ampule and sealed with rubber cement. The come up time was determined to be 1 min. Heat treatments were varried to obtain different degrees of heat injury among survivors. Cultures were heated at 62.8 C for

15, 20, 25 and 30 min. with one min. allowed for come up time. Ampules were cooled and held in an ice bath until used, generally within 10 min.

Detection of Heat-Injured Organisms. Four ampules were prepared and one heated for each of the desired times. After cooling, duplicate 1 ml samples were taken from each ampule and placed in 9 ml water blanks. Each tube was mixed and filtered through a membrane filter. Duplicate filters were were placed on TSB and TSBS3%. Plates were incubated for 48 hrs. at 32 C, and the colonies were counted. The filters from the TSBS3% then were transferred to fresh TSB plates. These plates were incubated for 48 hrs. at 32 C. and counted. The original TSB count was considered to represent the total population of each suspension. The TSBS3% count indicated the uninjured population. After counting the TSBS3% plates, each colony was marked by perforating the filter near the colony with a sterile needle. The colonies thus marked, were disregarded when counting the colonies after the transfer TSB incubation. This gave a direct count of the recovered organisms from the new colonies which were produced during the second incubation on TSB. Figure 1 is a flow sheet giving the steps in determining heat injury and studying the surviving organisms.

Theoretically, the original TSB count and the transfer TSB count should be equal to each other. Significant differences between the original TSB count and the transfer TSB count for each heating time were identified with the paired t test (32). A trend was noted toward decreased count of organisms following the transfer of the filter from TSBS3% to fresh TSB and subsequent incubation compared to the original TSB count. However, the differences were not statistically significant. A possible explanation was that NaCl contact with the heated cells caused death after a period of time. The following experiment was performed to determine if salt induced death was the reason for the lower counts.

THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.

THIS IS AS RECEIVED FROM CUSTOMER.

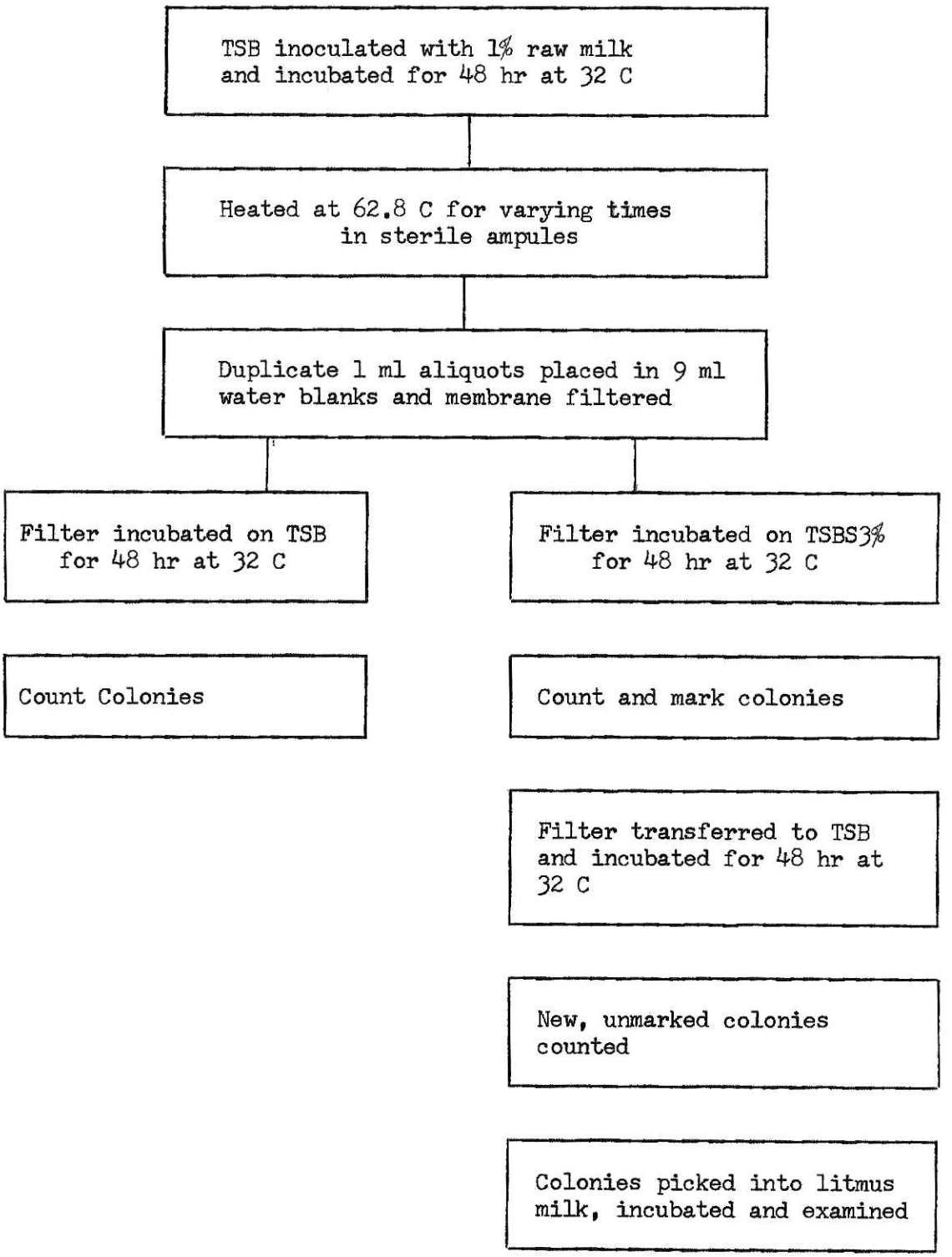


Figure 1. Flow sheet of the membrane filter technique for the detection and isolation of heated organisms

Determination of Effect of TSBS3% Contact with Heated Cells

Cultures were placed into 6 ml ampules in 5.2 ml lots with a 10 cc syringe. In a 62.8 C water bath, the come up time was determined to be 2.5 min. Ampules were held for 32.5 min. The ampules were cooled in an ice bath and held until used, generally within 10 min. Five 48 mm petri plates were prepared with an absorbent and 2 ml of media. TSB was placed in plate 1 and TSBS3% in plates 2 through 5. The ampule and contents were agitated on a Vortex mixer. One ml of culture was added to each of five water blanks. These were agitated and the contents filtered through membrane filters. The filter was rinsed with a second water blank. The filters were removed and placed on the prepared plates in a randomized order. The plates were incubated at 32 C. Plate 1 served as a control with a NaCl exposure time of zero and was incubated for 48 hr. the remaining filters were exposed to TSBS3% for varying times. Filter 2 was incubated for 6 hr. and filter 5 for 48 hr. Following the TSBS3% incubation, the filters were transferred to fresh TSB plates and incubated for 48 hr. at 32 C. Alternately, duplicate plates were allowed to incubate on TSBS3% for 96 hr. to determine if colonies would form on the filter in greater numbers than after 48 hr., indicating incomplete inhibition. The counts from the 96 hr. incubation on TSBS3% and the 48 hr. incubation on TSBS3% were compared with the paired t test to determine significant differences.

The data from the TSBS3% incubation for periods ranging from zero to 48 hr. were compared using the two-way analysis of variance to determine if salt exposure produced cell death instead of inhibition.

Description of Organisms

In some injury trials following the second incubation, both marked and unmarked colonies from the filters were picked into litmus milk and incubated for 48 hr. at 32 C. Culture tubes were observed for litmus milk reactions. Gram stains of each culture were made and examined microscopically for morphology. This allowed description of the types of organisms which were injured or uninjured by the heat treatment.

RESULTS AND DISCUSSION

Determination of Salt Sensitivity

Membrane filters from unheated cultures were incubated on TSBS media with NaCl content ranging from 0.5% (normal TSB) to 5.0%. Fifteen replications were performed. The results are given in Table 1. Two-way analysis of variance showed that the means of the count per ml for the six media were not all equal. The New Multiple Range Test showed 4% and 5% NaCl to be the significant treatments as the counts were reduced in these media. Any variation in the counts on all TSBS media up to 3% NaCl were shown to be due to sampling error. TSBS3% was considered to be the highest NaCl content which could be tolerated by the unheated cells and accordingly was used in all subsequent trials.

Determination of Heat Injury

Duplicate aliquots of heated cultures were filtered onto membrane filters. Filters were placed on TSB and TSBS3% and incubated for 48 hr. at 32 C. Colonies were counted and the filter from the TSBS3% plate was transferred to a fresh TSB plate and incubated for an additional 48 hr. at 32 C. All colonies on the TSBS3% plate were identified by perforating the filter near the colony with a sterile needle. These colonies were then easily differentiated from colonies which grew during the transfer incubation. The count data are summarized in Tables 2 through 5. Twenty-seven replications of each of the four heating times were performed. As noted in the Tables, some plates were uncountable due to spreaders, molds or contamination. These plates were not used in compiling data for statistical analysis. Table 2 gives the data of the trials from heating for 15 min. at 62.8 C. The TSB counts represented both heat

Table 1. Determination of salt sensitivity of mixed bacterial cultures.

Trial number	Bacterial count expressed as 10^8 per ml. Percent NaCl in TSBS					
	.5 (TSB)	1	2	3	4	5
1	32	27	23	28	21	21
2	10	12	11	13	6	3
3	20	19	18	17	9	2
4	48	46	43	47	40	36
5	23	21	21	20	15	12
6	37	34	36	35	30	22
7	28	27	28	26	21	19
8	21	20	21	22	17	15
9	17	16	14	16	10	8
10	42	41	39	41	25	22
11	21	19	20	20	16	14
12	35	33	31	34	27	21
13	34	33	33	32	28	24
14	27	28	26	27	22	26
15	38	37	35	37	34	30
Means	28.9 a	27.5 a	26.6 a	27.7 a	21.4 b	17.7 c

abc Means with different letters are significantly different (P=.05).

injured and uninjured organisms. The counts on TSBS are from uninjured bacteria with the difference representing injured cells unable to develop on the TSBS. The variation among trials is not expected due to differences in initial inocula. The extent of cell injury also varied among trials. For the 27 trials the mean count decreased from 110.8 to 23.2 per ml indicating 79.2% injured cells. The remaining 20.8% retained their salt tolerance and thus had no injury.

When the filters from TSBS were transferred to TSB the counts increased and reached the same general range as the original TSB counts. The means for the 27 trials showed no significant differences ($P = .05$). This indicated that the injured cells had recovered and produced colonies. These colonies were distinguishable from the original uninjured growth and could be counted separately.

Table 3 shows data from heating cultures for 20 min. at 62.8 C. The results are essentially the same as in Table 2. No significant differences were noted between the original TSB count and the transfer count indicating recovery of injured organisms. Sub-lethal injury was suffered by 81.7% of the population while 18.3 had no injury. The difference between 15 min. and 20 min. exposure had little effect on the count levels shown in Tables 2 and 3.

Data from heating cultures at 62.8 C for 25 min. are given in Table 4. Injured cells accounted for 75.2% of the total viable cells. The remaining 24.8% incurred no injury during the heat treatment. No significant difference existed between the original TSB count and the transfer count.

A significant difference between the original TSB count and the transfer count of cultures heated at 62.8 C for 30 min. was noted. These data are given in Table 5. The difference is possibly related to the longer heating time, which gave a more severe heat injury. This is not borne

out by the percent of injury figures, however. The injured population accounted for 78.2% and the remaining 21.8 were uninjured.

Comparisons among the four heating times are shown in Table 6. Some variation is noted in the percentage of injured cells. This variation was not significant (P = .05). The transfer count decreased with increasing heating time. This was not unexpected since a more intense heat treatment would cause more bacterial death. The original TSB count, with the exception of 15 and 20 min. heating times which were nearly equal in count, decreased as the severity of the heat treatment increased.

Consideration was given to the possibility that some interaction between heat and salt was responsible for the original TSB count and the transfer TSB count being unequal after heating for 30 min. The heated cells were possibly killed by salt concentrations which would not affect unheated cells. To test this possibility the following test was performed.

Effect of TSBS3% Contact with Heated Cells

Cultures were heated for 30 min. at 62.8 C, filtered, placed on TSBS3% and incubated for varying periods at 32C. After the desired period of time, the filters were transferred to fresh TSB plates and incubated at 32 C for 48 hr. Colony counts were made following incubation. Twelve replications were performed and the data are given in Table 7. The test data were examined with the two-way analysis of variance for significant differences in count caused by varying exposure to TSBS3%. Decreasing count with increasing NaCl exposure time would indicate that NaCl had a killing effect on heated cells. No significant differences were found with TSBS3% incubation times of 0 to 48 hr. This indicated that NaCl did not kill heated cells at the 3% level during such incubation periods.

Three additional trials were conducted to determine whether or not incubation time in excess of 48 hr. on TSBS3% would produce additional

Table 2. Effect of membrane filter transfer technique on bacterial counts per ml of heated cultures. (15 min. at 62.8 C)

Trial	TSB Count ∠1	TSBS3% Count ∠1	Transfer Count ∠2	Recovery Count ∠3
1	97	61	97	36
2	*	17	26	9
3	*	57	117	60
4	147	10	136	126
5	53	60	79	19
6	174	20	186	166
7	17	8	21	13
8	138	1	111	110
9	*	17	39	22
10	*	12	66	54
11	40	9	54	45
12	20	18	25	7
13	43	33	42	9
14	44	10	39	29
15	24	10	22	12
16	37	12	33	21
17	107	19	98	78
18	74	67	69	2
19	356	16	340	324
20	98	20	84	64
21	113	18	106	88
22	232	22	196	174
23	46	8	43	35
24	224	31	188	157
25	162	20	158	138
26	127	17	118	101
27	175	34	165	131
Means	110.8 a	23.2	98.4 a	75.2

∠1 Colony counts on membrane filter after 48 hr. at 32 C

∠2 Colony counts on membrane filter from TSBS following additional 48 hr. incubation after transfer to TSB

∠3 Colonies which were unmarked following final incubation

* Uncountable plates due to spreaders, molds or unexplained contamination: data not used to calculate paired t test.

a Means with the same letter are not significantly different (P = .05).

Table 3. Effect of membrane filter transfer technique on bacterial counts per ml of heated cultures. (20 min. at 62.8 C)

Trial	TSB Count ∠1	TSBS3% Count ∠1	Transfer Count ∠2	Recovery Count ∠3
1	53	75	97	22
2	*	24	35	9
3	*	22	170	148
4	*	9	31	22
5	151	13	187	174
6	8	12	14	2
7	267	8	107	99
8	*	15	61	46
9	*	21	54	33
10	45	11	45	34
11	22	16	39	23
12	32	13	34	21
13	20	6	17	11
14	200	15	189	174
15	153	15	149	134
16	66	48	69	21
17	270	15	258	243
18	143	14	100	86
19	73	18	65	47
20	184	25	178	153
21	38	10	31	21
22	170	45	154	109
23	156	17	142	125
24	110	18	103	85
25	61	20	54	34
26	161	32	153	121
27	73	17	66	49
Means	111.6 a	20.5	96.4 a	75.9

∠1 Colony counts on membrane filter after 48 hr. at 32 C

∠2 Colony counts on membrane filter from TSBS following additional 48 hr. incubation after transfer to TSB

∠3 Colonies which were unmarked following final incubation

* Uncountable plates due to spreaders, molds or unexplained contamination: data not used to calculate paired t test

a Means with the same letter are not significantly different (P = .05).

Table 4. Effect of membrane filter transfer technique on bacterial counts per ml of heated culture. (25 min. at 62.8 C)

Trial	TSB Count ∠1	TSBS3% Count ∠1	Transfer Count∠2	Recovery Count∠3
1	45	60	84	24
2	*	24	162	138
3	*	29	176	147
4	34	9	31	22
5	145	17	45	28
6	*	75	95	20
7	10	11	19	8
8	260	18	87	69
9	61	15	50	35
10	42	14	38	24
11	17	13	40	27
12	23	6	19	13
13	40	20	37	17
14	18	5	16	11
15	41	15	37	22
16	85	11	83	72
17	57	36	55	19
18	197	13	180	167
19	83	19	71	52
20	133	15	102	87
21	160	21	159	138
22	30	9	23	14
23	119	29	115	86
24	143	21	129	108
25	98	16	94	78
26	40	17	39	22
27	145	30	149	119
Means	84.4 a	21.0	79.0 a	58.0

∠1 Colony counts on membrane filter after 48 hr. at 32 C

∠2 Colony counts on membrane filter from TSBS following additional 48 hr. incubation after transfer to TSB

∠3 Colonies which were unmarked following final incubation

* Uncountable plates due to spreaders, molds or unexplained contamination: data not used to calculate paired t test

a Means with the same letter are not significantly different (P = .05).

Table 5. Effect of membrane filter transfer technique on bacterial counts per ml of heated cultures. (30 min. at 62.8 C)

Trial	TSB Count∠1	TSBS3% Count∠1	Transfer Count∠2	Recovery Count∠3
1	44	36	47	11
2	*	23	116	93
3	89	15	62	47
4	55	25	31	6
5	95	15	26	11
6	177	14	140	126
7	72	28	60	32
8	*	8	101	93
9	*	19	80	61
10	*	16	35	19
11	10	13	16	3
12	45	13	26	13
13	27	17	29	12
14	15	4	16	12
15	44	15	47	32
16	47	13	44	31
17	56	27	53	26
18	162	12	137	125
19	77	13	67	54
20	122	14	108	94
21	97	16	93	77
22	36	11	32	21
23	73	12	66	54
24	130	15	117	102
25	84	12	81	69
26	39	15	29	14
27	138	41	136	95
Means	74.9 a	16.3	66.4 b	50.1

∠1 Colony counts on membrane filter after 48 hr. at 32 C

∠2 Colony counts on membrane filter from TSBS following additional 48 hr. incubation after transfer to TSB

∠3 Colonies which were unmarked following final incubation

* Uncountable plates due to spreaders, molds or unexplained contamination: data not used to calculate paired t test

ab Means with different letters are significantly different (P = .05).

Table 6. Effect of four different heating times on percentage of injured cells, total TSB count and transfer TSB count

Heating time (62.8 C)	Data from 27 trials		
	Injured cells	TSB count	Transfer count
(Min.)	(%)		
15	79.2	110.8	98.4
20	81.7	111.6	96.4
25	75.2	84.4	79.0
30	78.2	74.9	66.4

Table 7. Effect of exposure time to TSBS3% on bacterial counts per ml following transfer to TSB and 48 hr. incubation at 32 C. Cultures were heated for 30 min. at 62.8 C.

Trial	House incubation on TSBS3% before transfer to TSB				
	0	6	18	24	48
1	85	87	81	85	84
2	83	81	80	85	80
3	180	242	257	260	275
4	61	50	66	70	67
5	84	81	78	77	75
6	99	93	97	102	95
7	50	49	43	46	52
8	160	164	159	155	152
9	82	81	84	77	74
10	20	23	16	18	24
11	192	198	202	193	182
12	58	53	49	47	46
Means	104.5 a	100.2 a	101.0 a	101.3 a	100.5 a

a Means with the same letter are not significantly different (P = .05).

colonies in the presence of NaCl. The three trials were in conjunction with trials 7, 9, and 12 (data in Table 7). A sixth replicate of the above series was prepared and the filter was incubated on TSBS3% for 96 hr. before transfer. The data from this experiment are shown in Table 8. No significant growth of cells occurred on TSBS3% after 48 hr. This showed that 3% NaCl did inhibit heat-injured cells. The cells were not able to repair the injury when in the presence of NaCl and could not grow into visible colonies even after extended incubation. The count following transfer of the filter after 96 hr. from TSBS3% to TSB and subsequent 48 hr. incubation indicates that no lethal effect was present even after extended periods of exposure to NaCl. No significant differences ($P = .05$) existed between 48 hr. TSBS3% incubation followed by 48 hr. TSB incubation and 96 hr. TSBS3% incubation followed by 48 hr. TSB incubation. The incubation on media containing 3% NaCl did not affect the injured population except for temporarily inhibiting colony formation while on that medium.

The insignificant ($P = .05$) variation in the percent of injury after heating for 15, 20, and 25 min. at 62.8 C is probably due to daily variations in inocula and culture growth. Using a mixed flora, the growth patterns, organism balance and heat sensitivity would be expected to change from day to day.

Effect of Technique Using Other Milk Sources

The foregoing data were obtained from milk inocula from mixed bulk milk of one producer. In order to determine if different sources of milk would produce different results, trials were run using two other sources. Results are presented in Tables 9 and 10. A larger percentage of injury was noted in these trials than in previous trials. When cultures were heated at 62.8 C for 30 min. cultures from milk of the University herd showed 83.6% heat injury and that from Mid-America Dairymen showed 86.3%.

Table 8. Effect of 96 hr. incubation of membrane filter in TSBS3% on colony counts per ml of cultures heated at 62.8 C for 30 min.

Trial	Colony counts per ml on membrane filters after:			
	48 hr. TSBS3%	96 hr. TSBS3%	48 hr. TSBS3% + 48 hr. TSB	96 hr. TSBS3% + 48 hr. TSB
7	8	7	52	50
9	13	14	74	68
12	11	10	46	47
Means	10.6 a	10.3 a	58.6 b	55.0 b

ab Means with the same letter are not significantly different (P = .05).

Table 9. Effect of membrane filter technique on bacterial counts of cultures heated at 62.8 C for 30 min. (Milk inocula source: Kansas State University herd milk)

Trial	TSB Count∠1	TSBS Count∠1	Transfer Count∠2	Recovery Count∠3
1	90	14	86	72
2	71	11	69	58
3	107	19	104	85
Means	89.3 a	14.7	86.3 a	71.6

∠1 Colony count per ml on membrane filter after 48 hr. at 32 C

∠2 Colony count per ml on membrane filter from TSBS following additional 48 hr. incubation after transfer to TSB

∠3 Colonies which were unmarked following final incubation

a Means with the same letter are not significantly different (p = .05).

Table 10. Effect of membrane filter technique on bacterial counts of cultures heated at 62.8 C for 30 min. (Milk inocula source: Mid-America Dairymen, Inc.)

Trial	TSB Count∠1	TSBS Count∠1	Transfer Count∠2	Recovery Count∠3
1	114	14	109	95
2	123	19	99	80
Means	118.5 a	16.5	104.0 a	87.5

∠1 Colony count per ml on membrane filter after 48 hr. at 32 C

∠2 Colony count per ml on membrane filter from TSBS following additional 48 hr. incubation after transfer to TSB

∠3 Colonies which were unmarked following final incubation

a Means with the same letter are not significantly different (P = .05).

Even with the small number of trials these were considerable increases over results from the main trials. Since no salt sensitivity trials were run on cultures from these sources, it is possible that the flora was more salt sensitive. Other data were essentially the same as in the previous work. The membrane transfer technique gave the same recovery as before with no significant difference between the TSB counts and transfer counts on the heated cultures. The effectiveness of the recovery technique was not affected by the milk source of the flora.

Plates I and II show the results of the transfer technique. Plate I shows a membrane filter (approximately 2.2 diameters enlargement) following 48 hr. at 32 C on TSBS3%. Four colonies are visible. Plate II shows the same filter following transfer and incubation on TSB for 48 hr. at 32 C. In addition to the four colonies, eighteen new colonies developed from recovered cells. Two of the original colonies remained the same size and two increased in size. One mold grew during the transfer incubation.

The marking technique worked very well in allowing colonies to be differentiated as injured and uninjured groups. Other methods of marking colonies were tried without success. Stains either washed off the colonies or the dyes killed the organisms. The punch marking technique did not photograph well and cannot be seen in the photographs.

Description of Heated Organisms

Segregated and differentiated colonies were picked from TSBS3% and from transferred filters into litmus milk and incubated at 32 C for 48 hr. Culture characteristics were noted and gram stains were made to determine the basic types of organisms present.

With the cultures inoculated with milk from the original source, the injured and subsequently recovered organisms were 100% gram positive cocci. Litmus milk reactions were acid production, coagulation, reduction, and the

EXPLANATION OF PLATE I

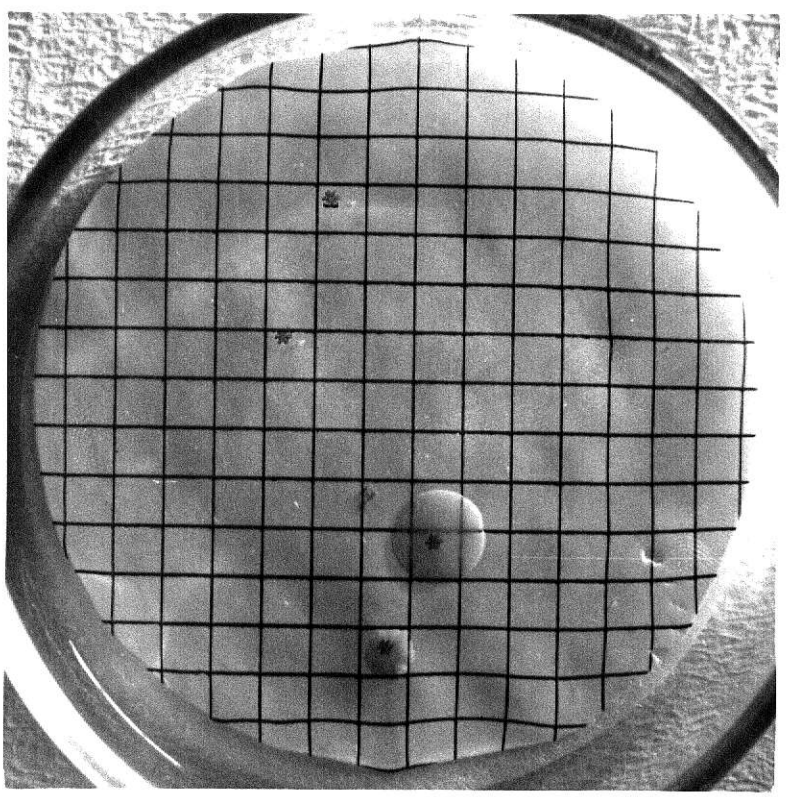
Membrane filter with developed colonies (2.2 diameter enlargement)

* Colonies which grew during the 48 hr. TSBS3% incubation

THIS BOOK CONTAINS NUMEROUS PICTURES THAT ARE ATTACHED TO DOCUMENTS CROOKED.

THIS IS AS RECEIVED FROM CUSTOMER.

PLATE I

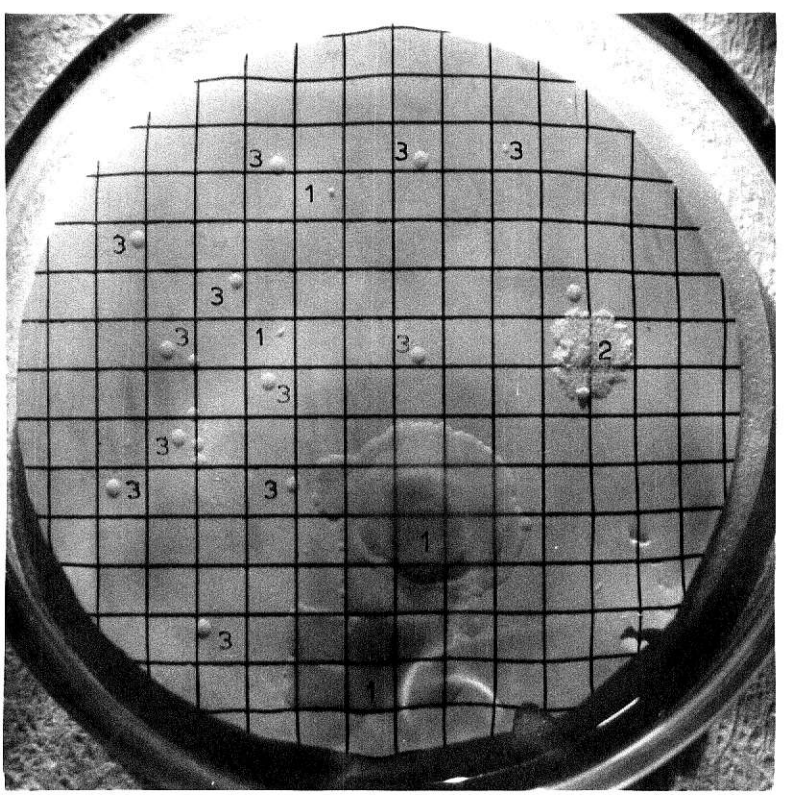


EXPLANATION OF PLATE II

Same membrane filter shown Plate I, after transfer incubation

1. Colonies which grew on TSBS3% during initial incubation
2. Mold
3. Colonies which grew after transfer to TSB

PLATE II



proteolysis typical of Streptococcus faecalis var. liquefaciens. No confirmatory tests were made to ascertain the actual genus and species. Cells which survived heating uninjured were gram positive rods, small gram negative rods and gram positive cocci. Gram positive rods showed rapid proteolysis of litmus milk. Spores were observed microscopically and the organism was classified as a Bacillus type. The small gram negative rods were gas producing, lactose fermentors in litmus milk and resembled coliform types. The gram positive cocci again resembled S. faecalis var. liquefaciens in litmus reactions. Pseudomonads and other psychrotrophic types normally associated with raw milk were not present in either injured or uninjured groups and were apparently killed in the heating treatment.

SUMMARY AND CONCLUSIONS

A technique was developed for recovery and isolation of heat-injured bacteria. It utilized the membrane filter procedure for making bacterial counts. Heated cultures were filtered and incubated on trypticase soy broth containing 3% NaCl (TSBS3%) for 48 hr. at 32 C. Resulting colonies were marked by punching holes in the filter near the site of each colony. Subsequent transfer of the filter and incubation on trypticase soy broth (TSB) produced new colonies which were distinctly separate from the marked colonies. The major advantage of the technique is that colonies produced by injured and subsequently recovered cells can be distinguished from those developing from uninjured cells. Uninjured cells which had no loss of NaCl tolerance produced colonies even in the presence of NaCl. Injured cells lost their salt tolerance and would not grow on media containing high levels of NaCl. Subsequent incubation on media with very low NaCl content (TSB) allowed the injured cells to recover and produce colonies which were distinct from those of the uninjured cells. The amount of heat injury could be quantitated directly by counting the cells which grew on the transfer TSB incubation but not in the original TSBS3% incubation.

The percentage of injured cells from a mixed flora inoculum incubated at 32 C for 24 hr. in TSB ranged from 75.2 to 81.7% of those cells which survived heating at 62.8 C for 15, 20, 25, or 30 min.

In using a mixed flora, it is necessary to recognize that different types of organisms have different NaCl tolerances. This may account for the fact that the transfer TSB count tended to be lower (but not in most cases significantly so) than the original TSB count of a duplicate aliquot of the sample originally incubated on TSBS3%. The mixed flora, however, is thought to be very important for studies on milk or food organisms

since rarely do foods contain pure cultures of microorganisms. The membrane filter technique provides a method of selecting those types of organisms susceptible to sub-lethal heat injury.

It is believed that the technique can be applied to pure culture studies by first determining the organisms sensitivity to some agent (i. e. NaCl, etc.) and then using differential media to segregate the injured and uninjured organisms.

ACKNOWLEDGMENTS

The author would like to express his sincere appreciation to Dr. T. J. Claydon, Department of Dairy and Poultry Science, for his patience, understanding and direction during this investigation and in the preparation of this thesis.

Grateful acknowledgment is given to Dr. J. J. Iandolo, Division of Biology, for suggestions throughout the course of the study and to Dr. Ross Mickelsen, Department of Dairy and Poultry Science, who served on the author's advisory committee.

Appreciation is also given to Dr. C. L. Norton, Head of the Department of Dairy and Poultry Science, and to the faculty, staff, and graduate students of the Department for their interest and cooperation.

Acknowledgment is given to Dr. George A. Milliken, Department of Statistics and Computer Science for suggestions in the statistical analysis of data.

Sincere thanks are expressed to my parents, Mr. and Mrs. Harold A. Goff, for their encouragement and support during the completion of this study.

LITERATURE CITED

1. Allen, P. W. 1923. The attenuation of bacteria due to temperature shock. J. Bacteriol., 10:555.

2. American Public Health Association, 1966. Recommended methods for the examination of foods. 2nd ed. American Public Health Ass., Inc., New York, N. Y.

3. Anon. 1967. Techniques for microbiological analysis-ADM 40. Millipore Corporation, Bedford, Mass.

4. Ayers, S. H., and C. S. Mudge. 1920. Milk powder agar for the determination of bacteria in milk. J. Bacteriol., 5:565.

5. Baird-Parker, A. C., and Elizabeth Davenport. 1965. The effect of recovery medium on the isolation of Staphylococcus aureus after heat treatment and after the storage of frozen or dried cells. J. Appl. Bacteriol., 28:390.

6. Barber, F. W., C. P. Burke, and H. Fram. 1954. The millipore filter technique in the dairy industry. J. Milk Food Technol., 17:109.

7. Beuchat, L. R., and R. V. Lechowich. 1968. Effect of salt concentration in the recovery medium on heat-injured Streptococcus faecalis. Appl. Microbiol., 16:772.

8. Bowers, C. S., and J. G. Hucker. 1935. The composition of media for the bacteriological analysis of milk. N. Y. Agr. Expt. Sta. Tech. Bull. 188.

9. Bradfield, Alec. 1937. Bacterial content of milk as affected by the use of different plating methods. Vt. Agr. Expt. Sta. Bull. 417.

10. Busta, F. F., and J. J. Jeseski. 1963. Effect of sodium chloride concentration in an agar medium on growth of heat-shocked Staphylococcus aureus. Appl. Microbiol., 11:404.

11. Clark, Carol W., L. C. Witter, and Z. J. Ordal. 1968. Thermal injury and recovery of Streptococcus faecalis. Appl. Microbiol. 16:1764.

12. Dabbah, R., W. A. Moats, and J. F. Mattick. 1969. Factors affecting resistance to heat and recovery of heat-injured bacteria. J. Dairy Sci., 52:608.

13. Dahlberg, A. C. 1946. The relationship of the growth of all bacteria and coliform bacteria in pasteurized milk held at refrigeration temperature. J. Dairy Sci., 29:651.

14. Fryer, H. C. 1966. Concepts and methods of experimental statistics. Allyn and Bacon, Inc. Boston.

15. Garvie, E. I. 1955. The growth of Escherichia coli in buffer substrate and distilled water. J. Bacteriol., 69:393.

16. Hansen, N. H., and H. Riemann. 1963. Factors affecting the heat resistance of non-sporing organisms. J. Appl. Bacteriol., 26:314.

17. Heinmets, F., W. W. Taylor, and J. J. Lehman. 1954. The use of metabolites in the restoration of the viability of heat and chemically inactivated Escherichia coli. J. Bacteriol., 67:5.

18. Hurwitz, C., C. L. Rosano, and B. Blattberg. 1957. A test for the validity of reactivation of bacteria. J. Bacteriol., 73:743.

19. Iandolo, J. J., and Z. J. Ordal. 1966. Repair of thermal injury of Staphylococcus aureus. J. Bacteriol., 91:134.

20. Jackson, H., and M. Woodbine. 1963. The effect of sub-lethal heat treatment on the growth of Staphylococcus aureus. J. Appl. Bacteriol., 26:152.

21. Kaufmann, O. W., L. G. Harmon, O. C. Pailthorp, and I. J. Pflug. 1959. Effect of heat treatment on the growth of surviving cells. J. Bacteriol., 78:834.

22. Maxcy, R. B. 1970. Non-lethal injury and limitations of recovery of coliform organisms on selective media. J. Milk Food Technol., 33:445.

23. Nelson, F. E. 1940. The effect of the new standard milk agar on the plate count of dairy products. J. Bacteriol., 39:263.

24. Nelson, F. E. 1943. Factors which influence the growth of heat treated bacteria. I. A comparison of four agar media. J. Bacteriol., 45:305.

25. Nelson, F. E. 1944. Factors which influence the growth of heat treated bacteria. II. Further studies on media. J. Bacteriol., 48:473.

26. Ordal, Z. J. 1970. Current developments in detection of microorganisms in foods: Influence of environmental factors on detection methods. J. Milk Food Technol., 33:1.

27. Pariza, M. F., and J. J. Iandolo. 1969. Coagulase production by injured Staphylococcus aureus during recovery. Appl. Microbiol., 17:836.

28. Phelum, J. F. 1936. Some effects of the proposed new bacteriological techniques. J. Dairy Sci., 19:385.

29. Rosenthal, L. J., and J. J. Iandolo. 1970. Thermally induced intracellular alteration of ribosomal ribonucleic acid. J. Bacteriol., 103:833.

30. Russell, A. D., and Diann Harries. 1968. Damage to Escherichia coli on exposure to moist heat. Appl. Microbiol., 16:1394.

31. Sherman, J. M. 1916. The advantage of carbohydrate medium in the bacterial examination of milk. J. Bacteriol., 1:481.

32. Snedecor, G. W., and W. G. Cochran. 1967. Statistical methods. 6th ed. The Iowa State University Press. Ames, Iowa.

33. Speck, M. L. 1970. Selective culture of spoilage and indicator organisms. J. Milk Food Technol., 33:163.

34. Stiles, M. E., and L. D. Witter. 1965. Thermal inactivation, heat injury, and recovery of Staphylococcus aureus. J. Dairy Sci., 48:677.

35. Yale, V. W. 1938. Standard agar counts as compared with those on improved agars at 32 C. Am. J. Pub. Health, 28:148.

36. Youssef, E. H., J. D. Ortego, and J. H. Gohlson. 1971. Effect of heat treatment on the growth of selected psychrophillic bacteria in milk. Abstract in J. Dairy Sci., 54:448.

THE RECOVERY AND ISOLATION OF HEAT-INJURED BACTERIA BY A MEMBRANE FILTER TECHNIQUE

by

JAMES HAROLD GOFF

B. S., Kansas State University, 1969

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

in

Food Science

Department of Dairy and Poultry Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1971

The purpose of this study was to evaluate the membrane filter technique as a method for isolating and enumerating sub-lethally heat-injured and uninjured bacteria. The recognized differences in salt tolerance caused by sub-lethal injury also were used to segregate injured and uninjured cells in studies made on broth cultures containing mixed flora. The mixed flora were provided by inoculating trypticase soy broth (TSB) with 1% raw milk and incubating at 32 C for 48 hr.

Salt tolerance of the unheated culture was determined to be 3% sodium chloride. The cultures were heated at 62.8 C for varying times, and duplicate aliquots were membrane filtered. Duplicate filters were placed in plates containing absorbent pads saturated with 2.0 ml of media. Media used were TSB and TSB containing 3% sodium chloride (TSBS3%). Incubation time was 48 hr. at 32 C. Colonies that developed were usually small, but they were readily detected by suitable spot lighting.

The TSB count indicated the total population which survived the heating process and included injured and uninjured cells. The injured cells were not able to grow in TSBS3%. This count indicated only those cells which were uninjured. The colonies which grew on TSBS3% were marked by perforating the filter near the site of the colony with a sterile needle. The filter was then transferred aseptically to a fresh TSB plate and incubated for an additional 48 hr. at 32 C. The heat-injured cells, which had been suppressed by the presence of 3% sodium chloride, grew and produced new colonies which were easily differentiated from the colonies produced by the incubation on TSBS3%. The number of heat-injured cells was determined by the count of colonies which appeared after the transfer incubation on TSB.

The proportion of heat-injured organisms among survivors of heating at 62.8 C for 15, 20, and 25 min. was rather constant and varied from 75.2 to 81.5%.

A trend was noted in comparing the original TSB count and the transfer TSB count. The transfer count was slightly lower than the original TSB

count, but the difference was not significant (P = .05) for the first three heating times. At 30 min. heating time the difference was significant (P = .05). Further tests, in which heated cells were incubated on TSBS3% for varying times before transfer to TSB, showed that the reduction in count was not due to interaction between the effects of heating and salt contact.

Some of the colonies which were isolated and differentiated by the technique were examined for type characteristics. The organisms which recovered from heat injury were 100% of a type tentatively identified as Streptococcus faecalis var. liquefaciens on the basis of litmus milk reactions and gram stain morphology. Uninjured cells were gram positive cocci, gram positive rods and gram negative rods.