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Potential for broad applications of flow cytometry and fluorescence techniques in microbiological and somatic cell analyses of milk

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Abstract

Monitoring the quality and safety of milk requires careful analysis of microbial and somatic cell loading. Our aim was to demonstrate proof of the principle that flow cytometry (FCM), coupled with fluorescence techniques for distinguishing between cell types, could potentially be employed in a wide variety of biological assays relevant to the dairy industry. To this end, we studied raw milk samples and ultraheat-treated milk, into which known numbers of bacteria or mouse cells were inoculated. For bacterial analyses, protein and lipids were removed, whereas only centrifugal lipid clearing was needed for somatic cell analyses. Cleared samples were stained with fluorescent dyes or with bacterial-specific fluorescent-labeled oligonucleotides and analyzed by FCM. A fluoresceinated peptide nucleic acid probe enabled efficient enumeration of bacteria in milk. Dual staining of samples with fluorescent dyes that indicate live (5-cyanol-2,3-ditolyl tetrazolium chloride, CTC or SYTO 9) or damaged cells (oxonol or propidium iodide, PI) enabled determination of viable bacteria in milk. Gram-positive and -negative bacteria were distinguished using hexidium iodide and SYTO 13 in dual staining of cleared milk samples. An FCM-based method gave a good correlation ($r = 0.88$) with total microscopic counts of somatic cells in raw milk. The FCM method also correlated strongly ($r = 0.98$) with the standard Fossomatic method for somatic cell detection. We conclude that FCM, coupled with fluorescence staining techniques, offers potentially diverse and rapid approaches to biological safety and quality testing in the dairy industry. Potential application of flow cytometers to a broad range of assays for milk biological quality should make this instrumentation more attractive and cost effective to the dairy industry and indeed the broader food industry.

Keywords: Flow cytometry; Milk; Bacteria; Somatic cells; PNA probe; Fluorescent in situ hybridization (FISH); SYTO dyes

1. Introduction

Biological monitoring of raw milk, which involves analysis of microbial and somatic cells, is essential for milk and dairy quality assurance. Milk microbiology impacts on issues of shelf life of dairy products as well as on the determination of the type of product for which raw milk is to be used (Muir, 1996). A high biological count in raw milk alerts the dairy processor to possible problems with product safety (Sørhaug and Stepaniak, 1997). Currently, detection and enumeration of microbial contaminants rely heavily on conventional culture methods such as direct microscopy,

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plate counts and most probable numbers, or application of indirect methods such as bioluminescence and redox staining. Direct microscopic counts or automated instruments are applied to somatic cell counts in raw milks. Generally, the methods are time consuming or they do not provide complete quality and safety assurance (Vasavada, 1993), or instrumentation is limited in its range of application.

There is a need within the dairy industry for a single-instrument/technology-based system that enables assays of somatic and microbial cells including detection, enumeration and differentiation of microbial contaminants. Flow cytometry (FCM) offers the possibility for single-instrument, rapid analyses of milk. Automatic FCM-based methods have been applied to analysis of raw milk (Suhren and Walte, 1998), but the specialised type of instrument (Bactoscan-FC) and analytical method used is limited to total bacterial counts. The cost of flow cytometer instrumentation is inhibitory to the food and dairy industry if only single-assay types can be carried out. However, if multiple types of assays can be performed in a rapid and relatively simple manner, the value of a rapid instrumentation-based approach to food microbiology should become attractive. In fact, flow cytometers offer much greater potential for detecting and enumerating total and subgroups of microorganisms present in raw and processed milks. For example, we have developed a rapid method using FCM, which can be used to detect bacteria in processed milk as well as raw milk (Gunasekera et al., 2000). We have also demonstrated the use of a green fluorescent protein reporter system in conjunction with fluorescence microscopy and FCM to detect viable but nonculturable bacteria in heat-treated milk (Gunasekera et al., 2002). Others have shown that by applying fluorescent antibody technology, it is possible to detect low numbers of specific microorganisms such as pathogens using FCM (Pinder and McClelland, 1994; Clarke and Pinder, 1998). The ability to use a single instrument for numerous rapid microbiological and somatic cell assays has obvious advantages for the dairy industry.

Here, we show proof of the principle that FCM and fluorescence technologies can be applied to a broader range of microbiological analyses of milk. Evidence is presented for the feasibility of using fluorescent oligonucleotide-based techniques for bacterial cell counts. We also demonstrate the application of fluorescent stains and FCM for determining the viability of bacteria, Gram stain analyses and somatic cell counts in milk, within short time frames suitable for use in dairy laboratories.

2. Materials and methods

2.1. Bacterial strains, somatic cells and growth conditions

Bacterial strains used for inoculating phosphate-buffered saline controls or milk were *Pseudomonas fluorescens* B52 (Richardson, 1981), *Escherichia coli* XL1-Blue MRF$^-$ and *Staphylococcus aureus* NCTC 4163 (Gunasekera et al., 2000). These were chosen as examples of bacteria relevant to milk quality and safety. Bacteria were grown in Tryptone Soya Broth (Oxoid, Sydney, Australia) at 28 °C on a rotary shaker at 180 rpm for 16 h. Standard plate counts of bacteria were carried out as described previously (Gunasekera et al., 2000). Mouse hybridoma (somatic) cells were grown in RPMI 1640 standard cell culture medium (supplied by Life Technologies Australia, Sydney, Australia) supplemented with 10% fetal calf serum, 0.5% penicillin G, 0.5% L-glutamine and 0.5% sodium pyruvate. Cells were maintained in tissue culture flasks (25-ml canted-neck flasks with 0.2-μm vented filter caps), subcultured every other day and incubated in a 5% vol/vol CO_2 atmosphere at 37 °C. Cells were harvested by centrifugation at $250 \times g$ for 10 min, washed in phosphate-buffered saline and inoculated in known numbers into buffered saline or milk.

2.2. Milk, milk clearing and fluorescent staining of bacteria and somatic cells

Milk was either ultra-heat treated (UHT), or raw milk obtained daily from Perfection Dairies, Sydney, Australia. For total count assay, milk samples were cleared of proteins and/or lipids using enzymes, detergent and centrifugation as described previously (Gunasekera et al., 2000). For bacterial analyses, protein and lipids were removed, whereas only centrifugal lipid clearing was needed for somatic cell analyses. Cleared milk pellets were resuspended in phosphate-buffered saline (Gunasekera et al., 2000) prior to staining and analysis. For viability testing and

Gram differentiation, we used modified milk clearing procedure. Savinase (1 μl; EC 3.4.21.52; 16L Type EX, activity 16 KNPU/g; Novo Nordisk Bioindustrial) was added to 100 μl of milk, which was incubated at 30 °C for 45 min. NaCl (900 μl of 0.15 M) was added and samples centrifuged at 10,000 × *g* and 22 °C for 5 min. Digested proteins and the top layer containing lipids were drawn off with a micropipette and the bacterial pellet was resuspended in 100 μl of 0.15 M NaCl.

Staining for total bacteria using SYTO BC was as described previously (Gunasekera et al., 2000). SYTO 9 (Molecular Probes, Bioscientific, Sydney, Australia) was used as described by the manufacturer. Propidium iodide (PI; Sigma-Aldrich, Sydney, Australia) was used as described previously (Deere et al., 1998; Attfield et al., 2000). 5-Cyanol-2,3-ditolyl tetrazolium chloride (CTC; Polysciences, Bioscientific) was dissolved at 100 mmol in filter-sterilized distilled water and added to resuspended cells and cleared milk samples at a final concentration of 5 mmol. Bis-1,3-dibutylbarbituric acid trimethine oxonol (OXN) (Molecular Probes, Bioscientific) was prepared and used as described previously (Attfield et al., 2000; Deere et al., 1998). A dual-fluorescence Gram Stain Kit employing hexidium iodide and SYTO 9 (Molecular Probes, Bioscientific) was used as instructed by the manufacturer. Acridine orange (AO) (Sigma-Aldrich) was added to resuspended cleared milk at a final concentration of 0.01 mg/ml. Fluoresceinated oligonucleotide probes used for fluorescent in situ hybridizations (FISH) were EUB338, complementary to a 16S rRNA region (5′-GCTGCCTCCCGTAGGAGT-3′) conserved in all eubacteria and Eubacteria Flu-Peptide Nucleic Acid (PNA1) (Boston Probes, USA). Milk samples were cleared of proteins and/or lipids and FISH was carried out as described by Wallner et al. (1993).

2.3. Preparation of samples for viability testing

For viability testing, cultures grown in Trypton Soya Broth, as described in Section 2.1, were diluted and resuspended in PBS. Samples of 1 ml were heat killed (80 °C for 10 min) and mixed with live bacteria in different ratios to obtain 100%, 80%, 60%, 40%, 20% and 0% live bacteria in a total population of 10^7/ml, and inoculated into UHT milk. Milk samples were processed as described above and stained with SYTO 9 and PI before analysis using FCM.

2.4. Comparison of somatic cell numbers obtained from FCM with Fossomatic instrument

Raw milk samples ($n = 20$) were analysed for somatic cells using flow cytometry, and compared with values obtained from the Fossomatic 400 (Foss Electric, Denmark). Foss instrument analyses were carried out by the Dairy Technical Services, Victoria, Australia using standard methods (Zeng et al., 1997).

2.5. Flow cytometry, microscopy and data analysis

FCM was performed using a FACSCalibur (Becton Dickinson, Sydney, Australia) sorting flow cytometer equipped with a 15-mW argon ion laser emitting 488 nm light. Detectors were forward-angle light scatter

Table 1
Flow-cytometer settings used for assaying bacterial viability, Gram differentiation and somatic cells in milk samples

Assay (stains)	Threshold	Detector settings					% Compensation	
	FL1	FSC	SSC	FL1	FL2	FL3	FL2–FL1	FL3–FL2
Total bacteria (FISH)	235	E02	551	659	605	650	00.0	0.0
Total bacteria (SYTO BC)	416	E02	505	638	590	646	78.1	0.0
Viability (PI, SYTO 9)	94	E02	505	638	590	646	49.3	8.7
Viability (CTC, OXN)	289	E02	505	674	613	764	99.0	3.0
Gram differentiation (HI, SYTO 9)	94	E02	505	638	590	646	49.3	8.7
Somatic cell counts (AO)	289 (FSC)	E-1	211	288	258	288	0.0	0.0

All gains were logarithmic. FSC, forward-angle light scatter; SSC, side-angle light scatter; FL1, green fluorescence channel; FL2, yellow/orange fluorescence channel; FL3, red fluorescence channel. FISH refers to fluorescent in situ hybridizations with eubacterial-specific fluoresceinated oligonucleotide or peptide nucleic acid probes (PNA).

(<15°), side-angle light scatter (>15°), FL1 (515–565 nm), FL2 (565–605) and FL3 (>605). Instrument settings used are shown in Table 1. Sheath fluid was Osmosol (LabAids, Sydney, Australia). For each sample, 10,000 events were captured. Data were analysed using Cellquest (Becton Dickinson) or WinMDI, Joseph Trotter, Salk Institute for Biological Studies, La Jolla, CA) software. Populations were confirmed by gating, sorting and subsequent analysis by epifluorescence microscopy (Gunasekera et al., 2000). Direct cell counts were carried out using a counting chamber and bright-field microscopy as described previously (Cruickshank et al., 1975). Bacterial suspensions were serially diluted and inoculated to obtain known numbers of bacteria in the range of 10^6–10^2 in the milk samples. Statistical analysis of

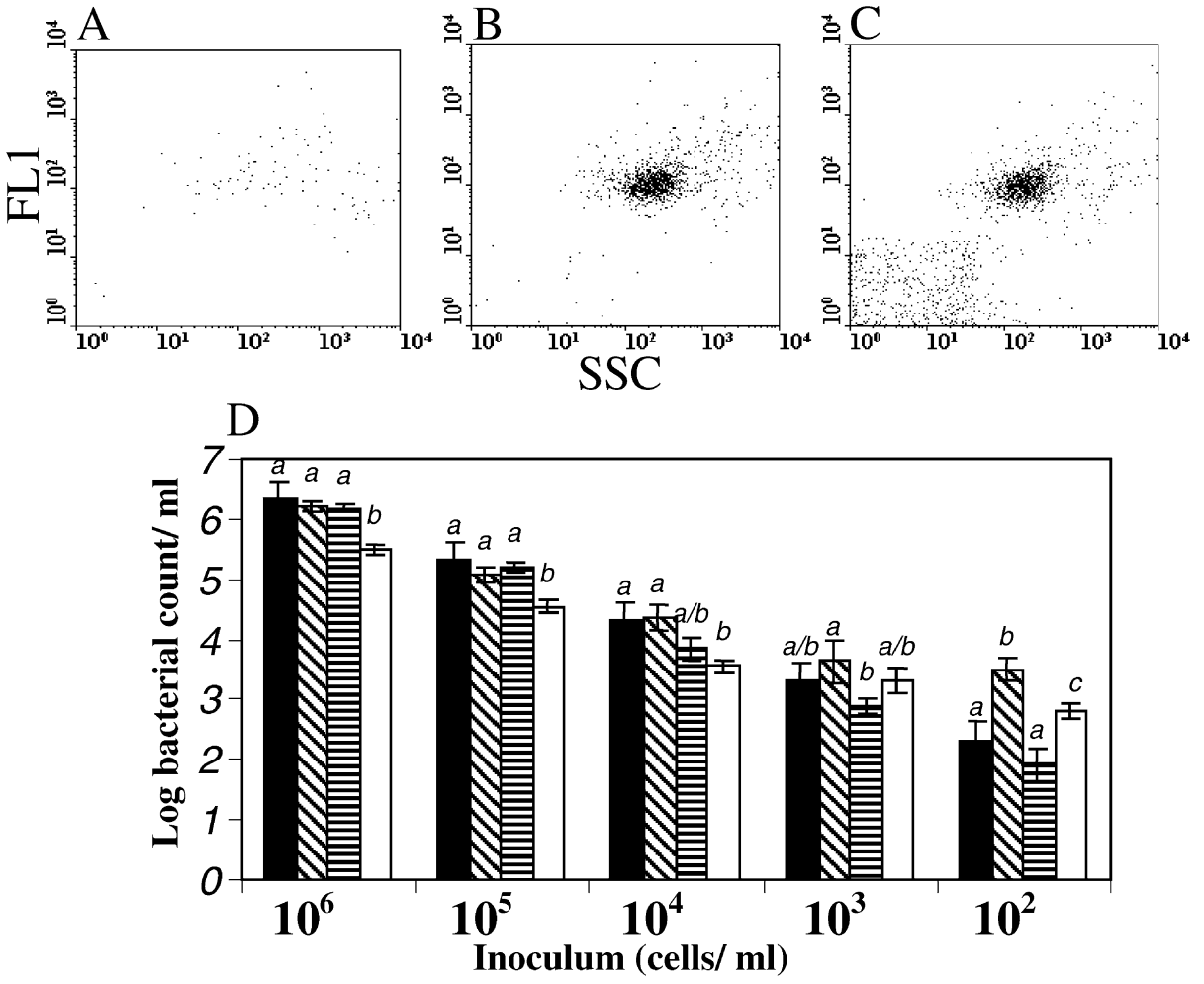

Fig. 1. Detection of bacteria in milk by fluorescent PNA probing and flow cytometry. A fluoresceinated PNA probe complementary to a highly conserved sequence encoding eubacterial 16S rRNA was used to tag *E. coli* cells. (A) UHT milk, cleared of lipids and protein micelles, with no added bacteria; (B) *E. coli* in phosphate-buffered saline; (C) *E. coli* in UHT milk cleared of lipids and protein micelles; (D) comparison of inoculated *E. coli* counts in UHT milk (bacteria inoculated into UHT milk after enumeration using direct microcopy), ■; nucleic acid staining by SYTO BC-FCM, ▧; standard plate counts, ▤; or PNA-FCM, □. Settings used on the FACSCalibur cytometer are shown in Table 1. FL1 refers to green fluorescence, SSC refers to side-angle (>15°) light scatter. Significant differences ($p<0.05$) between means are indicated by letters above histogram bars. Where the letters are the same, there is no significant difference between the means of the bacterial counting methods.

variance (ANOVA) and Tukey-HSD multiple range tests were used to detect significant differences among different bacterial detection and enumeration methods using the SPSS statistical software (SPSS for Windows Ver. 10).

3. Results

3.1. Fluorescent in situ hybridization for detecting bacteria in milk

In previous work, we developed an FCM-based method for enumerating total bacteria in milk samples using a nucleic acid binding stain SYTO BC (Gunasekera et al., 2000). The reported assay correlated well with standard plate count and total microscopic counts at $\geq 10^4$ cells/ml. Correlations were poorer at lower cell densities due mostly to the nonspecific binding of SYTO BC to protein particles remaining in cleared milk. FISH is a technique employing fluorescent-labeled oligonucleotides that bind with high specificity to homologous-defined nucleic acid sequences (Delong et al., 1989; Amman et al., 1995). FISH potentially offers a more stringent means of distinguishing bacteria from nonbacterial particles in milk. Therefore, FISH methods employing fluor-tagged oligo-

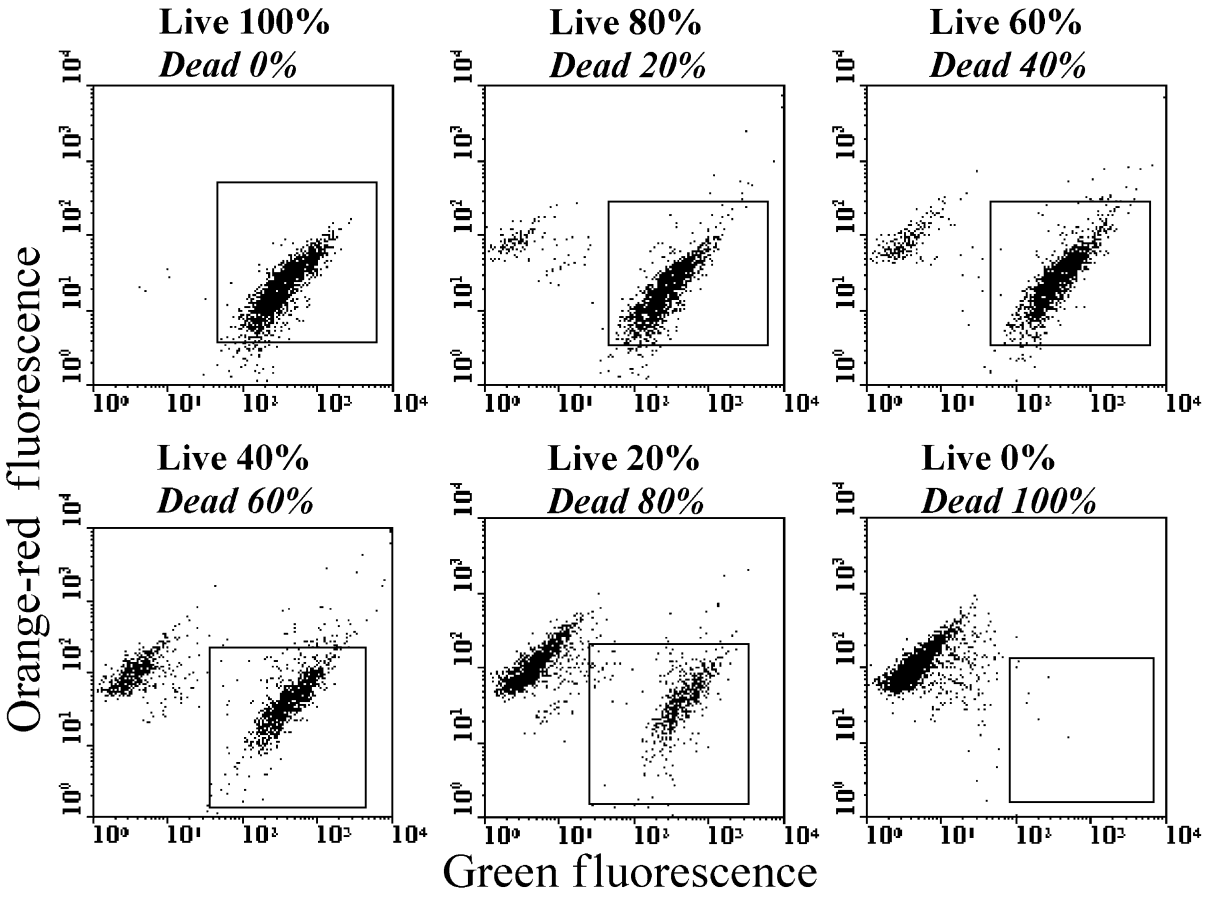

Fig. 2. Detection of live *S. aureus* in milk using flow cytometry. UHT milk samples were inoculated with live and heat-killed (70 °C for 15 min) *S. aureus* in different ratios. Milk was cleared of lipids and proteins and subsequently stained with SYTO 9 (green fluorescence) and PI (dead cell stain giving orange/red fluorescence). FACSCalibur cytometer settings were as detailed in Table 1. Boxed regions define the live bacterial populations.

nucleotides were compared with the previously reported SYTO BC staining method (Gunasekera et al., 2000). UHT milk was inoculated with *E. coli* cells and then cleared of protein and lipids. Fig. 1A–C shows that populations of *E. coli* cells added to milk, which was then cleared of fat and protein micelles, can be fluorescent tagged using a peptide nucleic acid (PNA) probe with an oligonucleotide sequence for eubacteria and clearly defined by FCM. The definition of the population of bacteria by cytometry was greater with the PNA probe than with the DNA oligonucleotide probe (data not shown).

The accuracy of the fluor-PNA-based technique relative to number of bacteria inoculated into milk by direct microscopic counting, standard plate counts and to the previously described SYTO BC-FCM method was tested by comparing bacterial counts obtained by the three methods. At bacterial counts $\geq 10^4$ cells/ml, the SYTO BC method was in close agreement with standard microscopy and plate counts (Fig. 1D), which is in agreement with previously published data (Gunasekera et al., 2000). The FISH method significantly underestimated bacterial counts relative to the other methods when bacteria were at densities above 10^4 cells/ml of milk. At 10^3 cells/ml, the data given by the FISH method were in reasonable agreement with standard microscopy and plate count data. However, at the lowest cell number tested, the FISH method significantly overestimated cell count, but it was closer to microscopy and plate counts than the SYTO BC staining method (Fig. 1D). Total time for the FISH assay procedure was approximately 3 h, which compares with 45–60 min for the SYTO BC stain procedure (Gunasekera et al., 2000).

3.2. Analysis of bacterial viability in milk

Microbial spoilage of dairy products is dependent upon the loading of viable bacteria in milk. We therefore developed a rapid assay for analysis of the viability of bacterial cells in milk. The assay of

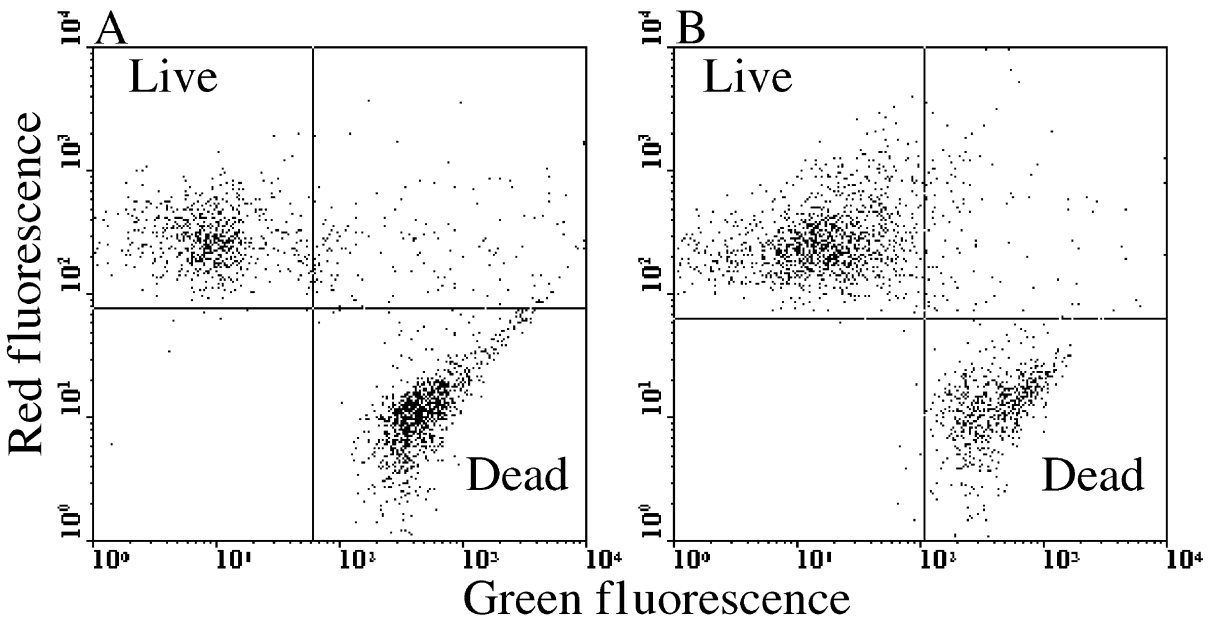

Fig. 3. Detection of live *P. fluorescens* in milk using flow cytometry. Mixtures (1:1) of live and heat-killed (70 °C for 10 min) bacteria were suspended in (A) phosphate-buffered saline, or (B) UHT milk. Milk was cleared of lipids and proteins prior to staining with CTC (5-cyanol-2,3-ditolyl tetrazolium chloride), which indicates cells with active dehydrogenases (red fluorescence), and oxonol, which indicates cells with compromised membrane potential (green fluorescence). FACSCalibur cytometer settings were as given in Table 1.

viability depends upon the ability to detect cellular functions or activities. Mixtures of live and heat-killed *S. aureus* were inoculated into UHT milk. The milk samples were then cleared of fat and protein micelles and dual stained with SYTO 9, which stains live and dead cells, and PI, which enters cells only if they have compromised membrane permeability. FCM analysis, with instrument settings as indicated in Table 1, showed two clear subpopulations of cells. Live cells stained green fluorescent with SYTO 9, whereas dead cells stained orange-red fluorescent with PI. The relative numbers of cells detected within these subpopulations related directly to the ratios of live and heat-killed cells inoculated into the UHT milk (Fig. 2). Similar data were obtained when *E. coli* was used as the inoculum (data not shown). Assay time was 40 min.

An alternative assay of viability involved the use of CTC, which enters cells and is reduced to form formazan crystals that fluoresce red. As a counterstain for dead cells, we used OXN, which concentrates in cells to give green fluorescence only when membrane potential has been compromised or lost. Fig. 3 indicates that two subpopulations of cells were clearly distinguished by FCM when UHT milk, into which live and dead cells of *P. fluorescens* were inoculated, was cleared and dual stained with the CTC and OXN. Assay time was 40 min.

3.3. Determination of Gram-positive and -negative bacteria in milk

As a preliminary test for the ability of FCM to distinguish between the types of bacteria present in milk samples, we investigated the ability to carry out Gram differentiation by FCM. The Gram Stain kit used relies on selective uptake of red fluorescent hexidium iodide into Gram-positive cells, but its exclusion by healthy Gram-negative bacteria that are counterstained with green fluorescent SYTO 9 (Haugland, 1996). FCM analysis showed two distinct populations of inoculated bacterial cells in cleared UHT milk, with *S. aureus* (Gram-positive cocci) appearing as red fluorescent cells and *P. fluorescens* (Gram-negative rods) as green fluorescent cells (Fig. 4). The time taken for the assay was 40 min.

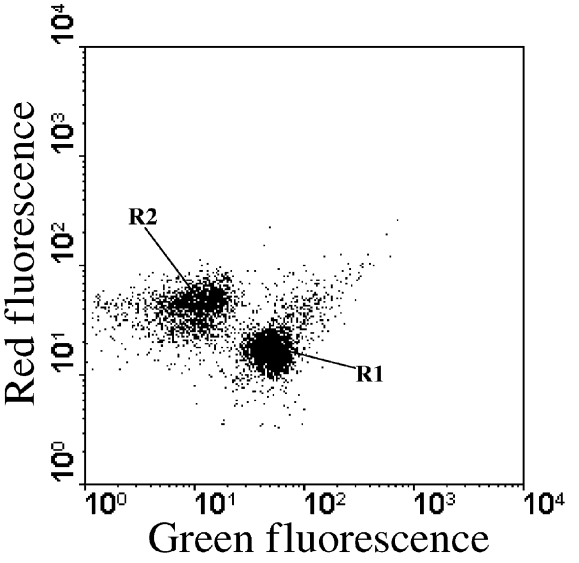

Fig. 4. Flow-cytometric analysis of bacterial Gram reaction in milk. *P. fluorescens* (Gram-negative; R1) and *S. aureus* (Gram-positive; R2) cells were inoculated into UHT milk. Milk was cleared and stained with hexidium iodide (red fluorescence) and SYTO 9 (green fluorescence).

3.4. Detection and enumeration of somatic cells in milk

Somatic cell analysis is important with regards to raw milk quality and identification of potential dairy cow infections, because high somatic cell counts can result from, for example, mastitis (Heeschen and Reichmuth, 1995). To determine the feasibility of using FCM for somatic cell enumeration, we first tested UHT milk inoculated with mouse hybridoma cells. Fig. 5A–C shows that populations of somatic cells were distinguished from milk proteins using a combination of FCM and fluorescent staining with AO. There was no significant difference ($p = 0.05$) between direct microscopy and flow cytometry methods for counting mouse cells in UHT milk across the range of 10^3–10^6 cells/ml (data not shown). There was a good correlation between the FCM method and standard direct microscopic counts for somatic cells in raw milk samples (that had not been inoculated with mouse cells) ($r = 0.88$, $n = 30$) across concentrations between 10^3 and 10^6 cells/ml (Fig. 5D).

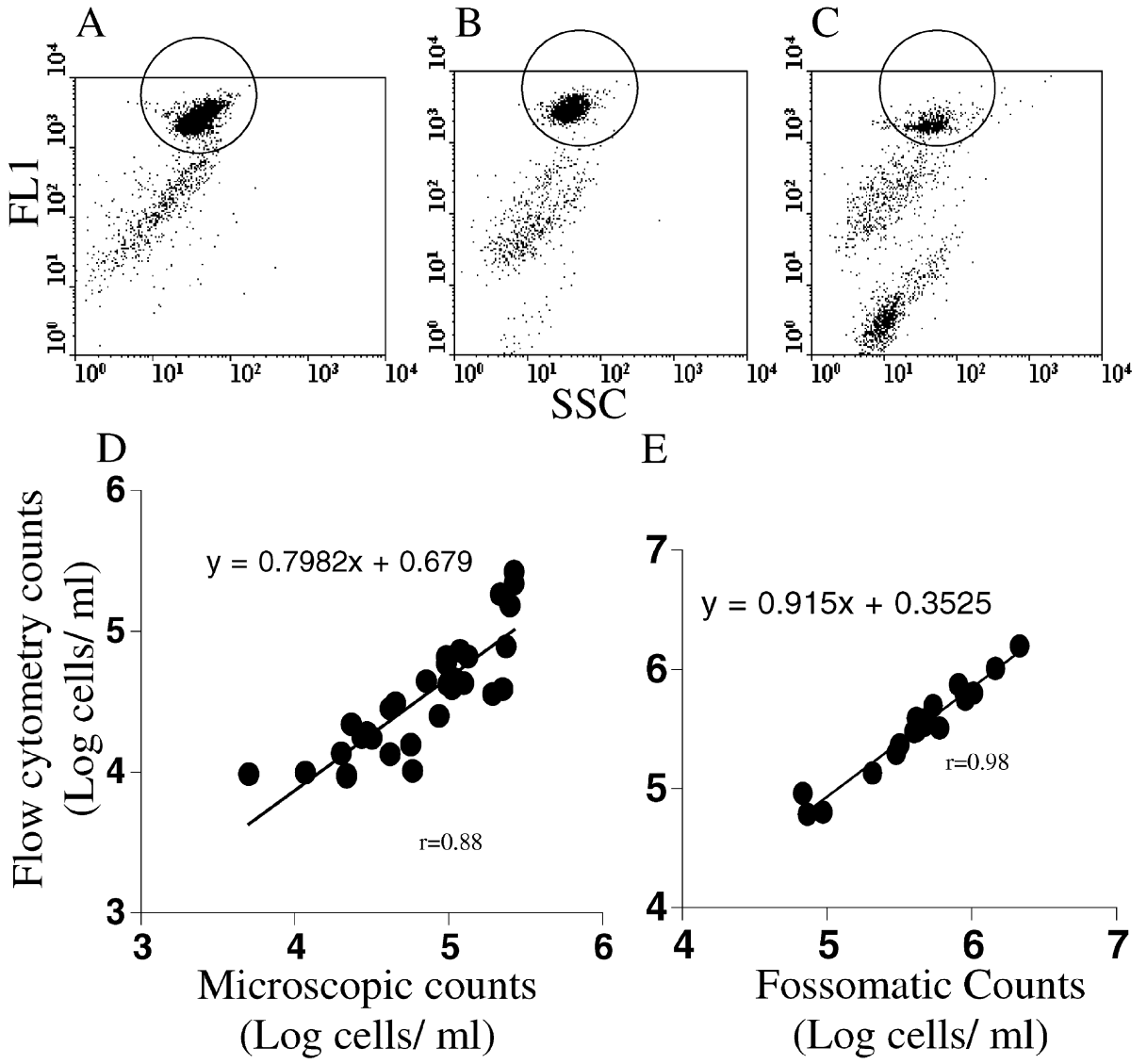

Fig. 5. Detection of somatic cells in milk by flow cytometry. Mouse hybridoma cells were inoculated into (A) phosphate-buffered saline, (B) UHT milk and (C) raw milk. Samples were cleared of lipids then stained with acridine orange. Cells highlighted by a circle are somatic cells. Settings used for analysis by the FACSCalibur cytometer are given in Table 1. (D) Correlation between somatic cell counts in raw milk samples (not inoculated with mouse cells) by direct microscopy and the acridine orange/flow-cytometric method. (E) Correlation between somatic cell counts in raw milk samples by Fossomatic instrument and flow cytometry methods. Assays of individual samples were performed in triplicate. FL1 refers to green fluorescence, SSC refers to side-angle (>15°) light scatter.

The cell count measured by FCM showed good agreement ($r = 0.98$) with the cell count data obtained using the Foss instrument (Fig. 5E). The total time for the somatic cell assay procedure was 15 min, and 24 samples could be processed simultaneously in 30 min.

4. Discussion

The purpose of this study was to demonstrate the principle that FCM coupled with fluorescence techniques can be applied to a variety of biological assays of milk. The proof of principle that numerous biological

assays can be performed using FCM is important if this type of instrumentation is to be utilised by the dairy and wider food industry. Based on the data, we conclude that FCM and fluorescence-labeling methods offer considerable potential for applications in aspects of dairy plant management, milk safety and quality testing. Most of the methods reported here can be carried out within 1 h using a single instrument. This time frame compares well with the microscopic, biochemical and culture-based methods currently in use. The FISH method takes 3 h, which is considerably more rapid than culture methodology. Fluorescent techniques in conjunction with FCM measure various physiological characteristics of individual cells (Davey and Kell, 1996). Although numerous viability probes have been discussed in the literature, direct application of these probes to analysis of viable bacteria in milk is limited due to presence of protein and fat particles in milk (Gunasekera et al., 2000). Therefore, removing milk particles without affecting bacterial viability is crucial for subsequent application of fluorescent probes to determine cell activity. This work demonstrates that the milk clearing method we have used, which takes <1 h (Gunasekera et al., 2000), does not interfere with the viability of the bacteria. Furthermore, even though some bacterial cells may not be culturable, they still can have biological function. We have shown previously that some bacterial cells can express protein even though they are nonculturable following pasteurisation of milk (Gunasekera et al., 2002). Therefore, regardless of culturability, the ability to determine whether bacteria are metabolically active by various methods such as PI, OXN, CTC staining, or rDNA probe is relevant to quality management. Moreover, developed assays using viability probes and FCM technology can be used in the dairy industry in controlling pasteurisation treatments in dairy plants or assessing viability and predicting the fermentation performance of starter cultures.

There are some specific limitations associated with the assays reported here. Intact Gram-negative bacteria exclude hexidium iodide, but the dye accumulates in damaged Gram-negative cells. Thus, the current fluorescent Gram stain is limited to the distinction of live cells in raw milk prior to any heating or other treatment that might cause damage to Gram-negative bacteria and result in a falsely high estimate of Gram-positive cells. Clearly, alternative Gram-specific dyes that are not affected by bacterial viability or vitality would be useful. With regards to total bacterial counts, we have demonstrated the accuracy of two different FCM-based methods for bacterial counts at levels between 10^6 and 10^2 cells/ml of milk. At bacterial levels above 10^4 cells/ml, which is a cut-off point for definition of good quality milk (Hubble, 1997), the SYTO BC nucleic acid staining procedure is sufficiently accurate. However, where greater sensitivity is required at bacterial densities below 10^4 cells/ml, we would recommend the demonstrated FISH-based FCM assay, since this gave the closest agreement with standard plate count and direct microscopic counting methods used traditionally in the dairy industry. Here, we have demonstrated the applicability of FISH to the microbiological analysis of milk by using a 16S rRNA-specific probe specific to eubacteria. A PNA probe was more suitable for analysis of milk than the equivalent DNA sequence, giving clearer definition of bacterial populations. This is not surprising, since PNA probes are known to hybridize more stably and efficiently than DNA probes (Ray and Nordén, 2000). In this regard, Matte-Tailliez et al. (2001) demonstrated the use of PNA probes for detection and identification of thermophilic lactobacilli. Using FISH technology and FCM methods, we have also demonstrated specific detection and enumeration of *Pseudomonas* spp. in milk (Gunasekera et al., unpublished). Therefore, FCM in conjunction with FISH can be easily adapted for detection of important spoilage microorganisms or specific pathogens related to the dairy industry.

Flow cytometry and appropriate fluorescence methods can play an important role in biological safety and quality issues of dairy and other food and beverage industries (Attfield et al., 1999; Veal et al., 2000; Boyd et al., 2002). However, uptake of the technology by industry will depend upon making it cost effective to industry sectors. The ability to carry out multiple types of assays will help in this regard. It is apparent that total bacterial and somatic cell counts can be carried out with a sensitivity that should be able to satisfy legislative needs (Suhren and Walte, 1998; Gunasekera et al., 2000; this study). Whilst we and others have demonstrated broader potential for experimental microbiological assays of milk using flow cytometry and fluorescence techniques, there

remains a need to demonstrate this in practice. The purpose of this current work was to demonstrate varied possibilities of flow-cytometric analyses of milk. Future work will involve analysis of raw and processed milk samples for natural bacterial flora under conditions more relevant to industrial situations. Moreover, issues of skills required by dairy plant operatives and costs of assay materials such as dyes and PNA probes will also impact on the economics of FCM-based techniques and require evaluation in dairy plants or other food manufacturing/testing environments. We have found that qualified microbiologists can be adequately trained in the use of flow cytometers such as the FACSCalibur and that specialist flow cytometrists are not required for the types of assays we have described. The potential that FCM has to provide multiple, same-day results should provide impetus for this technology to be more fully evaluated for applications in food and beverage microbiology.

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References

Amman, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews 59, 143–169.

Attfield, P.V., Gunasekera, T., Boyd, A., Deere, D., Veal, D.A., 1999. Applications of flow cytometry to microbiology of food and beverage industries. Australasian Biotechnology 9, 159–166.

Attfield, P.V., Kletsas, S., Veal, D.A., van Rooijen, R., Bell, P.J.L., 2000. Use of flow cytometry to monitor cell damage and predict fermentation activity of dried yeasts. Journal of Applied Microbiology 89, 207–214.

Boyd, A.R., Gunasekera, T.S., Attfield, P.V., Simic, K., Vincent, S.F., Veal, D.A., 2002. A flow-cytometric method for determination of yeast viability and cell number in a brewery. FEMS Yeast Research (in press).

Clarke, R.G., Pinder, A.C., 1998. Improved detection of bacteria by flow cytometry using a combination of antibody and viability markers. Journal of Applied Microbiology 84, 577–584.

Cruickshank, R., Duguid, J.P., Marmion, B.P., Swain, R.H.A., 1975. Medical microbiology, 12th ed. The Practice of Medical Microbiology, vol. 2. Churchill Livingstone, Edinburgh, United Kingdom, p. 306.

Davey, H.M., Kell, D.B., 1996. Flow cytometry and cell sorting of heterogenous microbial populations: the importance of single cell analyses. Microbiological Review 60, 641–696.

Deere, D., Shen, J., Vesey, G., Bell, P., Bissinger, P., Veal, D., 1998. Flow cytometry and cell sorting for yeast viability assessment and selection. Yeast 14, 147–160.

Delong, E.F., Wickham, G.S., Pace, N.R., 1989. Phylogenetic stains—ribosomal RNA-based probes for the identification of single cells. Science 243, 1360–1363.

Gunasekera, T.S., Attfield, P.V., Veal, D.A., 2000. A flow cytometry method for rapid detection and enumeration of total bacteria in milk. Applied and Environmental Microbiology 66, 1228–1232.

Gunasekera, T.S., Sörensen, A., Attfield, P.V., Sörensen, S., Veal, D.A., 2002. Inducible gene expression by non-culturable bacteria in milk after pasteurisation. Applied and Environmental Microbiology 68, 1987–1993.

Haugland, R., 1996. Handbook of Fluorescent Probes and Research Chemicals. Molecular Probes, Oregon.

Heeschen, W.H., Reichmuth, J., 1995. Mastitis—the disease under aspects of milk quality and hygiene. Kiel Milchwirtschaftliche Forschungsberichte 47, 221–237.

Hubble, I.B., 1997. Testing and reporting of raw milk quality. Australian Journal of Dairy Technology 52, 102–108.

Matte-Tailliez, O., Quenee, P., Cibik, R., van Opstal, J., Dessevere, F., Firmesse, O., Tailliez, P., 2001. Detection and identification of lactic acid bacteria in milk and industrial starter culture with fluorescently labeled rRNA-targeted peptide nucleic acid probes. Lait 81, 237–248.

Muir, D.D., 1996. Shelf-life of dairy products: 2. Raw milk and fresh products. Journal of the Society of Dairy Technology 49, 44–48.

Pinder, A.C., McClelland, R.G., 1994. Rapid assay of pathogenic *Salmonella* organisms by immunofluorescence flow cytometry. Journal of Microscopy 176, 17–22.

Ray, A., Nordén, B., 2000. Peptide nucleic acid (PNA): its medical and biotechnological applications and promise for the future. FASEB Journal 14, 1041–1060.

Richardson, B.C., 1981. The purification and characterisation of a heat-stable protease from *Pseudomonas fluorescens* B52. New Zealand Journal of Dairy Science and Technology 16, 195–207.

Sørhaug, T., Stepaniak, L., 1997. Psychrotrophs and their enzymes in milk and dairy products quality aspects. Trends in Food Science and Technology 8, 35–41.

Suhren, G., Walte, H.-G., 1998. First experiences with automatic flow cytometric determination of total bacterial count in

raw milk. Kiel Milchwirtschaftliche Forschungsberichte 50, 249–275.

Vasavada, P.C., 1993. Rapid methods and automation in dairy microbiology. Journal of Dairy Science 76, 3101–3113.

Veal, D.A., Deere, D., Ferrari, B., Piper, J., Attfield, P.V., 2000. Fluorescence staining and flow cytometry for monitoring microbial cells. Journal of Immunological Methods 243, 191–210.

Wallner, G., Amann, R., Beisker, W., 1993. Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry 14, 136–143.

Zeng, S.S., Escobar, E.N., Popham, T., 1997. Daily variations in somatic cell count, composition, and production of alpine goat milk. Small Ruminant Research 26, 253–260.