ASSESSMENT OF DAIRY PRODUCT QUALITY BY SELECTIVE INCUBATION AND RAPID TECHNIQUES

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(ABSTRACT)

A study was conducted to investigate the use of bacterial numbers and metabolites for their value in predicting potential shelf-life of pasteurized fluid milk. The study consisted of two phases. In the first phase, chemical inhibitors were evaluated for their ability to allow growth of gram-negative psychrotrophic bacteria while inhibiting growth of gram-positive organisms. Crystal violet, benzalkonium chloride, a mixture of crystal violet and benzalkonium chloride, and alkylaryl sulfonate were tested. These were incubated with milk samples separately inoculated with gram-negative and gram-positive bacteria, at 21°C for 18 Alkylaryl sulfonate allowed growth of gram-positive organisms. hours. Crystal violet inhibited gram-positive organisms but was not as effective as the mixture of crystal violet and benzalkonium chloride. The inhibitor of choice was benzalkonium chloride as it inhibited growth of all grampositive organisms tested and allowed growth of the gram-negative psychrotrophic organisms.

In the second phase of this study rapid detection or enumeration techniques were evaluated for their value in predicting potential shelflife of pasteurized fluid milk. Milk samples were stored at 7°C and tested weekly until determined to be unacceptable by daily sensory evaluation. Samples were subjected to 18 hours incubations at 21°C of milk alone, milk with benzalkonium chloride, milk and nutrient broth, milk and nutrient broth with benzalkonium chloride, and milk and a dairy gram-negative broth.

After incubation the following tests were conducted: modified psychrotrophic bacteria count (mPBC), impedance detection time, catalase detection by headspace pressure and flotation time (Catalasemeter), endotoxin detection, and direct epifluorescent filter technique. A11 rapid detection techniques had significant (p<0.001) correlations to shelf-life at time-zero and these correlations to shelf-life were improved after many of the preliminary incubations were conducted. For plate count techniques (mPBC and PmPBC) the highest correlation to shelf-life (-0.89) was after a preliminary incubation of milk in nutrient broth. For the impedance detection time the highest correlation to shelf-life (0.91) was after a preliminary incubation of milk in dairy gram-negative broth. For catalase detection the highest correlation (-0.84 and 0.77) for headspace pressure and flotation time, respectively, was after a preliminary incubation of milk and benzalkonium chloride. For the direct epifluorescent filter technique the highest correlation (-0.78) was after a preliminary incubation of milk and nutrient broth. For the endotoxin detection the highest correlation (-0.85) was after a preliminary incubation of milk or milk in benzalkonium chloride. The recommended shelf-life program developed involves a preliminary incubation (18 h, 21°C) of 10 ml milk followed by the mPBC, and resulted in a correlation to shelf-life of -0.88.

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INTRODUCTION

Milk contains 87.0% water, 3.9% fat, 4.9% lactose, 3.5% proteins, 0.7% ash, and a trace amount of vitamins. Milk therefore is an optimum medium for bacterial growth. Raw milk is pasteurized to lower the total number of bacteria and eliminate pathogenic bacteria. Some bacteria are present in milk which grow at refrigeration temperatures and spoil the product. Most bacteria contaminate the milk by post-pasteurization contamination. In order to predict the shelf-life of milk, it is necessary to have a test that indicates to what extent post-pasteurization contamination has taken place so that problems may be corrected. Two problems arise when determining the degree of post-pasteurization contamination. First, results need to be obtained quickly so that effective, corrective measures can be taken if a problem is detected. Second, the initial contamination may be so low that accurate detection is nearly impossible. Therefore, a need exists for a test which is accurate, reliable and gives results within a time period to allow for effective, corrective measures to be taken. The objective of this research was to develop a selective preliminary incubation which can be followed by a rapid detection or enumeration technique to accurately predict the potential shelf-life of pasteurized fluid milk.

LITERATURE REVIEW

Keeping Quality

Farm bulk tanks, every-other-day pick-up at farms, 5 day-a-week operation, discontinued home delivery, and purchasing only on shopping days have increased milk's age before consumption (78). Shelf-life, or keeping quality of milk, has been defined as the number of days required to attain an unsatisfactory flavor (42). Baker (4) also defined a "consumable life" as the period between processing and when milk becomes unacceptable to consumers, but he also indicated that there is no adequate working definition of shelf-life. By these definitions, we see that the flavor of milk is very important in assessing quality. Bandler (5) stated that milk flavor is the most important test of quality while Baker (4) suggested that organoleptic evaluation is the most useful means of assessing keeping quality.

A code date is placed on a milk carton to indicate the last day of sale (112). Consumers expect that, if the milk is properly refrigerated, the milk will be acceptable up to and hopefully beyond this code date. However, Hankin, et al. (43) report that keeping quality is unrelated to manufacturer's code date. Temperature of storage is very important in determining keeping quality (8,78). After the milk has left the plant, proper refrigeration is the exception rather than the rule (21). Overstocked showcases and consumer abuse give rise to elevated storage temperatures which decrease the shelf-life of the product. Hankin et al. (43) reported a shelf-life of 17.5, 12.1, and 6.9 days at 1.7, 5.6, and

10.0°C, respectively. Other data suggested 31 days at 0°C, 6 days at 7°C (29), 11 days at 4.5°C, and 9 days at 7.0°C (48). Boyd et al. (19) stated that at 4.4°C, a flavor score of 37+ indicates a shelf-life of 13-18 days and a standard plate count of 500,000 cfu/ml indicates a shelf-life of 8-11 days, but storage at 0.6°C extends the shelf-life 11-14 days. Schröder et al. (104) reported that raising the temperature from 5 to 11°C reduced shelf-life from 28 to 6 days for lab pasteurized milk and 13 to 5 days for commercially pasteurized milk.

Psychrotrophic Bacteria

The bacteria that ultimately cause quality degradation of milk are those which are capable of growth at refrigeration temperatures. This group of bacteria was previously known as "psychrophilic" or cold-loving. This term is misleading because these bacteria are not cold-loving but cold-enduring, therefore they are now known as "psychrotrophic" bacteria. Psychrotrophs have been defined as bacteria which grow relatively well at refrigeration temperatures (23,51,107, 114,123). Several studies have also shown that psychrotrophs are the main causes of low shelf-life in milk (51,96). Boyd (19) stated that flavor deterioration correlated with psychrotrophic growth and that the main bacteria isolated from milk with a poor keeping quality were gram-negative. Witter (123) also reported that psychrotrophs are primarily responsible for limiting keeping quality in milk and that gram-negative, non-sporeforming rods comprised most of He further stated that most of these were of the the isolates. Pseudomonas species. Gyllenberg et al. (39) reported that pseudomonads

may influence keeping quality. White et al. (118) found that 90% of their 315 milk samples containing psychrotrophs were <u>Pseudomonas</u>.

Psychrotrophs are present in water, soil, and on dirty equipment (23,123), but, for the most part, do not survive pasteurization (23). They gain entry into the milk through post-pasteurization contamination (51,60), which may result from improperly cleaned and sanitized equipment, the air, or filling equipment (23,44). Even though it has been reported that 5-20 x 10^6 (91), $>10^6$ (80), and $>10^7$ cfu/ml (68,104) are required before organoleptic changes are detected, low numbers of initial contamination will lead to a decreased keeping quality. Cousin (23) stated that at 7.2°C, 1 psychrotrophic cfu/ml will multiply to 10⁶ psychrotrophic cfu/ml in 8 days or less. Mikolajcik (67) reported an increased psychrotrophic count after 14 and 28 days storage at 7°C in heated milk with low initial counts. Carey (21) states that psychrotrophs increase 5 logs from buy to pull date. Psychrotrophs also secrete heatstable enzymes such as proteinases and lipases which will affect the keeping quality (47,59).

Bacterial Enumeration

Microbiological tests performed on milk supply information on sanitary practices as well as keeping quality. One traditional method of bacterial enumeration is the plate count. The theory behind a plate count is that a single bacterial cell will grow to form a visible colony after a period of time (108). This theory is somewhat questionable, however, as colonies may arise from clumps, aggregates or chains of bacteria rather

than a single cell (49). One plate count which is currently conducted on raw and pasteurized milk is the Standard Plate Count (SPC) (95) during which plates are incubated at 32°C for 48 hours. The Standard Plate Count has been proven to be valuable for indicating sanitary practices but of little value in predicting the keeping quality of milk (3,7,19,34,94,119).

Since the main spoilage organisms in milk are the psychrotrophic bacteria, new plate count techniques were tested for their ability to enumerate these bacteria (41). Randolph et al. (92) lowered the incubation temperature to 27°C but found this to be no better than 32°C for enumerating psychrotrophs. Roughley et al. (100) lowered the incubation temperature to 30°C and increased the incubation time to 72 hours and achieved higher counts which were assumed to be due to psychrotrophs. Atherton et al. (3) reported higher counts at 25°C than at 32°C. In trying to shorten the length of the psychrotrophic bacteria count (95) from 10 days, Oliveria and Parmelee (76) decreased the incubation temperature to 21°C and the incubation time to 25 hours. The correlation coefficients of their modified Psychrotrophic Bacteria Count (mPBC) to the standard Psychrotrophic Bacteria Count (PBC) were 0.992 in raw milk and 0.996 in pasteurized milk. A plate count method which utilizes a dry film was developed by the 3M corporation and research has shown it to be comparable to plate count methods for determining bacterial numbers (15,36,65)

Shelf-life Test

If an ideal test for shelf-life of milk was available, it would have

the following characteristics: 1) accurate, 2) rapid and 3) simple (16). Many researchers have shown that bacterial enumerations are of little value for predicting shelf-life, especially on freshly pasteurized milk (4,11,12,14,43,117). Hankin et al. (43) stated that we need to reflect on the ultimate value of determining keeping quality based solely on bacterial tests and that the key to predicting keeping quality is not the total bacterial count but rather a measurement of the biochemical activity of particular organisms present in the sample. Barnard (6) says that we need more emphasis on product flavor and shelf-life since this is what consumers use to determine product acceptability.

Some researchers have found success in predicting shelf-life. Elliker et al. (26) used a 5 day, 7°C test to detect post-pasteurization contamination. White and Little (120,121) found that the modified Psychrotrophic Bacteria Count and Preliminary Incubation-Gram-negative agar plating techniques correlated well to shelf-life, and Langlois et al. (58) found good correlations between purchase age and flavor, days kept and standard plate count on purchase day, and days kept and standard plate count after 7 days storage. Another shelf-life test, the Moseley keeping quality test involves conducting the Standard Plate Count on the milk stored at 7.2°C for 5-7 days (95). This test has been shown to be useful in predicting shelf-life (28). Smith et al. (105) report a correlation of -0.70. The main problem with this test is that it requires 7-9 days before results are available. By this time the milk has already reached the consumer, and if a problem is realized, 7-9 days of low quality milk have been processed.

There is clearly a need for a more rapid test than is presently being used by the dairy industry to predict the shelf-life of milk. In order to predict shelf-life, Blankenagel (16) suggests that we need to use: 1) a preliminary incubation, 2) selective media, 3) testing for metabolites, 4) surface plating, or 5) a combination of two or more of these.

Preliminary Incubation

Preliminary incubation is used to increase the number of bacteria in a sample before testing. Johns (49) stated that encouraging growth of contaminants prior to testing increases the utility of the tests. A low temperature preliminary incubation has been used as a quality test for raw milk (95). Baker (4) stated that temperature of incubation and length of storage determine the predominate bacterial population. A low temperature preliminary incubation allows psychrotrophs to grow to a level which will enable them to out-compete the other bacteria present when a plate count is performed.

Different preliminary incubations have been employed for testing milk. Ryan et al. (101) found that the preliminary incubation count is of considerable value for raw milk quality control, Oehlrich (74) found good correlations (0.866 and 0.936, respectively) between an 18°C/45-hour count and the 7°C/10 day count for raw and pasteurized milk. Johns (50) used a 55°F, 18 hour preliminary incubation followed by a standard plate count for raw milk to determine quality, while Reinbold (93) used a horizontal preliminary incubation to increase oxygen availability. Bishop

and White (11) found that the best preliminary incubation for psychrotrophic bacteria was 21°C.

Selective Media

Since gram-negative psychrotrophic bacteria are the main spoilage organisms in milk, researchers have tried to select for these by using inhibitors against gram-positive organisms. Freeman et al. (33) used sodium desoxycholate, alkyldimethyl benzyl ammonium chloride, methyl dodecyl trimethyl ammonium chloride, alpha-bromolauric acid and alphabromomyristic acid to inhibit growth of gram-positive organisms. Asher and Sargant (2) utilized sodium desoxycholate in a keeping quality test. Waes and Bossuyt (115,116) utilized benzalkon-A and crystal violet in a preliminary incubation of 24 hours at 30°C to select for gram-negative organisms prior to ATP determination. They found that this method had an 86% agreement to the shelf-life test and a 92% agreement to the Moseley keeping quality test (116). Smith and Witter (106) found that crystal violet and neotetrazolium chloride inhibited gram-positive organisms without inhibiting gram-negative organisms. Gyllenberg et al. (40) used ammonium lactate-crystal violet agar to select for pseudomonads and found that it was as good as the Nacconol-TTC test. They also discovered that keeping quality was related to growth of bacteria which grow on this selective agar (39). Maxcy (63) used nutrient agar with alkylaryl sulfonate as selective media to test for post-pasteurization contamination. Phillips et al. (87) identified post-pasteurization contamination with a preliminary incubation of 21°C for 25 hours in

nisin:penicillin:crystal violet. Phillips and Griffiths (89) compared inhibitor systems to estimate gram-negative bacteria in milk and found that crystal violet:penicillin:nisin worked well to select for gramnegative organisms; benzalkonium chloride:crystal violet and selectocult (Benzalkon A and crystal violet) inhibited gram-positives and some gramnegatives; monesin worked well in plate media but only partially inhibited gram-positive organisms when used in growth medium; and neotetrazolium chloride and sodium desoxycholate allowed growth of some gram-positive organisms. Langeveld et al. (57) used benzalkon-A in a plate after a preliminary incubation of 24 hours at 25°C to detect gram-negative psychrotrophs and found that the agreement with shelf-life was fair but not complete.

Catalase Detection

Most of the psychrotrophic bacteria responsible for spoilage of milk produce catalase to some degree. Catalase will convert hydrogen peroxide water and oxygen. to This oxygen formation can be measured instrumentally. This reaction has been used as a screening for mastitis (113,122) and contamination in metalworking fluids (35). Catalase detection has promise for shelf-life prediction because the instrumentation is simple to operate, rapid, and requires little equipment Hill et al. (46) used a catalase-based method and found a (122). correlation of catalase detection to the standard plate count of 0.99 but, as discussed previously, the Standard Plate Count does not adequately predict inherent quality and shelf-life. Phillips and Griffiths (90) used

catalase detection following a 25 hour, 21°C preliminary incubation in crystal violet:nisin:penicillin. They obtained a correlation to plate count of -0.74.

Endotoxin (Lipopolysaccharide) Detection

Detection of endotoxins has been used to test water and wastewater (24,27,53,54,109), detect bacteriuria (52), detect contamination of drugs, biological fluids (22,61,110), and meats (103). Cooper et al. (22) stated that the Limulus amoebocyte lysate test (LAL) is 10x as sensitive as the rabbit test for determining the concentration of gram-negative bacteria.

Since gram-negative bacteria exhibit an endotoxin activity, associated with the lipid A segment of their outer cell membrane, the LAL test could be used to detect the gram-negative psychrotrophs which ultimately spoil milk (24,70,111). Ohki et al. (75) demonstrated the cascade effect of the <u>Limulus</u> lysate and lipopolysaccharide which results in clotting.

The LAL has been used for quality of raw and pasteurized milk (71,72). Mikolajcik and Brucker (71) obtained correlations of lipopolysaccharide (LPS) concentration to the gram-negative bacteria count of 0.95 and 0.97 for pasteurized and raw milk, respectively. They also used the LAL after a preliminary incubation of 18 hours at 13°C to predict keeping quality. Bishop et al. (9) detected a relationship between endotoxin concentration and days storage, flavor score, and psychrotrophic count. Bishop and White (11) found that endotoxin levels have a predictive value for shelf-life with a correlation of -0.91, and Bishop and Bodine

(13) used a spectrophotometric LAL method to correlate LPS concentration to days storage and flavor intensity with correlations of 0.94 and 0.93, respectively.

Some LAL limitations are that the test requires expensive reagents, lacks sensitivity and is a complex test to perform (17). Mikolajcik and Brucker (69) stated that the LAL will detect $10^3 \cdot 10^4$ gram-negative bacteria per ml in milk which is not sensitive enough to detect the low levels of contamination which are normally present in freshly pasteurized milk. Researchers have tried to reduce cost and complexity while increasing sensitivity with microtiter methods for the LAL (45,64,66).

Direct Epifluorescent Filter Technique

The Direct Epifluorescent Filter Technique (DEFT) is a bacterial enumeration procedure in which a sample is filtered, the bacteria are stained, and then examined under an epifluorescent microscope. Viable cells will fluoresce orange and non-viable cells fluoresce green. The fluorescence is due to acridine orange binding to exposed phosphate groups on RNA, allowing dye polymerization producing a metachromatic (orange) fluorescence. If little RNA is present (as with non-viable cells), the acridine orange will bind to the double helix every third base pair, allowing no dye-dye molecular interaction. The acridine orange will then fluoresce orthochromatically (green) (99). Many researchers have used the DEFT for bacterial enumeration (77,84,98,124). Rodrigues and Kroll (99) used the DEFT for counting yeasts. Pettipher and Rodrigues (83) stated that the DEFT was effective with whole and skim milk, cream, whey and sweet cream butter, and for testing sterility of UHT milk. Pettipher (82,85) developed a method for using the DEFT to enumerate bacteria in milk samples. Peterkin and Sharpe (81) employed Tween 80 and protease to increase the filterability of milk. Pettipher et al. (82) were able to detect as low as 5 x 10^3 organisms/ml and obtained a correlation of 0.91 for DEFT count to plate count. Pettipher and Rodrigues (83) achieved a similar correlation of 0.93. Pettipher et al. (86) had six labs perform the DEFT and all had a correlation to plate counts of 0.90 but they also stated that the DEFT is slower than counting plates and requires some operator experience. Rodrigues and Pettipher (97) obtained a correlation of DEFT count to keeping quality of -0.72 after a 30°C preliminary incubation. Kroll and Rodrigues (56) obtained correlations to keeping quality of -0.73 to -0.83 after preliminary incubation.

Impedance Detection

Impedance is concerned with the resistance to the flow of an electrical current through a conducting material. It is composed of conductance and capacitance (25). Parsons and Sturges (79) stated that conductivity could be used to detect metabolic changes in the media. Allison et al. (1) stated that electrical conductivity of solutions depends upon concentration and mobilities of charged particles of varying sizes. As bacteria metabolize, they break down the larger molecules in the media into smaller charged molecules, which reduces the resistance to flow of the current. At a concentration of approximately 10⁷ cfu/ml, a sharp inflection in the flow results. The inflection represents the

detection time.

Measurements of impedance has been used to detect: postpasteurization contamination in milk (9), cream contamination (38), contamination by coliforms (30), and for raw milk screening (20,73) with good reproducibility and variability (32). Gnan and Luedecke (37) obtained a correlation of Impedance Detection Time (IDT) to standard plate count of -0.77 to -0.88. Firstenberg-Eden and Tricarico (31) obtained a correlation of IDT to total, mesophilic and psychrotrophic plate counts of -0.96, -0.95, and -0.96, respectively.

Martins et al. (62) found that impedance was superior to standard plate counts and psychrotrophic count for predicting shelf-life on the day of pasteurization as results could be obtained in 14 hours rather than 2 or 10 days. Kahn and Firstenberg-Eden (55) found that IDT could be used as a substitution for sensory evaluation for shelf-life determination. Phillips and Griffiths (88) discovered that 21°C was a better temperature than 30°C for shelf-life prediction. Bishop et al. (10) and Bishop and White (11) obtained correlations of IDT to shelf-life of 0.88 and 0.93, respectively.

Although some of the bacterial detection techniques have been successful at enumeration, they have proven unsuccessful at shelf-life prediction due to the low numbers of bacteria which are usually present in a freshly pasteurized milk sample. The rapid detection techniques are not sensitive enough to detect this low contamination level. A new shelflife test is needed which will allow for effective, corrective measures to be taken if a problem is realized. Therefore, the objectives of this

study are to develop a selective preliminary incubation which will allow growth of gram-negative psychrotrophic bacteria while inhibiting growth of gram-positive organisms. This preliminary incubation will then be conducted prior to developed rapid detection techniques to increase the number of gram-negative psychrotrophic organisms to a detectable level in order to predict the potential shelf-life of pasteurized fluid milk.

MATERIALS AND METHODS

PHASE ONE

Two-percent milk was bottled as 99 ml milk blanks, steamed 5 min., and held at 3°C. The bottles were warmed to room temperature and inoculated with 1 ml cultures each of Pseudomonas fluorescens ATCC 13525, Pseudomonas fragi ATCC 4973, Pseudomonas putida ATCC 12633, Pseudomonas aeruginosa ATCC 9721, <u>Bacillus</u> cereus ATCC 11778, <u>Escherichia coli</u> ATCC 12014, Staphylococcus aureus ATCC 25923, and Streptococcus sp. ATCC 12386 in nutrient broth. The inoculated bottles were allowed to incubate at 21°C for 48 h for the Pseudomonads and 32°C for 24 h for the other organisms to assure that each test organism was in logarithmic growth phase. After incubation a concentration of approximately 5×10^8 cells/ml was achieved. The milk cultures were then diluted in phosphate buffer to target concentrations of 10^4 , 10^2 , and 10^1 cells/ml and inhibitors were added to the milk cultures at levels to provide the following final concentrations: crystal violet 0.002% (w/v), benzalkonium chloride 0.10% (w/v), crystal violet: benzalkonium chloride 0.002% (w/v):0.03% (w/v), alkylaryl sulfonate 0.20% (w/v) and a control (no inhibitors added). The inoculated inhibitors were incubated at 21°C for 18 h prior to bacterial enumerations. The study was replicated three times.

Bacterial Enumerations

Bacteria were enumerated by the modified psychrotrophic bacteria count (21°C, 25 h) mPBC for <u>P. fluorescens</u>, <u>P. fragi</u>, <u>P. putida</u>, and <u>P. aeruginosa</u>; and standard plate count (32°C, 48 h) SPC for <u>B. cereus</u>,

<u>S. aureus, Streptococcus</u> sp., and <u>E. coli</u>.

Statistical Analysis

Analysis of variance, least squares mean, and contrasts were made using the Statistical Analysis System (102). The interactions of fixed effects with replicates were used to test the fixed effects for significance.

PHASE TWO

Milk Samples

Whole milk, packaged in plastic jugs (3.8 liters), was obtained from local retailers and stored at 7°C until determined to be unacceptable by daily sensory evaluation. Samples were analyzed at time zero and after preliminary incubations at 21°C for 18 hours.

Preliminary Incubations

Preliminary incubations were performed at 21°C for 18 hours. The following incubations were used: milk alone (control), milk in benzalkonium chloride (0.10% w/v final concentration), milk in nutrient broth (1:1), milk in nutrient broth with benzalkonium chloride (0.10% w/v final concentration) (1:1), and milk in a dairy gram-negative broth (1:1) containing tryptone (20.0 g/1), yeast extract (20.0 g/1), dextrose (4.0 g/1), sodium desoxycholate (0.5 g/1), and crystal violet (1.0 ml of 0.1% solution in ethyl alcohol).

Bacterial Enumerations

Bacterial enumerations conducted were: Psychrotrophic Bacteria Count- PBC (7°C, 10 d) (95), modified Psychrotrophic Bacteria Count- mPBC (21°C, 25 h) (95), Petrifilm modified Psychrotrophic Bacteria Count- PmPBC (21°C, 48 h) (95), and Moseley Keeping Quality test (7°C, 7 d then SPC (32°C, 48 h)) (95). All tests were performed at time zero. Modified psychrotrophic bacteria count and Petrifilm modified psychrotrophic bacteria count were also performed after preliminary incubations.

Impedance Detection

Impedance measurements were conducted with a Bactometer Microbial Monitoring System Model-120 (Vitek Systems, St. Louis, MO). Modified plate count agar (MPCA) containing (in grams per liter): yeast extract, 20; tryptone, 20; dextrose, 4; and agar, 10; and dairy gram-negative agar (DGNA) containing (in grams per liter): yeast extract, 20; tryptone, 20; dextrose, 4; sodium desoxycholate, 1; 0.1% crystal violet solution in ethyl alcohol, 2 ml; and agar, 10 were used for the impedance analysis. Modules used in the instrument were filled by pipeting 0.5 ml of MPCA or DGNA into each module well. The modules were gently agitated to allow for even distribution of the agar. The modules were then placed in individual bags which were sealed and stored in the refrigerator until needed. Modules were prepared at least every other week.

After each preliminary incubation, 0.5 ml of milk solution was placed into duplicate agar wells containing MPCA or DGNA. After the entry of the modules into the Bactometer Processing Unit, continuous automatic data

collection was carried out by the instrument. During the test, the impedance detection times (IDTs) of the sample were automatically determined by the instrument. The IDTs for the duplicate wells were averaged. This average IDT was compared to the shelf-life of the sample tested.

Catalase Detection

Catalase was detected using two methods, the first being the HMB-II (Biotech, Inc., St. Louis, MO). Ten ml of milk solution were placed into a vacutainer tube and 0.5 ml of a H_2O_2 reagent was added. The tube was stoppered, vented, vortexed and allowed to react for 15 minutes before being vortexed and head-space pressure measured with the HMB-II. Catalase was also detected using a Catalasemeter (BioEngineering Group, Ltd., New Haven, CO). Milk solution was stabilized with reagent before being drawn into a disk via capillary action. The disk was then dropped into a tube containing 5 ml of stabilized hydrogen peroxide reagent and flotation time measured with the Catalasemeter. Both catalase detections were conducted at time zero and after preliminary incubations. The HMB-II involved a preliminary incubation with continuous shaking (150 rpm).

Endotoxin Detection

Concentration of lipopolysaccharide (LPS) was determined using the <u>Limulus</u> Amebocyte Lysate Assay (LAL) (Marine Biologicals, Marmora, NJ). All pipets, pipet tips, dilution tubes, and testing tubes used were pyrogen-free. Sample dilutions were made in sterile, pyrogen-free water. Serial dilutions of samples were analyzed as follows: 0.1 ml lysate was mixed with 0.1 ml sample in a 10 x 75 mm glass tube and incubated in a waterbath at 37°C for 1 h. A tube was considered to be positive when the glass tube was inverted 180° and the clot remained intact. One-quarter dilutions were made on the highest ten-fold dilution which gave a positive LAL test. The test was then repeated. Negative controls and an endotoxin series (0.05, 0.025, 0.0125, and 0.006 ng/ml endotoxin) were also run with each test. The concentration of LPS (ng/ml) was determined by multiplying the lysate potency by 1/highest dilution which gave a positive LAL test.

Microscopic Counts

Microscopic counts were performed using the Direct Epifluorescent Filter Technique as follows: Trypsin (0.5 ml), 2 ml Triton X-100 (0.5% v/v), and 3 ml of milk sample were added to a test tube, vortexed and incubated at 50°C for 10 min. A nucleopore polycarbonate membrane filter (0.6 μ m pore size, 25 mm diameter) was mounted onto a filter tower. The filter was warmed by filtering 5ml Triton X-100 (0.1% v/v) at 50°C. The sample was then filtered. Five ml Triton X-100 (0.1%) at 50°C was added to test tube, vortexed and filtered. The tower was rinsed with 5 ml 0.1% Triton X-100 and filtered.

The filter was overlayed with 1.0 ml acridine orange (0.025% v/v), and allowed to stand for 30 sec., then filtered. The filter was rinsed with 2 ml of 0.1 M citric acid buffer (pH 3.0) and filtered. The filter was then rinsed with 1.0 ml isopropyl alcohol and filtered.

The filter was removed and allowed to air dry. A drop of non-

fluorescent immersion oil was placed onto a microscope slide and the filter was placed onto the oil. Another drop of immersion oil was placed onto the filter and a coverslip was placed onto the slide.

The slide was examined under an Olympus BH-2 fluorescent microscope (Optical Elements Corporation, Washington, DC) using a 100X Plan D objective. Orange-fluorescing clumps or bacteria were counted as follows:

Number of fields counted	<u>Avg. # of clumps/field</u>
15	0-10
10	11-25
6	26-50
3	51-75
2	76-100
dilute and repeat	>100

The DEFT count per ml of milk was obtained by multiplying the average number of clumps per field by the microscope factor. The microscope factor (MF) was determined as follows:

MF- <u>Area of membrane which sample was filtered (mm²)</u> Microscope field area (mm²) x sample volume (ml).

Shelf-life Determination

The shelf-life of each milk sample was determined by the method of Bishop, et. al. (10) amended as follows: The shelf-life of each milk sample was determined by daily sensory evaluation as performed by trained judges. When an objectionable flavor was detected, the previous day was considered to be the shelf-life of the product. An untrained panel of 10-20 judges also evaluated the milk on days 10 and 14. The untrained panel was used to mimic the typical consumer and days 10 and 14 were used as tasting days because these days fit our sampling procedure and panel availability. An objectionable flavor was a recognition of an off-flavor which would correspond to a "5" or lower on the ADSA score card for milk (11). The number of days must be used as <u>potential</u> shelf-life when making correlation comparisons to measured parameters. The word "potential" is used because this is the best possible shelf-life at 7°C storage. Temperature abuses would result in a lower shelf-life.

Statistical Analysis

Multiple correlation value analyses were made using the Statistical Analysis System (102).

RESULTS AND DISCUSSION

PHASE ONE

Analysis of variance (Table 1) illustrates the significance of each source of bacterial variation. This indicates: 1) a difference between growth of gram-positive and gram-negative organisms; 2) a difference among the bacteria within each gram reaction; 3) a difference among treatments (time zero before PI, inhibitors, and control - inoculated, uninhibited, and allowed to PI); 4) a difference in treatment reaction depending on gram; and 5) a difference in treatment reaction depending on bacteria.

A comparison of the least squares means of the gram-positive organisms after incubation (Table 2) reveals that benzalkonium chloride was the inhibitor of choice as it inhibited growth of gram-positive organisms while allowing uninhibited growth of the gram-negative organisms tested. At time-zero, the log bacteria count was 4.39. Crystal violet: benzalkonium chloride inhibited growth of gram-positive organisms as the log count decreased to 1.98. The data support previous work with crystal violet:benzalkonium chloride (89,116) in that crystal violet:benzalkonium chloride also had an inhibitory effect. Crystal violet displayed a slight inhibition. The log count remained at 4.39. Contrary to previous research (63), alkylaryl sulfonate, which is presently used in a dyereduction test for keeping quality estimation, allowed the growth of grampositive organisms not significantly different from the control. The log count was 5.70.

Comparison of least squares means for gram-negative organisms after incubation at 21°C for 18 h indicated that none of the inhibitors

Table 1. Analysis of bacterial variation (log plate counts) due to gram reaction, bacteria tested and treatments tested after incubation at 21°C for 18 h.

Source	df	Mean Square	F-Value
Rep ¹	2	1.48	8.84 ^a
Gram ²	1	253.11	6914.67ª
Rep*Gram	2	0.04	0.22
Bact(Gram)	6	2.50	5.04 ^a
Rep*Bact(Gram)	12	0.50	2.96ª
Tmt ³	5	37.44	67.90 ^a
Tmt*Rep	10	0.55	3.29 ^a
Gram*Tmt	5	36.29	97.05 ^a
Gram*Tmt*Rep	10	0.37	2.23
Bact*Tmt(Gram)	30	1.18	7.08ª
Error	60	0.17	••••

¹Replications

²Gram reaction

³Inhibitors, Time Zero, Control

^aSignificant (p<0.01)

Table 2. Least squares means and standard errors for logarithms of Grampositive and Gram-negative bacteria enumerated.

Treatment	Gram-positive	<u>Gram-negative</u>
Control	7.01	7.60
AS ¹	5.70	7.24
TZ ²	4.39	4.37
CV ³	4.39	7.24
CVBC ⁴	1.98	7.17
BC ⁵	0.89	7.17
Standard Error	0.20	0.16

¹Alkylaryl sulfonate

²Time zero

³Crystal violet

⁴Crystal violet:benzalkonium chloride

⁵Benzalkonium chloride

significantly inhibited growth of gram-negative organisms. The only significant value obtained was for time zero which indicates that the inhibitors allowed gram-negative organisms uninhibited growth during the incubation period.

Examination of benzalkonium chloride with comparisons to the other treatments (Table 3) shows a difference between benzalkonium chloride and all other treatments for the gram-positive bacteria, revealing that benzalkonium chloride was clearly the reagent of choice for inhibiting growth of gram-positive organisms. The only significant value obtained in the contrasts of benzalkonium chloride versus other treatments for the gram-negative bacteria was for benzalkonium chloride vs. time zero, again evidence that all inhibitors allowed uninhibited growth of the gramnegative bacteria. Therefore, benzalkonium chloride is clearly the inhibitor of choice for selective incubation of gram-negative bacteria.

PHASE TWO

Bacterial enumerations

Throughout the study, Petrifilm and agar methods appeared to perform equally well as evidenced by the high correlation value of r= 0.99 (Fig. 1). At time zero (without any preliminary incubation), psychrotrophic enumeration methods were significantly correlated to shelf-life (p<0.0001) (Table 4). The modified Psychrotrophic Bacteria Count (mPBC) and Petrifilm mPBC had correlation comparable to the standard Psychrotrophic Table 3. Contrasts of benzalkonium chloride versus other treatments for Gram-positive and Gram-negative bacterial growth (log).

<u>Contrast</u>		Gram-positive	Gram-negative
BC ¹ minus	5 TZ ²	-3.50ª	2.80ª
BC minus	CV 3	-3.50ª	-0.08
BC minus	CVBC ⁴	-1.09 ^b	-0.01
BC minus	AS ⁵	-4.81 ^a	-0.07
BC minus	Control	-6.12ª	-0.43

¹Benzalkonium chloride

²Time zero

³Crystal violet

⁴Crystal violet:benzalkonium chloride

⁵Alkylaryl sulfonate

^aSignificantly different from zero (p<0.01), Bonferroni t-test.

^bSignificantly different from zero (p<0.05), Bonferroni t-test.



Figure 1. Scattergram of the linear relationship of log modified Psychrotrophic Bacteria Count to log Petrifilm modified Psychrotrophic Bacteria Count (r=0.99, n=638).

Table 4. Quadratic correlation values for shelf-life estimation of pasteurized fluid milk using selective preliminary incubation at 21°C for 18 h.

	Time					
	Zero	Control	BC ¹⁰	Broth	BC Broth	DGNB ¹¹
PBC ¹	-0.78	ND ¹²	ND			
Moseley ²	-0.84	ND				
mPBC ³	-0.77	-0.88	-0.84	-0.89	-0.89	-0.88
PmPBC ⁴	-0.79	-0.88	-0.86	-0.89	-0.87	-0.89
IDTSL ⁵	ND	0.90	0.86	0.86	0.89	0.91
HMB-11 ⁶	-0.55	-0.74	-0.84	-0.62	-0.79	-0.68
CTLM ⁷	0.58	0.66	0.77	0.65	0.77	0.70
DEFT ⁸	-0.75	-0.76	-0.76	-0.78	-0.75	ND
LAL ⁹	-0.69	-0.85	-0.85	-0.81	-0.80	ND

¹Psychrotrophic Bacteria Count (7°C, 10 d) ²Moseley Keeping Quality test (PI 5-7 d at 7°C, then SPC -32°C, 48 h) ³modified PBC (21°C, 25 h) ⁴Petrifilm mPBC (21°C, 48 h) ⁵Impedance Detection Time (shelf-life procedure) ⁶Catalase detection by head-space pressure ⁷Catalasemeter - floatation method ⁸Direct Epifluorescent Filter Technique ⁹Limulus Amoebocyte Lysate assay ¹⁰O.10% benzalkonium chloride (final concentration) ¹¹Dairy gram-negative broth ¹²Not determined *All correlation coefficients were significant (p<0.0001).</pre> Bacteria Count (PBC) (-0.77, -0.79, -0.78, respectively); thus either of these two methods could be used in place of the PBC for estimating the quality of fluid milk products. The mPBC and the PmPBC have a definite time-saving advantage - 25 h for mPBC and 48 h for PmPBC versus 10 d for PBC, with the PmPBC method much easier to count due to larger, red colonies. The mPBC and PmPBC showed significant correlations to shelflife after each preliminary incubation. No difference existed between selective and non-selective preliminary incubations, probably due to the low temperature incubation of the plates (21°C). This low temperature would only allow growth of psychrotrophic bacteria. The highest correlation of a bacterial enumeration method to shelf-life was for preliminary incubation of the milk:broth mixture followed by mPBC (either agar or Petrifilm), which gave a correlation of -0.89 (Fig. 2).

Impedance detection

Impedance detection was highly correlated to shelf-life after all preliminary incubations (Table 4). There was no difference between selective and non-selective preliminary incubations, probably due to the 21°C incubation in the Bactometer Processing Unit. The highest correlation for IDT was 0.91 after a preliminary incubation of milk in dairy gram-negative broth (Fig. 3).

Catalase detection

Both methods of catalase detection gave low correlations to shelflife at time zero (Table 4). Testing after each preliminary incubation



Figure 2. Scattergram of the quadratic relationship of modified Psychrotrophic Bacteria Count to potential shelf-life of whole milk after preliminary incubation of milk in broth for 18 h at 21°C (r=-0.89, n=118).



Figure 3. Scattergram of the quadratic relationship of impedance detection time to potential shelf-life of whole milk after preliminary incubation of milk in dairy gram-negative broth for 18 h at 21°C (r=0.91, n=89).

improved the correlations to shelf-life, especially after selective preliminary incubation. The highest correlation for the headspace pressure detection (HMB-II) was -0.84 after a preliminary incubation of milk with benzalkonium chloride (Fig.4). The highest correlation obtained for the flotation method (Catalasemeter) was 0.77 after a preliminary incubation of milk either with benzalkonium chloride or broth with benzalkonium chloride (Fig. 5). This clearly illustrates the value of a selective preliminary incubation.

Direct Epifluorescent Filter Technique

For the DEFT, significant correlations to shelf-life were obtained at time-zero (-0.75) and after all preliminary incubations (-0.75 to -0.78) (Table 4). The highest correlation was with an incubation of milk and broth (Fig. 6). Use of benzalkonium chloride in the incubation produced little difference fact that incubations without benzalkonium chloride select for in the correlations, possibly due to the psychrotrophic bacteria and the main psychrotrophic bacteria in milk are gram-negative. These results are comparable to those of Kroll and Rodrigues (56) who found significant correlations to keeping quality of pasteurized cream.

Endotoxin Detection

The correlation to shelf-life for the LAL test at time-zero was unacceptable for practical use (-0.69) (Table 4). This correlation was improved after all preliminary incubations conducted. The highest



Figure 4. Scattergram of the quadratic relationship of head-space pressure (psi) to potential shelf-life of whole milk after preliminary incubation of milk in benzalkonium chloride for 18 h at 21°C (r=-0.84, n=97).



Figure 5. Scattergram of the quadratic relationship of flotation time (seconds) to potential shelf-life of whole milk after a preliminary incubation of milk in broth with benzalkonium chloride for 18 h at 21°C (r=0.77, n=119).



Figure 6. Scattergram of the quadratic relationship of log DEFT count to potential shelf-life of whole milk after a preliminary incubation of milk in broth for 18 h at 21°C (r=-0.78, n=80).

correlation was after an incubation in milk or milk with benzalkonium chloride (-0.85) (Fig. 7). These results are expected because the detected endotoxin is specific to gram-negative organisms. Psychrotrophs were selected with low temperature incubation and the vast majority of psychrotrophs in milk are gram-negative. Other researchers have used the LAL test to detect gram-negative bacteria (52,71), but little work has been done to compare LPS concentration to shelf-life. Bishop and White obtained a correlation of -0.91 when comparing a spectrophotometric endotoxin test to shelf-life (11). The research used a 3-dilution series gelation method, followed by quarter dilutions of the highest positive dilution (a total of 7 tests/sample) to obtain a LPS concentration.

Virginia Tech Shelf-Life Program

The recommended procedure for predicting the potential shelf-life of milk is the Virginia Tech Shelf-Life Program (Fig. 8). A 10 ml sample of freshly pasteurized milk is transferred to a test tube and incubated at 21°C for 18 hours. A 1:1000 dilution is then made on the milk sample and the dilution is plated as a modified psychrotrophic bacteria count, either agar or Petrifilm (21°C, 25h or 48h, respectively). If the total count is $\leq 1,000$ cfu/ml then a shelf-life of ≥ 14 days is expected. If the total count is 1,000 to 200,000 cfu/ml then a shelf-life of 10 to 14 days is expected. If the total count is $\geq 200,000$ cfu/ml then a shelf-life of ≤ 10 days is expected. Only one dilution is necessary as it is possible to count between 1 and 200 colonies on the single plate.



Figure 7. Scattergram of the quaratic relationship of log LPS concentration (ng/ml) to potential shelf-life of whole milk after a preliminary incubation of milk for 18 h at 21°C (r=-0.85, n=100).





Figure 8. The Virginia Tech Shelf-life Program.

CONCLUSIONS

All experimental preliminary incubations improved the shelf-life correlations for both plating techniques and rapid instrumentation. These methods gave correlations which were higher or equivalent to that of the Moseley Keeping Quality test, which has been reported as -0.70 (95), -0.77 (10), and -0.84 (this study). The major advantage of the methods tested is in their time-saving value. Results were obtained in 43 h for PI/mPBC, 66 h for PI/PmPBC, 19 h for the PI/catalse detection methods and 42 h for PI/impedance microbiology, as opposed to 7-9 d for the Moseley test. This time-savings will allow the dairy processor to obtain results within a time period which will allow for effective, corrective measures to be taken if a problem is detected. The method with the most advantages (rapid, inexpensive, simple, accurate) is a preliminary incubation of milk alone at 21°C for 18 hours, followed by the modified Psychrotrophic Bacteria Count, either agar or Petrifilm methods - the Virginia Tech Shelf-Life Program. This test uses a common plating technique and requires only 43-66 h. There is no large initial investment and the cost is approximately \$0.40 per sample. If a more rapid test (20-38 h) is desired, either catalase detection method (HMB-II or Catalasemeter), impedance microbiology (Bactometer), Direct Epifluorescent Filter Technique, or Limulus Amoebocyte Lysate assay may be used in conjunction with their preferred preliminary incubation for very good results (r= -0.84, 0.77, 0.91, -0.78, -0.85, respectively).

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