Journal of Food Protection Vol. 41, No. 4, Pages 277-283 (April, 1978)

Automated Impedance Measurements for Rapid Screening of Milk Microbial Content

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(Received for publication August 26, 1977)

ABSTRACT

The electrical impedance of media is altered with chemical changes brought about by microbial metabolism and growth. Time required to bring about readily detectable change (detection time — DT) is a function of the initial levels of microorganisms in the sample. DTs were compared to Standard Plate Counts for 407 milk samples — homogenized, low fat, skim and raw. Using the criterion that a sample of pasteurized milk with a DT of 7 h or less was indicative of a plate count of 10,000/ml or greater, 323 of 380 samples were correctly classified. For raw milk, the DT was 10 h to resolve samples into greater or less than 10,000 organisms per ml. Results of a preliminary study on estimation of psychrotrophs in pasteurized milk showed that impedance monitoring at 21 C provided a 22-h screen correctly classifying 88% of the samples into categories of more than or less than 1,000 organisms per ml. Better agreement (91%) in a shorter time (13.7 h) was obtained with a screen for 10,000 organisms. Finally, for the first 22 samples analyzed, keeping quality data on pasteurized milk have correlated better with post-pasteurization impedance measurements than with either post-pasteurization total counts or psychrotrophic counts.

The dairy industry has long been interested in the bacterial populations found in milk. In addition to the need to meet State regulatory standards, bacterial spoilage or line contamination caused by high microbial concentrations can be very expensive for milk producers, processing plants, and distributors, since it relates to the milk's keeping quality and consumer brand preferences.

Since the effect of spoilage is so important, the dairy industry would certainly be interested in a rapid method for determining the microbial population in milk products. Present microbiological techniques are not very practical as test results are usually not available until several days after products have been shipped to consumers (*3,11,13*). Plate count tests to determine total counts of organisms present in milk take 48 h. Methods for measuring psychrotrophs (organisms able to grow at refrigeration temperatures), as presently practiced, take 5-10 days. Furthermore, present keeping quality tests, which try to predict spoilage based on presence of psychrotrophic organisms, have two severe limitations. First, spoilage is not always directly related to the number of organisms present (*20*). Second, it appears that psychrotrophs are only part of the milk spoilage problem. Poor flavor and keeping quality can also be attributed to the presence of microbial enzymes and metabolic products (*6,17,18,19*) from organisms present before pasteurization even though the organisms themselves may be killed by pasteurization. Thus, present methods, although offering some useful information, are too slow and often too inaccurate to meet the needs of milk producers and processors.

An optimal microbiological test would provide counting and keeping quality estimates within a time period allowing for effectual corrective measures. This would enable raw milk to be rejected before accepting delivery. Line contamination could be detected and corrected quickly; poor quality final products could be shifted into other products, thus preventing marginal products from reaching the consumer and reducing spoilage costs. In addition, an optimal method should be easy to use and should cost no more than present methods. Keeping these objectives in mind, we investigated impedance techniques, which provide rapid microbiological results for other food products (*10*). It was hoped that development of rapid methods would be of use to the dairy industry.

The impedance method is based on the observation that organisms growing in a liquid culture medium produce chemical changes which alter the electrical resistance (impedance in an AC circuit) of the solution. With a sensitive impedance monitor, the impedance changes caused by the growing organisms can be detected as the organisms reach the instrument's threshold. The time of detection can then be used to roughly estimate the concentration of organisms initially present in the milk sample. Furthermore, since impedance measurements may detect activity not only from organisms present in the milk but also from enzymes remaining from bacteria killed by pasteurization, impedance monitoring may provide a new means of predicting keeping quality. The results of our work to date are summarized in this paper.

MATERIALS AND METHODS

Impedance measurements

The impedance monitoring instrument used in all experiments was the Bactometer[1] 32 Microbial Monitoring System (Fig. 1) described elsewhere (5). The system was operated at 2 KHz, Gain 9 and all data were displayed via a strip chart recorder. Detection time was defined as the time required to produce an accelerating impedance change of 0.8%.

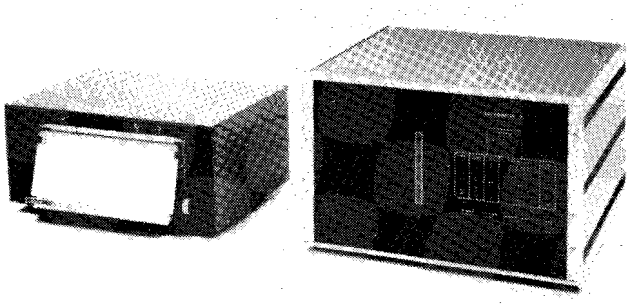

Figure 1. *Bactometer[1] 32 Microbial Monitoring System and strip chart recorder.*

Samples and media were aseptically added to 20-ml vials equipped with stainless steel electrodes descending from the cap. These vials were placed in a basket (Fig. 2) with electrical connection to the instrument. The basket of vials was then put in a standard incubator. Samples incubated at 21 C were monitored in modules (Fig. 3), containing sample chambers with electrodes for eight samples, and plugged directly into the instrument's incubator section. All samples, whether in vials or modules, had a corresponding reference of uninoculated medium.

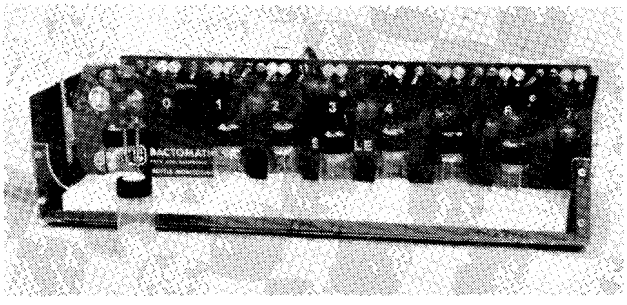

Figure 2. *Vials with vertical stainless steel electrodes in a rack which can be placed in a standard incubator and connected to the Bactometer 32 Microbial Monitoring System with an extension cable.*

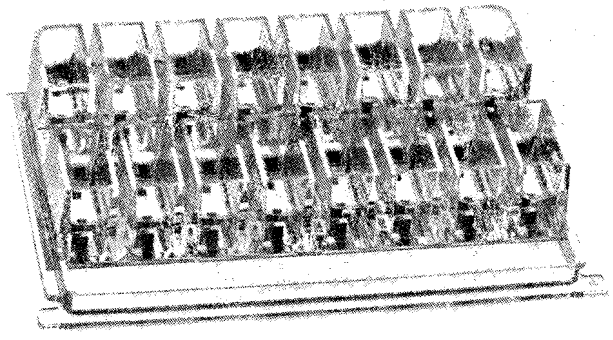

Figure 3. *Disposable module with stainless steel electrodes.*

[1]*Trademark, Bactomatic, Inc.*

The theory relating impedance detection times to initial microorganism concentration has been described by Hardy et al. (*10*) and is breifly summarized in Fig. 4. The upper half of the figure illustrates typical bacterial growth curves starting at two different concentrations (10^5 and 10^3 organisms/ml). The horizontal dashed line indicates the level of organisms where significant impedance changes are detectable. The lower half of the figure shows the impedance changes resulting from these two cultures. The response to the smaller initial concentration occurs later than the response to the larger initial concentration. In general, as long as the microbial growth rate is roughly the same from sample to sample, samples with high microbial numbers produce impedance changes before those with low numbers of organisms. Thus, for any prescribed concentration of organisms, a cutoff time can be defined such that an impedance change before the cutoff time indicates microbial numbers above the prescibed concentration and an impedance change after the cutoff time indicates microbial numbers below the prescribed concentration. This method was applied to impedance-based screens for both total mesophilic organisms and psychrotrophic organisms.

Microbiological methodology

Figure 5 illustrates a schematic diagram comparing the conventional method and the impedance method of estimating the number of organisms per ml of milk. In the conventional method 1 ml of milk was

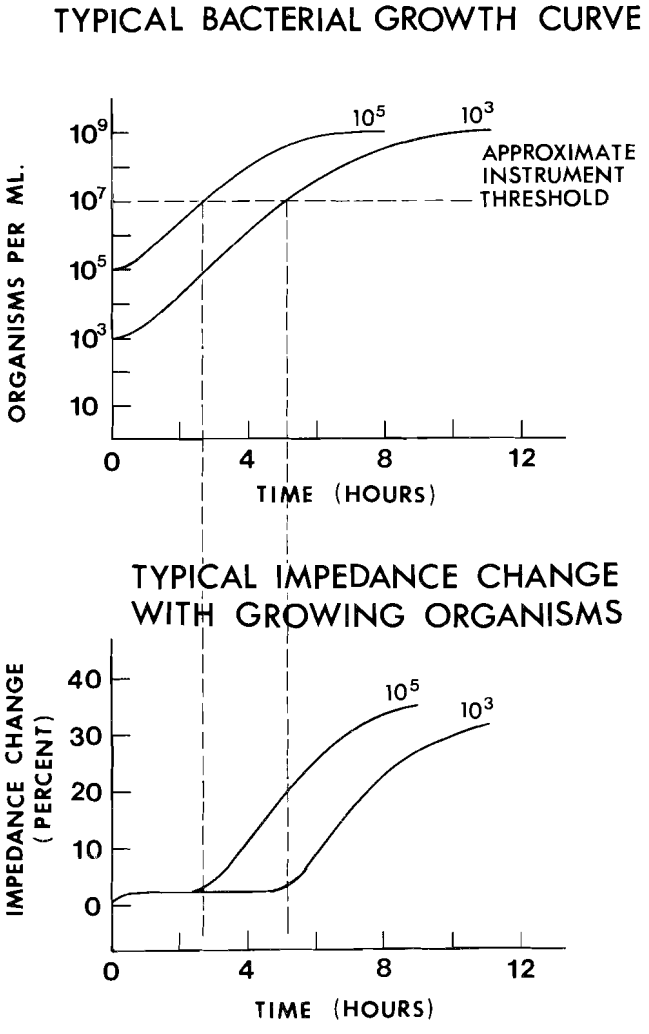

Figure 4. *Relationship between microorganism growth (top) and impedance response (bottom) which makes possible the method of estimating initial microorganism concentration from impedance response detection times.*

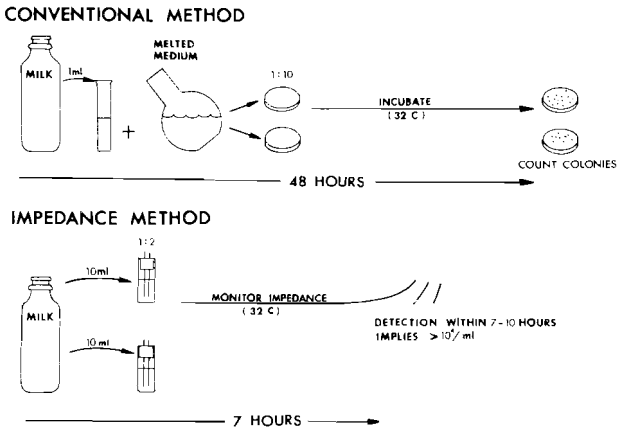

Figure 5. *Schematic representations of the conventional plate count and impedance methods for screening a milk sample.*

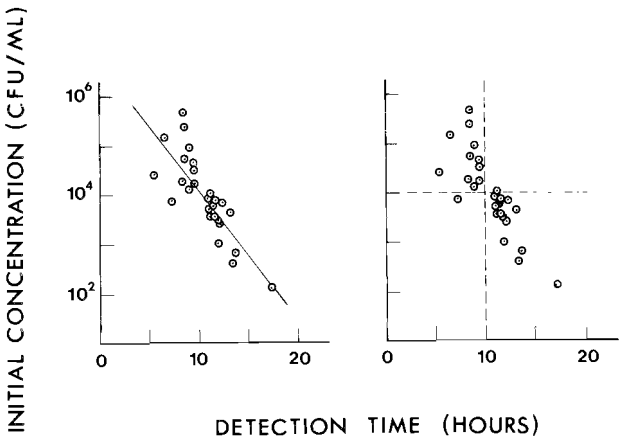

Figure 6. *Scattergram of impedance response detection times (averages of duplicate channels) for 27 raw milk samples graphed against initial microorganism concentration as determined by plate count at 32 C. The solid line on the left side is the least squares linear fit to the data. The broken lines on the right side illustrate a scheme by which samples could be classified as having more than or less than 10^4 organisms/ml.*

added to 9 ml of phosphate buffer, and 1 ml of this dilution was mixed with 10-15 ml of Standard Methods Agar to make a pour plate (*1*). For samples incubated at 32 C (but not at 7 C or 21 C), the standard method was modified by adding a 3-5 ml agar overlay to the pour plates. This eliminated spreading colonies at the expense of a slight reduction in surface colonies. Plates were done in duplicate and incubated under each of the following conditions: 32 C for 48 h (*1*), 21 C for 25 h (*16*), and 7 C for 10 days (*1*).

For the impedance method, 10-ml milk samples were added to an equal amount of trypticase soy broth containing 0.1% yeast extract (TSBY) in vials containing electrodes; or 1-ml milk samples were added to an equal amount of TSBY or Standard Methods Broth (SMB) in modules. The sample containers were connected to the instrument and monitored at 32 C for comparison with mesophilic organism counts and keeping quality and at 21 C for comparison with psychrotrophic counts.

Flavor scoring

Milk flavor was judged according to the method used by Hankin and Diilman (*8*), by a panel of four trained milk tasters consisting at any one time, of at least three persons uninformed as to the identity of the sample being tasted. A flavor score of 40 was deemed excellent, while 35 or less was considered unsatisfactory. Intertester reliability was high, with an average standard deviation of less than 0.7 unit. Testing was done every other day until day 8 and then daily until the sample spoiled.

Samples

For psychrotrophic counts and keeping quality testing, homogenized milk samples were obtained from local dairies, and plate counts and impedance monitoring were begun within 6 h of pasteurization. For total mesophilic organism screening, refrigerated samples of raw, skim, low fat and homogenized milk were obtained from 24 milk processing plants across the United States.

RESULTS AND DISCUSSION

Raw milk screen

Figure 6 shows a scattergram of impedance response detection times graphed against initial microbial concentration for 27 raw milk samples. Note that the shorter the detection time, the greater is the initial concentration. (These data have a correlation coefficient of −0.8 between detection time and the logarithm of the initial concentration.) The solid diagonal line on the left side of the figure is the least squares linear fit to the data (regression line). The slope of this line indicates that the doubling time for the total population of microorganisms in the mixture of raw milk and TSBY during impedance monitoring is approximately 70 min.

The broken lines on the right side of the figure show a classification by which these raw milk samples could be classified as having more or less than 10,000 organisms/ml. The horizontal dashed line represents the 10,000 organism/ml level. The vertical dashed line at 10 h represents the cutoff time that best distinguishes samples containing greater than 10,000 organisms/ml from those with fewer than 10,000 organisms/ml. Therefore any sample with detection time before 10 h would be classified as having over 10,000 organisms/ml and any sample with detection after 10 h would be classified as having less than 10,000 organisms/ml. The two broken lines separate the samples into four quadrants. For samples in the upper left and lower right quadrants, the impedance and plate count classifications agree. For these data, 25 out of 27 samples, or 92.6%, yielded agreement between the impedance and plate count classifications. Samples in the upper right quadrant were classified above 10,000 organisms/ml by plate count but below 10,000 organisms/ml by the impedance technique (false negatives). Samples in the lower left quadrant were classified below 10,000 organisms/ml by plate count but above 10,000 organisms/ml by impedance (false positives). Moving the vertical cutoff line forward or backward in time will reduce false negatives at the expense of increasing false positives and vice versa.

Because the number of samples is small, this agreement may be fortuitously high; however, it compares favorably with the work of Gnan and Luedecke, who reported 99% agreement between impedance and plate count classifications for raw milk using similar methods and a larger number of samples (*7*).

Total mesophile screen

Figure 7 shows similar scattergrams for various types of pasteurized milk. Shown with their regression lines are

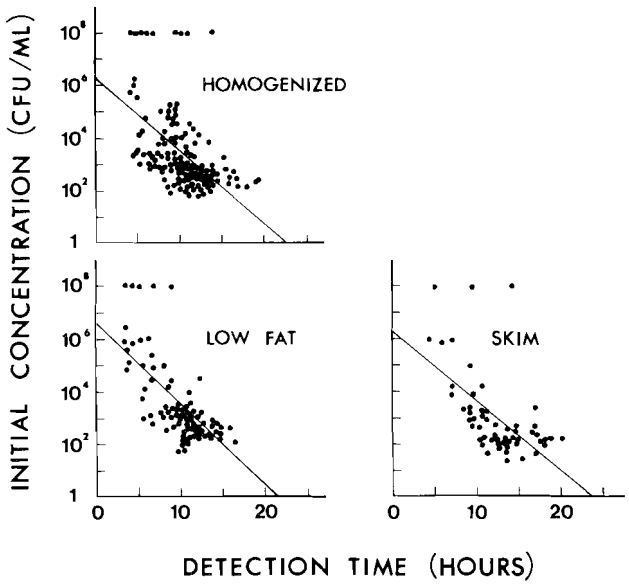

Figure 7. *Scattergrams of impedance response detection times (averages of duplicate channels) graphed against initial microorganism concentration as determined by plate count at 32 C for 191 homogenized, 119 low fat, and 70 skim milk samples. The solid lines are the least squares linear fits to the data.*

the data from 191 samples of homogenized milk, 119 samples of low-fat milk and 70 samples of skim milk. Among these were samples held at refrigeration temperatures from a few hours to as long as 12 days after pasteurization. All of the samples with more than 100,000 organisms/ml were of this latter category.

Although the slopes of the regression lines differ slightly between homogenized, low fat, and skim milk, they do not differ significantly. Hence, these data have been combined and the 380 data points are displayed together in Fig. 8. The correlation coefficient for these data is –0.60, indicating a good deal more spread in these pasteurized milk data than with, for example, frozen vegetable data, which showed a correlation coefficient of –0.85. The coefficient of determination (0.36) indicates that only 36% of the variance of the

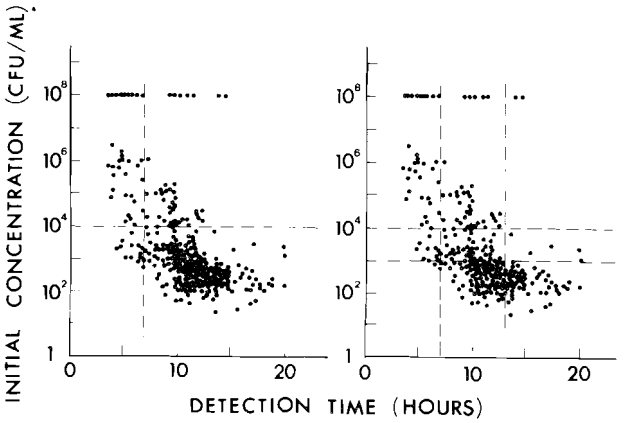

Figure 8. *Scattergram of impedance response detection times (averages of duplicate channels) graphed against initial microorganism concentration as determined by plate count at 32 C for 380 samples of pasteurized milk. The dashed lines indicate two-way (left side) and three-way (right side) classification schemes applied to these data.*

detection time is associated with variability in the log of the plate count. Nevertheless, the correlation between log plate count and detection time is significantly different from zero ($p < .001$).

The left side of this figure shows a two-way classification similar to that shown for raw milk in Fig. 6. For the pasteurized milk shown here, the best cutoff time to classify samples as having more than or less than 10,000 organisms/ml is 7 h. On 323 (85%) of the 380 samples the impedance and plate count methods agreed. There were 4% false positives and 11% false negatives.

The data points at the 10^8 organisms/ml level in Fig. 7 and 8 correspond to samples where the plates were too numerous to count, thus indicating an initial concentration above 10^6 organisms/ml. The fact that many of these very high count samples were not detected until after 10 h indicates one of the necessary precautions needed with impedance screening. When initial concentrations of microorganisms exceed the instrument's threshold level of 10^7/ml, the initial accelerating impedance change is lost or obscured by the impedance changes resulting from the initial thermal equilibration. If the initial response is missed, a secondary response about 6 h later is frequently detected. Presumably, concentrations in excess of 10^7 organisms/ml would be rare in fresh milk, and were they to occur, there would be a good chance that such high-count milk could be caught by inspection when setting up the samples for testing.

On the right side of Fig. 8 is an example of the three-way screen. By selecting two cutoff times, one can have classifications for low, intermediate, and high count product. For example, in Fig. 8 cutoff times of 7 h and 13 h may be used to divide all samples into those containing greater than 10,000 organisms/ml (with detection times under 7 h), those containing greater than 1,000 organisms/ml but fewer than 10,000 organisms/ml (with detection times greater than 7 but under 13 h), and those containing fewer than 1,000 organisms/ml (detection times greater than 13 h). The agreement between impedance and plate count classification is 50, 80 and 32% for samples over 10,000 organisms/ml, between 10,000 and 1,000 organisms/ml and below 1,000 organisms/ml, respectively. Although the agreement for each classification is low using this scheme, the chance of a serious misclassification is surprisingly low. Thus 98% of all samples are either classified correctly or into the neighboring category. Bray et al. (*4,12*) have proposed a three-way classification scheme where a number of subsamples of a batch is tested and the batch is rejected if any one subsample is high count or if a high proportion of them are intermediate. In this particular illustration, the screen yields a much higher percentage of intermediate samples than might normally be expected, probably because milk samples of various ages and origins have been included.

The impedance-based screen provides a rough estimate of total mesophilic count in pasteurized milk samples within about 7 h (or within 13 h if a lower

organism demarcation level or a three-way screen is used). Even though this is considerably faster than the total mesophilic plate count, requiring 48 h of incubation, it probably is not fast enough to allow a milk processor to test his milk before shipment. Most milk processors will have already sent their milk out before 7 h have elapsed from pasteurization. The value of the 7-h screen, however, is that potential problems will be detected almost 2 days earlier, thus saving large quantities of milk from being processed under less than ideal conditions.

Psychrotroph screen

The number of psychrotrophic organisms in a milk sample is a frequently used predictor of the sample's keeping quality. The conventional procedure for psychrotrophic counts requires 10 days of incubation at 7 C. Last year, Oliveria and Parmelee (*16*) reported that milk psychrotrophs grow well at 21 C whereas the mesophiles grow very slowly at this temperature. They found that incubation at 21 C for 25 h was equivalent to incubation at 7 C for 10 days. Our investigation utilizing 21 milk samples (some of which were incubated various periods to provide 74 sets of plates counted by each method) has supported their findings (see Fig. 9). These observations suggest that a rapid test for psychrotrophs at 21 C could be done with impedance measurements.

Figure 10 presents a scattergram of detection times for milk samples incubated at 21 C graphed against psychrotrophic counts as determined by the conventional 10-day method. The 69 points shown on this scattergram were obtained from 21 milk samples analyzed after various periods of refrigeration for some samples to provide a wider range of psychrotrophic counts.

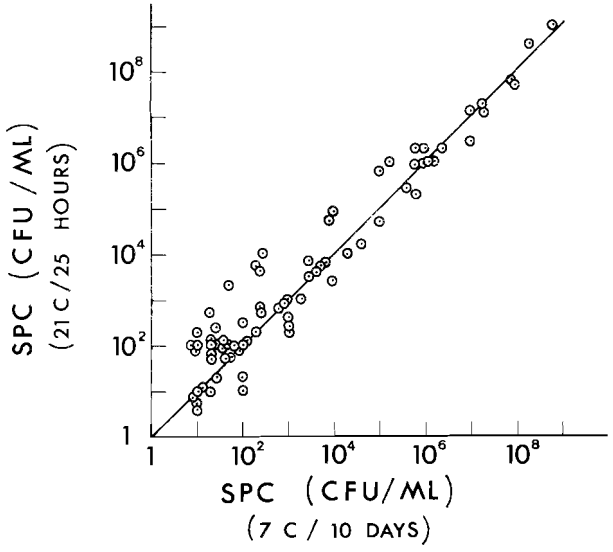

Figure 9. *Scattergram of psychrotrophic counts obtained from incubating plates at 21 C for 25 h graphed against counts obtained from the same samples when plates were incubated at 7 C for 10 days. The solid line represents the locus of points where equal counts are achieved. The 74 sets of plate counts were obtained from 21 milk samples after varying periods of refrigeration. The correleation coefficient is 0.97.*

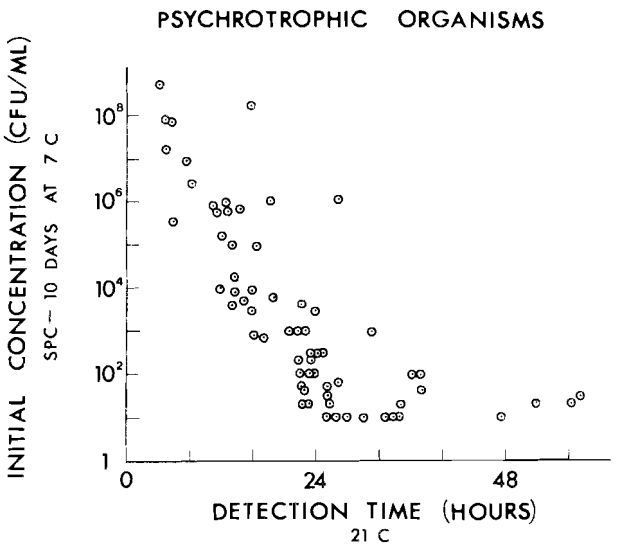

Figure 10. *Scattergram of impedance response detection times (earliest of duplicate channels) graphed against initial psychrotrophic count as determined by incubating plates 10 days at 7 C. Impedance monitoring was performed with SMB in modules at 21 C.*

The correlation coefficient for these psychrotrophic counts is –0.73. One source of spread in these data is the total population growth rate, which at 21 C (data shown in Fig. 10) is slower than the mesophilic organisms grown at 32 C (data shown in Fig. 8). The slopes of the regression lines for these two sets of data indicate a doubling time of about 2 h for the former and about 1 h for the latter. This results in a greater variation in detection time for the same relative variation in growth rate. In addition, the impedance changes were much more gradual, leading to less well-determined detection times.

For classification above or below 1,000 psychrotrophs/ml, a cutoff time of 21.3 h produced the maximum agreement between the impedance and plate count classifications (61 out of 69 samples or 88%). For classification above or below 10,000 psychrotrophs/ml, a cutoff time of 13.7 h was best (63 out of 69 samples or 91% agreement). A screening test for these higher concentrations of psychrotrophic organisms may find use in conjunction with an initial period of preincubation. Compared with the 10-day conventional test, these impedance-based screens (14 or 21 h) offer a considerable reduction in the time required to get test results.

Shelf life prediction

It is widely assumed that the keeping quality of milk is influenced by a great number of factors, many bearing upon milk's microbial content and the conditions that impede or further growth of the milk's endogenous flora. In spite of our knowledge of the microbiology of milk and of milk products, keeping quality is difficult to predict on the basis of laboratory estimates of the microbial content of freshly pasteurized milk (*9*).

Some probable reasons for this difficulty stem from the complexity of those factors leading to poor keeping

quality. The microbial flora of raw milk can influence the keeping quality of the pasteurized product in at least four ways: First, the microbial flora can contain thermoduric psychrotrophic organisms which survive pasteurization and then go on to cause spoilage even under the best of storage conditions. It has been estimated that as few as 10 thermoduric psychrotrophs in a quart of milk can cause spoilage within a few days (*3*). Second, microbial flora can be a source of enzymes surviving pasteurization and going on to cause continuing biochemical change. Third, there is some evidence to show that the microbial flora of raw milk might influence the rate of growth of organisms either surviving pasteurization or appearing as post-pasteurization contaminants (*18*). Presumably, metabolites produced by the raw milk flora can either augment or inhibit the post-pasteurization flora. Finally, certain metabolites in the raw milk, not destroyed by pasteurization, contribute directly to off-flavors and poor quality.

In addition to these factors are those contributing to errors in estimating microbial content. The population that will be seen by microbiological testing will depend very greatly upon the diluent used, the medium in which the sample is grown (*14,15,21*) and the temperature and length of time of incubation (*2*). Add to this the degree to which psychrotrophs form dense clumps, resistant to breaking up (*21*), and it is not surprising to find errors of up to two orders of magnitude in psychrotroph estimations.

An investigation of impedance response parameters (detection time, response strength, etc.) and their relationship to milk keeping quality has just begun. So far, 22 milk samples have been analyzed, and these samples have shown only a 6-day variation in shelf life. (Shelf life has been defined as the number of days from pasteurization until an unsatisfactory flavor score [$\leqslant 35$] occurs.) In Table 1 are shown the mesophilic plate count, psychrotrophic count, and impedance response detection times for 10 samples whose shelf lives were the shortest or the longest of the samples analyzed. The detection times presented in this table result from incubation at 32 C. In general, these detection times appear to reflect the values of the shelf life, the second sample being the graphic exception. The detection times, in fact, seem to correlate better with the shelf life than do the standard plate count and psychrotrophic count, at least for these few samples. Confirmation of this correlation will require the analysis of a much larger number of samples with a much larger spread in shelf life duration. These early data, however, show promise of a 9-14-h impedance-based keeping quality prediction.

TABLE 1. *Comparison of shelf life, impedance response detection time, standard plate count and psychrotrophic count for 10 milk samples.*

Shelf Life (days)	Detection time[1] (hours)	Mesophilic plate count[2] (cfu/ml)	Psychrotrophic count[3] (cfu/ml)
9	9.4	400	20
9	12.2	7000	30
10	9.6	400	10
10	9.4	200	100
10	10.4	200	—
14	11.1	300	10
14	10.9	400	100
14	11.5	100	30
15	11.4	100	10
15	10.3	200	100

[1]Earliest detection of duplicate vials with TSBY at 32 C.
[2]Incubation at 32 C for 48 h.
[3]Incubation at 7 C for 10 days.

ACKNOWLEDGMENTS

The laboratory assistance of Sheryle Hodapp and Mark Selby, the technical assistance of Robin Olsen and Loren Vanderbeek, and the secretarial assistance of Lu Yutzy are gratefully acknowledged.

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