The *Limulus* Amoebocyte Lysate Assay and the Direct Epifluorescent Filter Technique as Estimators of Potential Shelf-life of Pasteurized Fluid Milk

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**ABSTRACT**

The *Limulus* Amoebocyte Lysate Assay, Direct Epifluorescent Filter Technique and modified Psychrotrophic Bacteria Count were used to indicate potential shelf-life of pasteurized fluid milk. Commercial whole milk samples, stored at 7°C, were analyzed for bacterial and biochemical parameters, as well as for potential shelf-life by daily sensory evaluation. Each sample was evaluated before and after the following preliminary incubations: milk alone, milk with benzalkonium chloride, milk and broth, and milk and broth with benzalkonium chloride. The *Limulus* Amoebocyte Lysate Assay, Direct Epifluorescent Filter Technique, and modified Psychrotrophic Bacteria Count in conjunction with the preliminary incubations, produced relatively high correlations to shelf-life (-0.78, -0.85, and -0.86 respectively). Thus, these bacterial detection techniques could be used as rapid methods of shelf-life estimation.

In order to predict the shelf-life of milk, we must detect the bacteria which ultimately cause its quality degradation. It has been shown that these are the gram-negative psychrotrophic bacteria. The detection of gram-negative endotoxins and direct microscopic counts have been used to indicate bacterial numbers (4,5,6,8,10), and to predict shelf-life of milk (1). A limitation of these methods has been sensitivity. The numbers of psychrotrophic bacteria initially present in milk are so low that these methods are unable to accurately detect them. Byrne et.al. successfully used a selective preliminary incubation with benzalkonium chloride to increase the gram-negative psychrotrophic bacteria (2) and also developed a rapid test for shelf-life prediction using this preliminary incubation (3). The purpose of this investigation was to evaluate the *Limulus* Amoebocyte Lysate Assay, Direct Epifluorescent Filter Technique, and modified Psychrotrophic Bacteria Count, after preliminary incubations, for their ability to predict shelf-life of pasteurized fluid milk.

**MATERIALS AND METHODS**

**Milk Samples**

Whole milk, packaged in plastic jugs (3.8 l), was obtained from local retailers and stored at 7°C until determined to be unacceptable by sensory evaluation (n=100).

**Preliminary incubations**

Preliminary incubation were performed at 21°C for 18 h. The following incubations were used: milk alone (control), milk in benzalkonium chloride (0.10% w/v final concentration), milk in nutrient broth (1:1), and milk in nutrient broth with benzalkonium chloride (0.10% w/v final concentration) (1:1).

**Bacterial enumerations**

The modified Psychrotrophic Bacteria Count (mPBC) using *Petrifilm* (21°C, 48h) (3,7,9) was performed at time-zero and after preliminary incubations.

**Endotoxin detection**

Concentration of lipopolysaccharide (LPS) was determined using the *Limulus* Amoebocyte 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 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 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 (DEFT) (6) as follows:

Sample preparation: 0.5 ml trypsin, 2 ml Triton X-100 (0.5% v/v) and 3 ml of milk were added to a test tube, vortexed, and incubated at 50°C for 10 min.

Filtration: A nuclepore polycarbonate membrane filter (0.6 μm pore size, 25 mm diameter) was mounted onto a filter tower. The filter was warmed by filtering 5 ml Triton X-100 (0.1% v/v) at 50°C. The sample was then filtered. 5 ml Triton X-100 (0.1%) at 50°C was added to the test tube, vortexed, and filtered. The tower was rinsed with 5 ml 0.1% Triton X-100 and filtered.

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

Mounting of membrane filter: 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.

Counting: 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:

<table>
<thead>
<tr>
<th>Number of fields counted</th>
<th>Avg. # of clumps/field</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0-10</td>
</tr>
<tr>
<td>10</td>
<td>11-25</td>
</tr>
<tr>
<td>6</td>
<td>26-50</td>
</tr>
<tr>
<td>3</td>
<td>51-75</td>
</tr>
<tr>
<td>2</td>
<td>76-100</td>
</tr>
<tr>
<td>dilute and repeat</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

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:

\[ MF = \frac{\text{Area of membrane which sample was filtered (mm}^2\text{)}}{\text{Microscopic field area (mm}^2\text{)} \times \text{sample volume(ml)}} \]

Shelf-life determination

The shelf-life of the milk was determined by sensory evaluation as previously described (1). This value must be used as “potential” shelf-life when making correlation comparisons to measured parameters.

Statistical analysis

Multiple correlation value analyses were made using the Statistical Analysis System (SAS Institute, Inc., Box 800, Cary, NC 27511).

RESULTS AND DISCUSSION

Bacterial counts

At time-zero (TZ) the mPBC had a good correlation to shelf-life (-0.84) (Table 1). After preliminary incubation the correlations to shelf-life improved, especially after a preliminary incubation of milk alone (-0.86) (Table 1). These results are comparable to those obtained by Byrne et al. (3) even though time-zero results from more samples produced a lower correlation value of -0.79.

Endotoxin detection

The correlation to shelf-life for the LAL test at time-zero was fair (-0.69) (Table 1). This correlation was improved after all preliminary incubations were conducted. There were no differences between preliminary incubations in milk and milk with benzalkonium chloride (-0.85) (Table 1). These results are expected because the endotoxin is specific for gram-negative organisms. We selected for psychrotrophs with our low temperature incubation and the main psychrotrophs in milk are gram-negatives. Other researchers have used the LAL test to detect gram-negative bacteria (4,6), but little work had been done to compare LPS concentration to shelf-life. Bishop and White obtained a correlation of -0.913 when comparing a spectrophotometric endotoxin test to shelf-life (7). In our test we 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.

Microscopic counts

For the DEFT, good correlations to shelf-life were obtained at time-zero (-0.75) and after all preliminary incubations (-0.75 to -0.78) (Table 1). There was little difference in using benzalkonium chloride in our incubation. This may again be due to the fact that the low-temperature incubations without benzalkonium chloride select for psychrotrophic bacteria and the main psychrotrophic bacteria in milk are gram-negatives. These results are comparable to those of Kroll and Rodrigues (5) who found good correlations to keeping quality of pasteurized cream.

CONCLUSIONS

The correlations to shelf-life for the modified Psychrotrophic Bacteria Count were consistent with previously published data (3). The Limulus Amoebocyte Lysate Assay had good correlations to shelf-life, especially after preliminary incubation (-0.85) (Table 1). The Direct Epifluorescent Filter Technique also had good correlations to shelf-life, the best being after preliminary incubation of milk in broth (-0.78) (Table 1). These tests are rapid detection techniques (19 h for LAL and DEFT, 66 h for mPBC) which could be used to predict the shelf-life of pasteurized fluid milk.

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