Effect of Agitation on Bacterial Aggregates in Pure Cultures and Raw Milk

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ABSTRACT

A comparison of the effects of various mechanical agitation treatments on bacterial aggregates was performed on 8 pure cultures and 27 raw milk samples. Although both syringing and blending produced significant increases in total counts and psychrotroph counts, blending for 2 min gave the greatest increase in count. Use of the direct epifluorescent filter technique (DEFT) confirmed that syringing and blending reduced bacterial clump size to approximately 2 cells. These agitation treatments markedly improved the correlation between DEFT counts and plate counts.

The presence of bacterial clumps in milk and their susceptibility to disruption can have a significant effect on total plate counts. Although Reuter and Quente (14) concluded that flow processes in the dairy industry, such as batch stirring or circulatory pumps, do not influence bacterial counts, Luck and Walthew (10) have reported a 51% increase in total count from the action of a positive displacement pump. The influence of vibration appears to have a variable effect on total counts. Jurczak et al. (7) found that over a 15-min interval frequencies of 25 and 150 Hz increased the total count 25 and 8%, respectively, whereas frequencies of 50 and 100 Hz decreased the count.

Agitation of milk by high speed blending for 30 s produced a 44% increase in counts (20). If the milk was first stored at 5°C for 4 d and then blended, a 170% increase was obtained. Te Whaiti and Fryer (17) achieved a 290% increase after blending for 2 min. This study also showed that agitation by Stomacher, compressed air and syringing of washed cell clumps of Pseudomonas sp. produced an increase in total counts. Syringing was found to be more effective than blending, with a 650% increase compared to a 500% increase after blending. A comparison of methods of agitation by Stomacher, Osterizer, Waring Blender and shaking with and without beads (6) showed that highest plate counts of Bacillus cereus, Streptococcus faecalis and Staphylococcus aureus resulted after 2 min of blending.

Most clumps and chains were completely disrupted by this treatment. On the other hand, Yersinia enterocolitica, included as a control organism because its cells do not aggregate, gave plate counts that were not significantly different after all treatments. Law et al. (8) found that syringing had a variable effect on plate counts of refrigerated milks, with increases in counts ranging from <2 to >10 times the control count. Another study on agitation using a pure culture of S. faecalis and shaking with glass beads showed little effect on total count (9). Similarly, Richard (15) and Richard et al. (16), using preincubation and shaking, vortex mixing, homogenization and sonication, found no significant differences in plate-loop counts with raw milk samples.

Lee and Calcott (9) reported that 4 min of high speed blending successfully disrupted chains of S. faecalis, with an estimated 8% loss of viability. This study also showed that temperature stress (i.e., freezing and thawing) after blending caused no extra release of K+ above that previously observed with blending alone.

Te Whaiti and Fryer (17), when studying the effect of growth temperature on clump size of 9 pseudomonad strains in reconstituted skim milk (RSM), found that clumps were formed during exponential growth, with clump size at 4.5°C being twice that at 22°C. In addition, 5- to 10-fold difference in count between blended and unblended samples of refrigerated raw milk was recorded upon receipt at a dairy plant. Law et al. (8) found that increases in total count caused by syringing were greatest in those milks that had been stored under refrigeration conditions for the longest periods of time.

The above evidence suggests that cold storage of raw milk increases the formation of bacterial aggregates, with mechanical stress producing a variable effect on clump disruption. Therefore, there is need to improve and standardize current bacterial cell count procedures used in the microbiological analysis of milk. This report considers the effect of syringing, blending and sonication treatments on...
cell counts. It utilized the direct epifluorescent filter technique (DEFT; 4,5,12,13) to enumerate cell and clump counts in both raw milk samples and RSM cultures of organisms commonly found in milk.

MATERIALS AND METHODS

Pure cultures

RSM broth (10 ml) was inoculated with cultures of Escherichia coli NCTC 8196, Pseudomonas aeruginosa NCTC 6750, Streptococcus cremoris strain EB9 or Staphylococcus aureus NCTC 6571 and incubated for 18 h at 30°C. Alternatively, RSM broth was inoculated with cultures of Serratia liquefaciens, Pseudomonas fragi ATCC 4973, Pseudomonas fluorescens or Lactobacillus helveticus and incubated for 18 h at 30°C. S. cremoris and L. helveticus belong to the collection of cheese starter cultures held at this laboratory. S. liquefaciens and P. fluorescens were isolated from raw milk and identified using the Miniqek numerical identification system for enteric bacteria and nonfermentative bacteria (BBL, Cockeysville, MD).

Raw milk

Raw milk was sampled (in 200-ml volumes) from either farm or milk plant bulk tankers as described by Standards Association of Australia (1). Samples were stored for up to 3 d at approx. 2°C until tested.

Agitation treatments

Agitation treatments were applied to 1:10 dilutions of raw milk or RSM cultures in 0.1% (wt/vol) peptone (Oxoid, Basingstoke, U.K.) in distilled water. The sample was shaken for 12 s, using 25 complete up-and-down movements over an approx. 30-cm span, before making the dilution. Control samples were agitated only by the shaking procedure. Further dilutions were made, if necessary, according to Standards Association of Australia (3) in the same dilute as that described above. The methods cited from Standards Association of Australia (1-3) are similar to procedures outlined by the American Public Health Association (11) except as otherwise indicated in the text.

Syringing. Diluted sample (10 ml) was syranged three times using a 5-ml disposable syringe with a 21 guage 38-mm needle (Terumo, Melbourne, Australia). The sample (5 ml) was drawn up by the syringe and expelled in less than 2 s above the surface of the remaining sample in the container (17,18).

Blending. Diluted sample (20 ml) was blended in 50-ml containers at 10,000 rpm for intervals of 30 s to 3 min with a Sorvall Omni-Mixer homogenizer, (Norwalk, VA).

Sonication. Sonication was performed at maximum energy using an Ultrasonic Disintegrator model A1806 (Ultrasonics Ltd., Nunawading, Australia) tuned to give maximum audible sound and cavitation. Diluted sample (10 ml) was sonicated with the micro-tip probe for intervals of 30 s to 3 min in a 50-ml Pyrex beaker in crushed ice.

Plate counts

Standard plate counts were performed by the method described by Standards Association of Australia (3) using Oxoid plate count agar. Plates were incubated at 30°C for 3 d before counting. Only pin-point colonies of L. helveticus developed after 3 d at 30°C. Consequently, these plates were incubated for an extra 2 d at 41°C. Triplicate plates were used in studies involving pure cultures; duplicate plates in studies with raw milk. Psychrotroph counts were determined according to Standards Association of Australia (2). Plates were incubated at 7°C for 10 d before counting.

Microscopic counts

Definition. Separate clumps were defined to be any cell or groups of cells of the same species separated by a distance equal to or greater than twice the smallest diameter of the 2 cells nearest each other (11).

Pure cultures. The effect of agitation on aggregation of cells in pure culture was studied using the direct microscopic method described by Standards Association of Australia (3). The average number of cells per clump was estimated from a minimum of 20 clumps.

Raw milks. Microscopic examination of milk samples was initially performed using the direct epifluorescent filter technique (DEFT) of Pettipher et al. (13). On publication of a modification of this method by Beck and Hehir (4), samples were then examined by the more recent method. Trypsin (1:250; Difco, Detroit) at a concentration of 368 μg/ml of distilled water was used to treat milk when using the protocol of Pettipher et al. (13). (This concentration was derived on the basis of the specific activity of trypsin against casein). When using either method after syringing or blending treatments, 10 ml of sample were treated with trypsin and Triton X (Ajax Chemicals, Sydney, Australia) before filtration. Milk samples were filtered under vacuum with the aid of an Amicon vacuum filtration manifold system VFMI (Danvers, MA). Mounted membranes were examined by a vertical fluorescence illuminator on an Olympus Vanox A-RFL microscope, (Tokyo, Japan).

Enumeration of S. cremoris

The influence of syringing and a 1-min blending treatment on chain length of S. cremoris was investigated. RSM broth (500 ml) was inoculated with sufficient culture to give an initial concentration of 10⁸ CFU/ml and incubated at 20°C. At hourly intervals, subsamples were withdrawn for total plate count testing (3). In addition, microscopic examinations of the cultures were made at 2-h intervals for 7 h duration.

Analyses

The effect of blending and syringing on total and psychrotroph counts was compared using analysis of variance applied to data that was transformed. A log (x + 1) transformation was applied to raw data to equalize variances. Pairwise comparison of treatments was made using the least significant difference test. Regression lines relating DEFT cell and clump counts to plate counts were fitted after each of the counts had been log transformed.

RESULTS

Effects of the various treatments on total plate counts of five gram-negative and three gram-positive cultures are shown in Table 1. A 2-min blending treatment resulted in the largest increase in mean count (51%). One- and three-minute blendings also increased counts, i.e., 35% and 27%, respectively. With the exception of S. liquefaciens, counts of all cultures increased with 1- and 2-min blending treatments.

Although 1-min sonication gave an increase in total count (37% overall), plate counts of 5 of 8 cultures were reduced (sometimes greatly) by sonication. With the exception of the S. cremoris culture, syringing had little effect on total count. The average increase was 6% and in some cases the total count was slightly reduced.

With any treatment, largest increases in count were found with S. cremoris, L. helveticus and S. aureus cultures. Blending and sonication treatments consistently reduced clumps of chains of S. cremoris and S. aureus to an average of 2 cells or less. After syringing, chains of S. cremoris averaged four cells in length and clumps of S. aureus were reduced to 2 cells or less.

S. cremoris cultures were examined throughout the growth cycle for total count and chain length following either syringing or a 60-s blending treatment. Through exponential and stationary phases of growth, syringed counts were consistently higher than control counts, and blended counts were greater than syringed counts (Fig. 1). Control samples showed that the average chain length of S. cremoris decreased from 22 cells at the beginning of log phase...
TABLE 1. The effect of syringing, blending and sonication treatments on plate counts of 5 gram-negative and 3 gram-positive bacteria incubated in reconstituted skim milk for 18 to 24 h.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Control count (CFU x 10^6)</th>
<th>Syringing</th>
<th>Blending 1 min</th>
<th>Blending 2 min</th>
<th>Blending 3 min</th>
<th>Sonication 1 min</th>
<th>Sonication 2 min</th>
<th>Sonication 3 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>92</td>
<td>-9</td>
<td>8</td>
<td>5</td>
<td>20</td>
<td>-9</td>
<td>30</td>
<td>41</td>
</tr>
<tr>
<td><em>S. liquefaciens</em></td>
<td>240</td>
<td>-21</td>
<td>-21</td>
<td>-25</td>
<td>-25</td>
<td>-54</td>
<td>-88</td>
<td>-99</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>150</td>
<td>-7</td>
<td>27</td>
<td>17</td>
<td>7</td>
<td>40</td>
<td>33</td>
<td>13</td>
</tr>
<tr>
<td><em>P. fragi</em></td>
<td>1100</td>
<td>0</td>
<td>36</td>
<td>18</td>
<td>-26</td>
<td>-24</td>
<td>-40</td>
<td>-88</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>1400</td>
<td>0</td>
<td>7</td>
<td>43</td>
<td>7</td>
<td>-58</td>
<td>-68</td>
<td>-73</td>
</tr>
<tr>
<td><em>S. cremoris</em></td>
<td>48</td>
<td>60</td>
<td>129</td>
<td>192</td>
<td>90</td>
<td>317</td>
<td>150</td>
<td>56</td>
</tr>
<tr>
<td><em>L. helveticus</em></td>
<td>1500</td>
<td>13</td>
<td>53</td>
<td>80</td>
<td>93</td>
<td>-27</td>
<td>-61</td>
<td>-89</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>360</td>
<td>11</td>
<td>39</td>
<td>89</td>
<td>53</td>
<td>105</td>
<td>128</td>
<td>128</td>
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<tr>
<td>Mean</td>
<td>610</td>
<td>6</td>
<td>35</td>
<td>51</td>
<td>27</td>
<td>37</td>
<td>11</td>
<td>-14</td>
</tr>
<tr>
<td>Standard error</td>
<td>219</td>
<td>9</td>
<td>16</td>
<td>24</td>
<td>17</td>
<td>44</td>
<td>32</td>
<td>30</td>
</tr>
</tbody>
</table>

* a- , decrease in count.

Figure 1. Effect of blending and syringing on chain length and standard plate count (SPC) of *S. cremoris* during growth. ○, control; ■, syringed; ▼, blended.

to 9 cells after 7 h of growth. After syringing or blending, chains had a more consistent length of 6 or 2 cells, respectively.

Preliminary work (data not shown) on the effect of treatments on 7 fresh milk samples showed that although blending for 1, 2 or 3 min produced increases in total count of more than 100%, greatest increases occurred after either a 2- or 3-min treatment (117 and 126%, respectively). No counts decreased through blending. Syringing caused a 50% increase in count, with one sample showing a decrease. On the other hand, sonication resulted in a decrease in count in most samples, the loss of cell viability being directly proportional to the duration of the treatment.

Based on preliminary findings with pure cultures and raw milk, a 2-min blending period was chosen. Counts after syringing and blending were compared using 27 1- and 2-d-old raw milk samples. Increases in total count and psychrotroph count with syringing and blending treatments were significant at the 1% level of significance (Table 2). Higher total and psychrotroph counts occurred with blending than syringing. There was large variability in the effect of agitation on bacterial counts among milk samples. Increases of greater than 150% were found on syringing and blending one sample, whereas, after applying the same treatment to another sample, increases in total count of only 30 to 60% were achieved. Six samples showed a decrease in bacterial count after treatment relative to the control. Statistical comparisons showed no significant difference (P>0.05) between 1- and 2-d-old milk. This result occurred with both total and psychrotroph counts.

To microscopically examine the effects of syringing and 2-min blending on bacterial clumps, the DEFt procedure was used on 8 raw milk samples following treatment (Table 3). In control milk, the size of bacterial clumps was variable, with the numbers of cells aggregating to form a clump ranging from 2 to 32. On syringing and blending, clumps were fragmented to form smaller units comprising an average of 3 and 2 cells, respectively. This figure is consistent between milk samples (standard error = 0.2 and 0.3, respectively). Correlation of plate counts with DEFt cell and clump counts increased with agitation of milk samples (Table 4).

**DISCUSSION**

A comparison of the effects of syringing, blending and sonication treatments on pure cultures and raw milk samples suggests that, of these three methods, bacterial cell
numbers were most reliably estimated after a 2-min blending treatment. This treatment gave the greatest increase in total count and consistently reduced clumps to an average of 2 cells.

Te Whaiti and Fryer (17) initially reported that syringing of raw milk samples was more effective than blending for increasing the total count. Recent work by these authors (personal communication) found that syringing is operator dependent, and that instead, a preliminary 60-s treatment with an Ultra-Turrax has become their preferred technique for clump disruption. The present study shows that increases in total plate counts of both pure cultures and raw milk samples were less with syringing than blending. For example, of all pure cultures, a large increase in total count following syringing only occurred for \( S. \) cremoris. Microscopic examination of this culture showed that, although syringing was successful in breaking chains, it was less effective than blending.

DEFT enabled microscopic examination of the influence of agitation on the aggregation of bacterial cells in raw milk samples. Plate count results were confirmed, with DEFT showing that blending was more efficient than syringing.

Since DEFT concentrates bacteria by membrane filtration, microscopic examination of aggregated bacterial cells in raw milk samples was possible.
tion, this procedure is more suitable than other microscopic procedures for enumerating bacteria in low count milk samples. Other workers (4,5,12,13) have found good correlation between DEFT cell and clump counts, and plate counts. In this study, correlation coefficients of 0.88 and 0.75 were calculated between plate and cell counts, and plate and clump counts, respectively. This compared with correlations of 0.94 and 0.91 between clump and plate counts as reported by Beck and Hehir (4) and Pettipher et al. (13), respectively, and 0.97 between total cell and plate counts as reported by Cousins et al. (5). Correlation between DEFT and plate counts was considerably improved by agitation. Correlation coefficients of 0.96 were found between plate and clump counts after both syringing and blending.

This laboratory's experience with DEFT indicates that large errors can be caused by aggregation of bacteria to form a few very large clumps on the membrane. If such a clump is not found in any of the fields selected for counting, the DEFT count will grossly underestimate the true count. Agitation results in the fragmentation of these larger units into single cells or pairs, which tend to be homogeneously distributed through the milk sample. As a consequence, fewer fields need be examined to estimate the total count of the sample.

Trends in the bacteriological quality control of milk and other dairy products are progressing toward tests which yield results rapidly, such as the DEFT procedure. It is recommended from this study that the enumeration of bacteria in pure culture or in milk samples by either standard plate count or DEFT be preceded by a 2-min blending procedure.

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REFERENCES