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Development of a Flow Cytometric Method To Analyze Subpopulations of Bacteria in Probiotic Products and Dairy Starters

Christine J. Bunthof* and Tjakko Abee

Laboratory of Food Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, 6700 EV Wageningen, The Netherlands

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Flow cytometry (FCM) is a rapid and sensitive technique that can determine cell numbers and measure various physiological characteristics of individual cells by using appropriate fluorescent probes. Previously, we developed an FCM assay with the viability probes carboxyfluorescein diacetate (cFDA) and TOTO-1 {1′-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3dihydro(benzo-1,3-oxazole)-2-methylidene]-1-(3′-trimethylammoniumpropyl)-pyridinium tetraiodide} for (stressed) lactic acid bacteria (C. J. Bunthof, K. Bloemen, P. Breeuwer, F. M. Rombouts, and T. Abee, Appl. Environ. Microbiol. 67:2326-2335, 2001). cFDA stains intact cells with enzymatic activity, and TOTO-1 stains membrane-permeabilized cells. Here we used this assay to study the viability of bacterial suspensions in milk, dairy fermentation starters, and probiotic products. To facilitate FCM analysis of bacteria in milk, a commercially available milk-clearing solution was used. The procedure was optimized to increase the signal-to-noise ratio. FCM enumerations were accurate down to a concentration of 10^5 cells ml^{-1}. The level of retrieval of *Lactobacillus plantarum* WCFS 1 suspended in milk was high, and viability was not affected by the procedure. The plate counts for cleared samples of untreated cell suspensions were nearly as high as the total FCM counts, and the correlation was strong ($r > 0.99$). In dairy fermentation starters and in probiotic products the FCM total cell counts were substantially higher than the numbers of CFU. Three functional populations could be distinguished: culturable cells, cells that are intact and metabolically active but not culturable, and permeabilized cells. The proportions of the populations differed in the products tested. This FCM method provides tools to assess the functionality of different populations in fermentation starters and probiotic products.

In the food industry lactic acid bacteria (LAB) are used for production of fermented foods, such as cheese, yogurt, wine, and fermented meat products. Starter culture strains are selected for their fermentation capacities and their flavor formation characteristics. Other important criteria are the robustness of the cells during processing and the maintenance of different strains in a mixed starter (5). LAB are also important as probiotics. Probiotics are live microbial food ingredients that are beneficial to health. Some important issues in research on and development of probiotics are growth and survival during production, shelf life, resistance to bile salts and acids, and adherence and survival in the gut (11, 14, 27). Obviously, enumeration and assessment of viability are crucial in research on and selection of fermentation starter bacteria and probiotics.

The method used most frequently for assessing the viability of microorganisms is the plate count technique. Plate counting requires an incubation time of one or more days before the results can be scored, which is a disadvantage. Also, bacteria may occur in chains and clumps, resulting in underestimation of the bacterial number. In addition, cell injury and dormancy may result in low viable counts (2, 18). Most importantly, the plate count method detects only the bacteria able to form colonies under the given circumstances on the medium that is used; it does not give a clue about the presence of bacteria that do not form colonies but are nevertheless metabolically active. Active but nonculturable starter cells might well contribute to fermentation. It has been established that when starter cultures are subjected to various forms of sublethal stress, some bacteria may be injured and fail to grow on a medium adequate for growth of unstressed cells but they are capable of growth when they are given a suitable environment (21, 33). Furthermore, it has been shown that permeabilization may lead to a higher acid production rate (21). In addition, many possible probiotic effects of bacteria depend on activity rather than culturability, and even dead cells can have some probiotic effect, such as immunomodulation (22, 25). Therefore, obtaining information about all individual bacteria and their physiological status is relevant. The aim of this work was to study bacteria in milk suspensions, fermentation starters, and probiotic products by flow cytometry (FCM).

FCM is a rapid and sensitive technique that measures each cell individually. Fluorescent stains are used with FCM to detect cells and to analyze population heterogeneity (31). For total cell enumeration permeant DNA stains are often applied. Specific bacterial species may be detected with antibodies or rRNA probes labeled with a fluorescent dye. A number of viability and metabolic activity probes are available to analyze physiological characteristics, such as membrane integrity, enzyme activities, membrane potential, intracellular pH, respiration, and antibiotic susceptibility (1, 4, 8, 16).

FCM has been used widely as a tool to investigate bacteria in laboratory cultures. Also, FCM has been used for analysis of bacteria in environmental samples, such as soil, air, and especially water (31, 32, 37), and in clinical samples, such as blood, urine, and feces (1, 31, 36). The potential of FCM for the food

* Corresponding author. Mailing address: Laboratory of Food Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands. Phone: 31 (317) 484983. Fax 31 (317) 484893. E-mail: Christine.Bunthof@micro.fdsci.wag-ur.nl.

industry has been recognized as well (19, 32, 35). Some applications have been developed for FCM analyses of food products, such as meat, fruit juices, eggs, and milk. When milk was considered, most attention was paid to detection of specific pathogens and to bacterial quality control. Detection of the pathogen *Listeria monocytogenes* in raw milk was accomplished by using enrichment in broth and immunofluorescence staining (10). A recently described approach for specific detection is the use of immunomagnetic separation and FCM, which was used for detection of *Escherichia coli* O157:H7 (29). For direct enumeration in milk, staining with ethidium bromide and mithramycin was tried; however, the sensitivity was too low to detect 6×10^7 CFU of added *E. coli* per ml because of high background signals (24). The sensitivity was increased by a sample-clearing procedure using Promega's milk-clearing reagents, which was used for immunofluorescence detection of *Salmonella enterica* serovar Typhimurium (20). Alternatively, enzymatic clearing with proteinase and savinase (for ultra-heat-treated [UHT] milk) or with savinase and Triton X-100 (for raw milk) was developed (13). This method was used to enumerate total bacteria in milk from a dairy plant with the fluorescent probe SYTO BC (Molecular Probes). For routine analyses of milk quality, an automated FCM instrument, the Bactoscan-FC (Foss Electric, Hiller/od, Denmark), was developed (3, 34). This automated instrument uses ethidium bromide for staining, and the disturbing milk components are reduced and dispersed by treatment with detergent and enzyme at 50°C (34).

FCM can also be used for analyses of viability and metabolic activity in food. This is particularly relevant for fermentation starter bacteria and probiotics. Previously, we used carboxyfluorescein diacetate (cFDA) and TOTO-1 {1′-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3dihydro (benzo-1,3-oxazole)-2-methylidene]-1-(3′-trimethylammoniumpropyl)-pyridinium tetraiodide} for FCM assessment of the viability of various LAB species (6). cFDA is a nonfluorescent esterase substrate that enters the cell by passive diffusion. Once the compound is inside, the acetate groups are split off by enzymatic hydrolysis, releasing the green fluorescent compound carboxyfluorescein (cF). cFDA is commonly used as a live stain (4, 8, 31). TOTO-1 is a nucleic acid dye that is excluded by intact cells and thus stains a cell only when the membrane is damaged. In another study we used the permeant and impermeant DNA stains SYTO 9 and propidium iodide of a *Bac*Light LIVE/DEAD kit (Molecular Probes, Inc., Eugene, Oreg.) for analysis of permeabilization and lysis of *Lactococcus lactis* under simulated cheese ripening conditions by FCM (7).

In this study we optimized a milk-clearing procedure for FCM analysis of bacteria in milk and investigated the viability of bacteria in dairy starters and probiotic products. Staining with cFDA and TOTO-1 revealed three populations: culturable cells, cells that are intact and metabolically active but not culturable, and permeabilized cells. The proportions of the populations in the products tested differed. This FCM method allows for determination of the functionality of probiotic subpopulations, and possible applications are discussed below.

MATERIALS AND METHODS

Bacterial strain and sample preparation. The test strain used in this study was *Lactobacillus plantarum* WCFS 1, which was obtained from the Wageningen Centre for Food Sciences, Wageningen, The Netherlands. Cells were cultured in MRS broth (Merck, Darmstadt, Germany) at 37°C and harvested in the mid-exponential growth phase at an optical density at 600 nm of 1.0 by centrifugation at 13,000 rpm for 2 min at 20°C (Biofuge Fresco Eppendorf centrifuge; Heraeus Instruments, Ostrode, Germany). This culture contained approximately 5×10^8 CFU/ml. The buffer that was used throughout the experiments was 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) with 50 mM KCl adjusted to pH 6.5 and filtered by using a 0.2-μm-pore-size filter. Cells were washed twice with buffer and resuspended to an optical density at 600 nm of 1.0. To prepare a cell suspension in milk, the cells from a buffer suspension were spun down and then resuspended and diluted in semiskimmed pasteurized milk. To permeabilize cell membranes and cause cell death, suspensions were heated at 70°C for 10 min.

Extraction procedure. A milk-clearing solution (Promega, Leiden, The Netherlands) was used for extraction of bacteria and reduction of milk particles. This solution is commercially available as Reagents A of the Enliten milk assay kit for total viable organisms by ATP measurement. It contains a nonionic detergent, a chelating agent, and a microparticulate carrier (polystyrene beads with diameters between 0.5 and 1.5 μm) (23). McClelland and Pinder (20) have shown that this clearing solution causes flocculation and coalescence of the micelles in milk. This increases the micelle size, and therefore the fat moves readily to the surface of the milk during centrifugation. The clearing solution also lyses the somatic cells present in milk (23), which are mainly mammalian cells that are transferred from the cow's udder into the milk during milking. Somatic cells were indeed observed in milk by fluorescence microscopy before clearing, but they were eliminated by the clearing procedure. The polystyrene beads sediment slightly slower than microbial cells during centrifugation and serve as a visual indicator, facilitating removal of the supernatant. FCM indicated that the polystyrene beads were uniform in size and that the concentration in the clearing solution was approximately 10^9 beads ml^{-1}.

At first, the extraction procedure recommended by the manufacturer was tried. In this procedure a 1-ml sample of a bacterial suspension in milk was transferred to a 1.5-ml Eppendorf tube, and 0.5 ml of the clearing solution was added. The tube was inverted 10 times for mixing, and this was immediately followed by centrifugation at 13,000 rpm at 20°C for 5 min. The result was a cell pellet covered with polystyrene beads at the bottom of the tube, a clear supernatant, and a cream pad on top. The cream and supernatant were removed with an aspirator, and the pellet was resuspended in MES buffer. This extraction procedure was optimized by adding two steps. First, since the polystyrene beads interfered with the FCM analyses, the beads were removed from the clearing solution before use by filtration with a 0.2-μm-pore-size filter. Second, since with the standard procedure some cream remained on the wall of the tube after aspiration, which resulted in higher background counts, an extra step was included. After aspiration of the supernatant and most of the cream, the remaining cream was removed with a cotton-tipped stick.

Fluorescent staining. The fluorescent stains used were cFDA, TOTO-1, and SYTO 9 (Molecular Probes Inc.). cFDA is an esterase substrate that yields the fluorescent compound cF upon hydrolysis by cellular esterases. cF is retained in cells with intact membranes and stains the cells fluorescent green. cFDA staining was done by incubation with 50 μM cFDA at 30°C for 10 min. TOTO-1 is a membrane-impermeant nucleic acid stain with high fluorescence enhancement that stains membrane-compromised cells yellow-green. Double staining with cFDA and TOTO-1 was done by incubation with 50 μM cFDA and 1 μM TOTO-1 at 30°C for 10 min. SYTO 9 is a membrane-permeant nucleic acid stain which was used for total cell enumeration with *L. plantarum* samples. This probe stains cells fluorescent green and is part of the LIVE/DEAD *Bac*Light kit. SYTO 9 staining was done by incubation of samples with 6.5 μM SYTO 9 at room temperature for 10 min.

Fluorescence microscopy. Microscope slides were analyzed by using an Axioskop epifluorescence microscope equipped with a 12-V, 50-W halogen lamp for transmitted light illumination, a 50-W mercury arc lamp for epifluorescence illumination, a fluorescein isothiocyanate filter set (excitation wavelength, 450 to 490 nm; emission wavelength, >520 nm), and a ×100 1.3-numerical-aperture Plan-Neofluar objective lens. Fluorescence microscopy allowed direct observation of the morphology of cells, the presence of chains and clumps, the labeling of cells, and the presence and labeling of background particles. The effect of the extraction procedure on the cells was evaluated by comparing labeled samples before extraction with labeled samples after extraction.

FCM. FCM analyses were performed with a FACSCalibur flow cytometer and data analysis software as described previously (6). Forward scatter (FSC), side scatter (SSC), and three fluorescence signals were measured. A 530-nm band pass filter (515 to 545 nm) was used to collect the green fluorescence (FL1), a 585-nm band pass filter (564 to 606 nm) was used to collect the yellow-orange fluorescence (FL2), and a 670-nm long pass filter was used to collect the red

fluorescence (FL3). Fluoresbrite PC Red polystyrene latex microspheres (PC Red beads) with a diameter of 1.75 μm (Polyscience Europe GmbH, Eppelheim, Germany) were included in the FCM samples for enumeration of cells. The stock suspension (8.547×10^9 microspheres per ml) was sonicated and vortexed prior to use. In the evaluation of the extraction procedure and in the experiment performed with mixtures of nontreated and heat-treated milk with *L. plantarum*, the FCM analyses were performed with the following detector settings: FSC, E01; SSC, 400; FL1, 450; FL2, 450; and FL3, 600 using logarithmic gains. The threshold was set at an FSC signal of 250. In the experiments with *L. plantarum* in buffer and in milk at concentrations of 10^5 to 10^9 cells ml^{-1}, the FL1 and FL2 detector settings were adjusted to between 400 and 600, depending on the labeling of the sample. FCM samples were adjusted to an event rate of 200 to 700 per s at the low flow rate (approximately 10 μl min^{-1}). When possible, FCM samples that contained approximately 10^6 cells per ml were prepared, and 10,000 to 20,000 cells were measured during 2 min of data acquisition. For FCM samples prepared from milk containing less than 10^7 cells per ml, longer data acquisition times were used (up to 10 min), and lower numbers of cells were measured; however, at least 3,000 were measured in all cases.

Data analysis. The bead and cell populations were identified by using dot plots (i.e., bivariate displays in which each dot represented one measured event). The bead population was easily identified by both scatter and fluorescence signals. Windows were defined in the dot plot of FSC and SSC and in the dot plot of green fluorescence and red fluorescence. For calculating the number of beads the red fluorescence histogram of the bead population was used. On average, 10% of the beads was present in the form of double beads, which gave rise to a small but distinct peak to the right of the main peak in the red fluorescence histogram.

In buffer samples the cells could easily be identified, and windows were defined in the dot plot of FSC and SSC and in the dot plot of green fluorescence and red fluorescence. These windows were also used for milk samples. The fluorescence windows were adjusted when the fluorescence signals of the cells from the milk samples were lower or higher than the signals in the buffer control sample. Cell counting was done by enumerating the events that were included both in the scatter window and in the appropriate labeled cell region. Cell concentrations were calculated by using the count for the PC Red beads in the FCM sample.

The background count was defined as the FCM count for a labeled sample without added bacteria or beads. This count included all events with FSC above the threshold level, which indicated the number of particles whose size was similar to that of cells or greater.

The accuracy of counts is indicated by the coefficient of variation (CV). In a count of n items, the associated standard deviation is $n^{[1/2]}$. The CV is the standard deviation divided by the mean. The CV is a common measure of precision (31)

Dairy starters. The FCM assay was used with three dairy starters: the cheese starter BOS and two yogurt starters, RR and ISt (CSK Food Enrichment, Leeuwarden, The Netherlands). BOS starter is used for production of Gouda cheese. It is a mixed culture containing mainly lactococci and some leuconostocs. After production, BOS starter is concentrated 40-fold, packed in plastic beakers, and stored at −40°C. This mother starter can be added directly to milk for cheese production. However, in cheese production on an industrial scale, low-fat milk is inoculated with the mother starter for production of a bulk starter. After incubation, the bulk culture is used for cheese fermentation. Therefore, we analyzed BOS starter directly (BOS mother starter) and after incubation (BOS bulk starter). Both yogurt starters, RR and ISt, are used commonly in The Netherlands. Both contain *Lactobacillus delbruecki* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*, but RR contains strains other than the strains contained in ISt. After production, these yogurt starters are concentrated 20-fold, packed in plastic beakers, and stored at temperatures below −40°C. In industrial yogurt production the starter is added directly to milk. Therefore, we analyzed the RR and ISt starters directly.

The starters were thawed in a 20°C water bath for 1 h. BOS bulk starter was produced by inoculation of low-fat milk with 0.0125% concentrated BOS mother starter and incubation at 20°C for 18 h. The concentrated starters were diluted to their original concentrations in semiskimmed milk. Samples were extracted with the optimized extraction procedure. Samples were diluted 10-fold because of the high number of cells. Then samples were incubated with 500 μM cFDA and 1 μM TOTO-1 at 30°C for 10 min. For easy total cell enumeration TOTO-1 staining of samples heat treated at 70°C was used. Labeling was evaluated by fluorescence microscopy. FCM analyses were performed with the following detector settings: FSC, E01; SSC, 400; FL1, 450; FL2,450; and FL3, 600 using logarithmic gains. The threshold was set at a SSC signal of 200. Before and after extraction samples were plated on tryptone soya agar (TSA).

Probiotic products. The FCM method was applied to three probiotic products: Orthiflorplus (Orthica B.V., Weesp, The Netherlands), Yakult (Yakult Europe B.V., Almere, The Netherlands), and Mona Vifit yogurt drink (Campina Melkunie, Woerden, The Netherlands). Yakult and Mona Vifit were purchased form a local supermarket, and Orthiflorplus was obtained from a local reform shop.

Orthiflorplus is a probiotic food supplement sold in sachets containing 3 g of powder that has to be suspended in water. According to the information leaflet, one sachet contains at least 3×10^9 "germs" consisting of approximately equal amounts of *Lactobacillus acidophilus*, *Lactobacillus casei*, *L. lactis*, *Enterococcus faecium*, and *Bifidobacterium bifidum*. Furthermore, it contains oligofructose and fibers. One sachet was suspended in 100 ml of tap water in a glass. After 10 min to allow the fibers to sink to the bottom, samples were taken from the almost clear liquid in the upper part of the glass. During this time span bacteria did not sink to the bottom, which was confirmed by plate counting; the number of CFU was as high as the number of CFU in samples taken immediately after stirring. FCM was performed immediately and after extraction of bacteria.

Yakult is a food supplement containing the probiotic *L. casei* Shirota; according to the supplier a 65-ml bottle contains 6.5×10^9 bacteria. Yakult is produced by fermentation of sterilized milk (made from low-fat milk powder) with dextrose. After fermentation the preparation is homogenized, and water and glucose syrup are added. Experiments were done on the day of purchase, 3 weeks before the best-used-before date. FCM was performed immediately and after extraction of bacteria.

Mona Vifit is a yogurt drink that contains three probiotic bacteria, *L. acidophilus*, *B. bifidum*, and *Lactobacillus rhamnosus* Goldin and Gorbach. The supplier recommends that a person drink at least a 200-ml portion every other day; this should contain approximately 4×10^9 *L. rhamnosus* Goldin and Gorbach cells. The amounts of the other bacteria are not mentioned on the package. Experiments were done on the day of purchase, 8 days before the best-used-before date. Samples were cleared prior to FCM analyses.

Fluorescent labeling, microscopy, and FCM analyses of the three probiotic products were done as described above for dairy starters. Before and after extraction samples were plated on TSA.

Plate counts. Data obtained by FCM were compared with plate counts. Samples were serially diluted in a peptone physiological salt solution, and 50-μl portions of the appropriate dilution (resulting in 50 to 300 colonies) were spiroplated by using an EddyJet (IUL Instruments, Barcelona, Spain). Samples of *L. plantarum* WCFS 1 were plated on MRS agar (Merck). MRS is a medium for lactobacilli (9, 15). The plates were incubated anaerobically at 37°C for 48 h. Samples of dairy starters and probiotic products were plated on TSA, a general nonspecific laboratory medium (Oxoid Ltd., Basingstoke, United Kingdom). The plates were incubated anaerobically at 30°C for 72 h to obtain total anaerobic mesophilic colony counts.

RESULTS

Extraction of bacteria from milk. *L. plantarum* WCFS 1 cells were harvested in the mid-exponential growth phase and suspended in semiskimmed pasteurized milk and in buffer (control) at a concentration of 5×10^6 cells ml^{-1}. Figure 1 shows dot plots obtained with SYTO 9 staining. A threshold for the FSC signal was used to include all particles that were similar in size to or larger than bacterial cells and to exclude smaller particles. The cell suspension in buffer was diluted fivefold in order to obtain an appropriate event rate, and the cell population was easily observed (Fig. 1A and B). However, the cell suspension in milk had to be diluted 10,000-fold in order to obtain an appropriate event rate, and thus the number of cells was too low to be detected. Milk particles gave a band in the dot plot of FSC and SSC signals that overlapped with the signal of bacterial cells (Fig. 1C). However, milk particles did not give much of a fluorescent signal (Fig. 1D). Milk contained more than 10^{10} particles with a FSC signal above the threshold level per ml (Table 1). Such a high particle density affects the detection system because there is a high chance of scattering and absorption of excitation and emission light by the particles. Furthermore, milk caused problems with the flow system. To

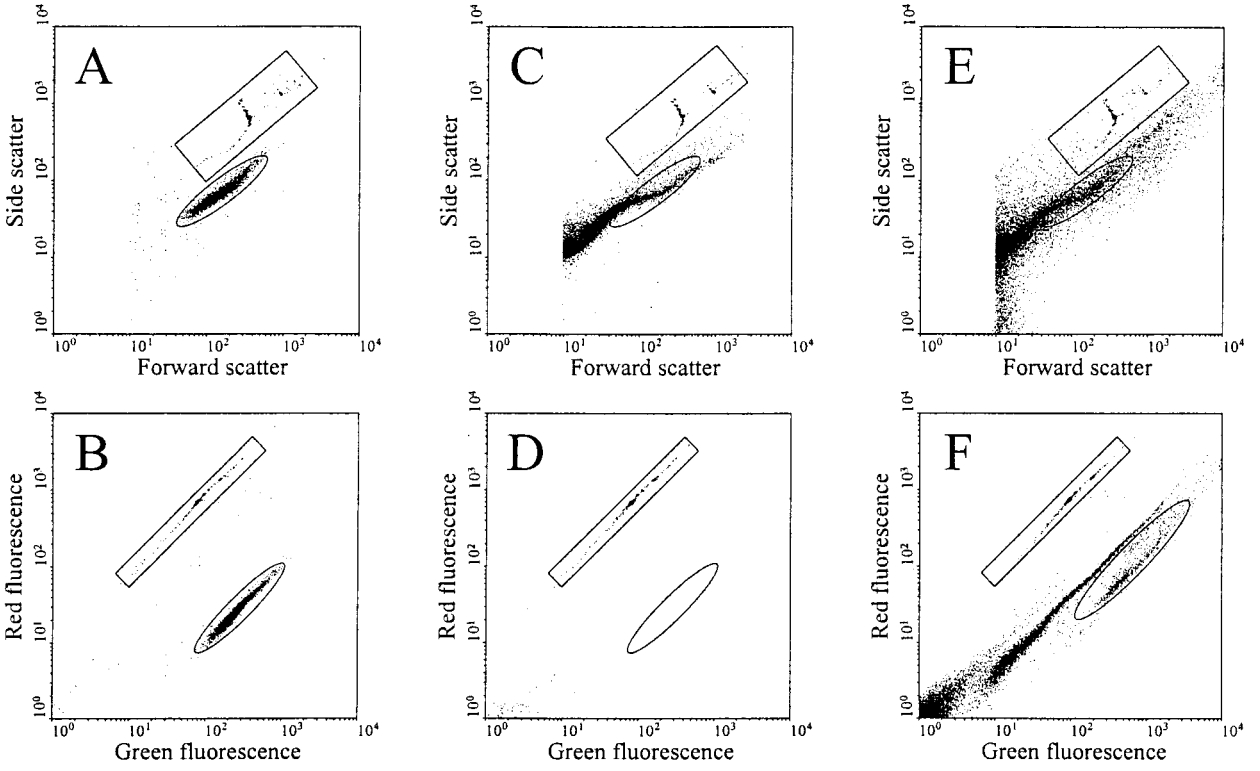

FIG. 1. Detection of bacteria in milk by FCM by using SYTO 9 staining. *L. plantarum* was suspended at a concentration of 5×10^6 cells ml^{-1} in either buffer or milk. Red fluorescent 1.75-μm polystyrene beads at a known concentration were included in the FCM samples to enable counting. Dot plots show the results obtained with 5 μl of an appropriately diluted sample; the upper dot plots (A, C, and E) show FSC and SSC, and the lower dot plots (B, D, and F) show green fluorescence and red fluorescence. The rectangles indicate the location of the bead population, and the ellipses show the location of the cell population. (A and B) Cell suspension in buffer, diluted fivefold. (C and D) Cell suspension in milk, diluted 10,000-fold. (E and F) Cell suspension in milk extracted by using the optimized procedure, diluted 10-fold.

facilitate counting of bacteria in milk, a procedure that reduced the signal from the milk itself was needed.

A milk-clearing solution used according to the protocol of the supplier reduced the number of counted events approximately 100-fold, to 2.3×10^8 events ml^{-1} (Table 1). Some of these events were remaining milk particles, but the majority were microcarrier polystyrene beads from the clearing solution. To decrease the number of particles detected by FCM, the protocol was adjusted. The clearing solution was filtered to remove the polystyrene beads before it was added to the milk. Plate counts indicated that the extraction yield did not change when a clearing solution without microcarrier beads was used (data not shown). Furthermore, the number of background counts from milk itself was reduced further by removal of cream that was stuck to the wall of the Eppendorf tube with a cotton-tipped stick. With this additional step the background counts were approximately 50-fold lower than the background counts obtained when the remaining cream was not removed (Table 1). In total, the optimized extraction procedure reduced the background counts from milk by a factor of approximately 10,000.

Figures 1E and F show dot plots obtained with a sample of *L. plantarum* in milk extracted with the optimized procedure. This sample needed to be diluted only 10-fold in order to obtain an appropriate event rate. The remaining particles from milk still overlapped with the region of cells in the dot plot of FSC and SSC (Fig. 1E). Some of the milk particles had very low fluorescence signals, while the other particles were stained (Fig. 1F). Compared to the fluorescence dot plot of the suspension in buffer, the fluorescence dot blot of the cells extracted from milk is shifted somewhat towards a higher signal. It is possible that the extraction procedure resulted in easier access for SYTO 9 by allowing faster diffusion across the membrane. However, most importantly, the reduction in the number of milk particles was sufficient, and the bacterial cells formed a distinguishable population in the fluorescence dot plot and could be enumerated.

TABLE 1. FCM of milk samples and optimization of extraction

Sample	Background count (bacteria/ml)[a]
Buffer	1.0×10^4
Milk	1.8×10^{10}
Milk, standard extraction with milk-clearing solution	2.3×10^8
Milk, extraction with filtered milk-clearing solution	9.4×10^7
Milk, optimized extraction procedure[b]	2.1×10^6

[a] The detector settings were as follows: FSC, E01; SSC, 400; FL1, 450; FL2, 450; and FL3, 600 (all with logarithmic amplification). The threshold was set at an FSC signal of 250.

[b] For the optimized extraction procedure we used milk-clearing solution free of polystyrene beads and removed the rest of the cream pad with a cotton-tipped stick.

Counting of bacteria in milk. First, the bacterial background in milk was determined. The milk that was used was semiskimmed pasteurized day-fresh milk that was purchased on the day of the experiment, so low viable counts were expected. The milk was cleared with the optimized extraction procedure. Samples were stained with SYTO 9 to obtain total counts. Also, samples were stained with cFDA and TOTO-1 to distinguish intact cells and cells with damaged membranes. The sum of cF-stained and TOTO-1-stained cells also gave the total number of cells. Samples were plated on MRS agar before and after extraction. The number of CFU before extraction was approximately 10^2 CFU ml^{-1} (5 to 10 colonies on plates), and after extraction the number of CFU was below the limit of detection. In samples of milk stained with cFDA and TOTO-1, no cells could be detected by FCM because there were no events in the region of cF-stained cells and in the region of TOTO-1-stained cells there was too much background to count cells at concentrations below 10^6 CFU ml^{-1}. With SYTO 9 staining the cell count was approximately 1×10^5 ml^{-1}. This was the number of events in a region in the dot plot of green fluorescence and red fluorescence, which was defined by using milk with added *L. plantarum*. However, it should be realized that some of the 10^5 events from milk in this region might have been particles other than bacteria. Thus, the bacterial background level in milk was, at most, 1×10^5 nonviable cells ml^{-1}.

Next, FCM counting and plate counting were performed with samples of *L. plantarum* suspended in milk at concentrations of 10^5 to 10^9 CFU ml^{-1} and, for comparison, samples of *L. plantarum* in buffer. The variation between replicates with FCM counting was considerably lower than the variation between multiple plate counts. With plating between 50 and 300 colonies were counted, and the CV was approximately 10%. With FCM at least 3,000 cells and a maximum of 20,000 cells were counted, and the CV was between 0.7 and 1.8%. Thus, FCM counts are more accurate than plate counts. FCM counts for cell suspensions in buffer with SYTO 9 staining exhibited a very strong correlation with plate counts and a regression line with nearly a 1:1 ratio (Fig. 2A). The suspensions of *L. plantarum* in milk were first cleared and then stained and analyzed by FCM. The FCM counts obtained with SYTO 9 staining (Fig. 2B), as well as the sum of the counts obtained with cFDA staining and TOTO-1 staining (Fig. 2C), strongly correlated ($r > 0.99$) with plate counts for extracted samples. The regression lines approximate the 1:1 ratio closely, indicating that nearly all of the cells counted by FCM formed colonies on plates. The percentage of permeabilized cells in these samples was less then 2%, as indicated by TOTO-1 staining.

The numbers of CFU of *L. plantarum* WCFS 1 after extraction were, on average, lower than the numbers of CFU before extraction. In this experiment the extraction yield (i.e., the ratio of the number of CFU after extraction to the number of CFU before extraction) was 0.71 ± 0.11 (95% confidence interval; $n = 22$). The extraction yield was not dependent on the concentration of cells. The average 29% loss of CFU could have been due to loss of cells during extraction and to sticking of cells during resuspension of the pellet.

Furthermore, viability staining of bacteria in milk was tested by using heat treatment. Milk containing 10^8 *L. plantarum* cells ml^{-1} was divided into two portions. One portion was not treated, and the other was incubated at 70°C for 10 min. This

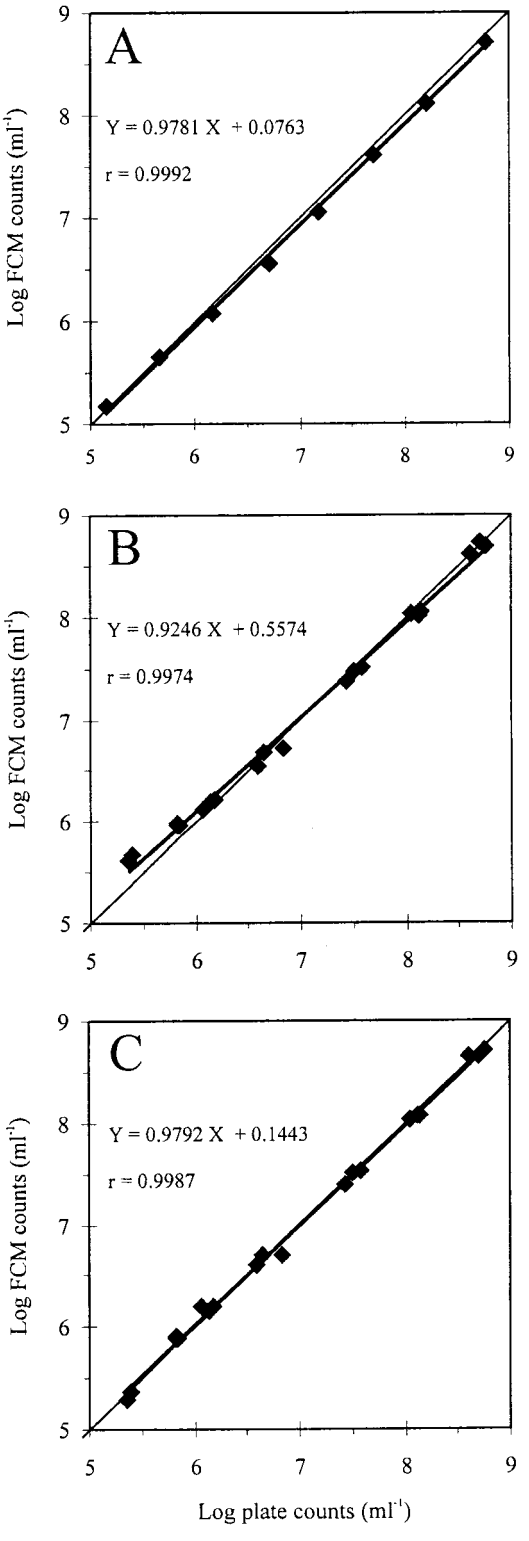

FIG. 2. (A) Correlation between plate counts and FCM counts obtained by SYTO 9 staining of *L. plantarum* in buffer. (B) Correlation between plate counts and FCM counts obtained by SYTO 9 staining of *L. plantarum* in milk extracted by the optimized procedure. (C) Correlation between plate counts and FCM counts obtained by double staining with cFDA and TOTO-1 of *L. plantarum* in milk extracted by the optimized procedure. The thin lines represent a 1:1 relationship. The thick lines show linear regression.

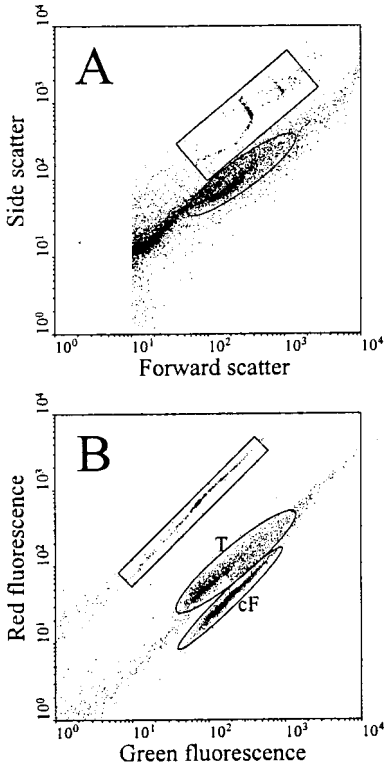

FIG. 3. Viability staining in milk by cFDA and TOTO-1 for a 1:1 mixture of nontreated and heat-treated (70°C) milk containing 10^8 *L. plantarum* cells per ml. (A) FCM dot plot of FSC and SSC. (B) FCM dot plot of green fluorescence and red fluorescence. The rectangles indicate the location of the bead population, and the ellipses indicate the location of the cell populations. The cF-stained population (cF) and the TOTO-1-stained population (T) are distinguished from each other by their locations in the dot plot of green fluorescence and red fluorescence.

heat treatment permeates cells and kills them. Mixtures of nontreated and heat-treated milk were prepared. These milk sample mixtures were cleared, stained with cFDA and TOTO-1, and analyzed by FCM. Figure 3 shows the results for the mixture containing 50% nontreated suspension and 50% heat-treated suspension. Nearly all of the cells in the nontreated suspension were intact and were thus stained by cFDA, while all of the cells in the heat-treated suspension were permeabilized and thus stained by TOTO-1. FCM counting showed that extraction was as efficient for heat-killed cells as it was for nontreated cells, and the correlation between FCM counts and the fractions of nontreated and heat-treated milk in the mixtures was very strong ($r > 0.99$).

FCM of dairy starters. The optimized extraction procedure and staining with cFDA and TOTO-1 were used for FCM analyses of dairy starters (Table 2). The extraction procedure cleared the background very well. cFDA and TOTO-1 gave well-stained cell populations (high fluorescence intensities). Total cell counts were determined in samples that were heat treated at 70°C. After heat treatment the cells had permeabilized membranes and were uniformly and intensely stained by TOTO-1. The total number of cells detected in untreated samples was the same as the total number of cells detected in heat-treated samples, indicating that all cells were also detected in the untreated samples. Note that the counts for BOS, RR, and ISt are the numbers present in the concentrated mother starters; BOS was concentrated 40-fold, and RR and ISt were concentrated 20-fold. The total FCM counts for the BOS, BOS bulk, and RR starters were approximately three times higher than the respective plate counts (Table 2). The total FCM counts for ISt were approximately five times higher than the plate counts. A considerable fraction of the cells lacked membrane integrity, as indicated by the 30 to 60% TOTO-1-stained cells. Still, the number of intact and enzymatically active cells, as indicated by cF staining, was substantially higher than the number of CFU.

FCM of probiotic products. Three probiotic products were selected for this study: a powder (Orthiflorplus), a drink (Yakult), and a yogurt (Mona Vifit yogurt drink). Some details about these products are described in Materials and Methods. The effects of the clearing procedure on the product matrix and on the bacteria were tested first by FCM and fluorescence microscopy. This showed that an Orthiflorplus suspension did not contain many particles other than bacteria, so clearing was not needed. Moreover, the procedure increased the number of cells with damaged membranes in Orthiflorplus substantially. In Yakult there were many particles besides bacteria, and most of these particles were somewhat smaller than the bacteria. Clearing did decrease the number of these particles, but it also damaged the Yakult bacteria. However, FCM analyses could be done without clearing of the samples. In Mona Vifit yogurt drink there was very high background. The clearing procedure decreased the background, but not as well as it did for milk. With this product the procedure hardly increased the number of bacteria with damaged membranes. We decided to perform FCM analyses of Orthiflorplus and Yakult with untreated samples, while Mona Vifit samples were cleared before use.

In each of the probiotic products the bacteria were heterogeneous with respect to membrane integrity. FCM with cFDA and TOTO-1 quantified the intact cells and cells with damaged membranes. Plate counting quantified the culturable cells, which constituted a subpopulation of the intact cells. The results are shown in Table 3. When the results of FCM and plate counting analyses were combined, three populations were evident. The first was the culturable population. The second was the population of cells that were intact and metabolically active but not culturable (FCM count with cFDA minus plate count).

TABLE 2. Bacterial numbers determined by plating and FCM in dairy starters

Dairy starter[a]	Plate count (bacteria/ml)	FCM count (bacteria/ml)		
		cFDA	TOTO-1	Total[b]
BOS	1.43×10^{10}	2.84×10^{10}	1.29×10^{10}	4.13×10^{10}
BOS bulk[c]	2.35×10^{8}	3.67×10^{8}	4.02×10^{8}	7.70×10^{8}
ISt	3.49×10^{9}	8.49×10^{9}	8.35×10^{9}	1.68×10^{10}
RR	5.69×10^{9}	7.55×10^{9}	1.10×10^{10}	1.85×10^{10}

[a] The starter cultures were cleared before analyses, and the average extraction yield was 0.78.

[b] Total counts were obtained by using heat-treated samples and TOTO-1 staining.

[c] BOS bulk starter was produced by inoculation of low-fat milk with 0.0125% concentrated BOS mother starter and incubation for 18 h at 20°C.

TABLE 3. Bacterial numbers determined by plating and FCM methods in probiotic products

Probiotic product	Plate count (bacteria/ml)	FCM count (bacteria/ml)		
		cFDA	TOTO-1	Total[a]
Orthiflorplus[b,c]	9.15×10^6	2.04×10^7	2.25×10^7	4.29×10^7
Yakult[b]	4.76×10^8	1.78×10^9	5.50×10^7	1.83×10^9
Mona Vifit[d]	1.93×10^7	9.85×10^8	1.43×10^8	1.13×10^9

[a] Total counts were obtained by using heat-treated samples and TOTO-1 staining.
[b] Orthiflorplus and Yakult were used directly (without clearing).
[c] One sachet of Orthiflorplus (3 g) was suspended in 100 ml of tap water.
[d] Mona Vifit was cleared, and the extraction yield was 0.82.

The third was the permeabilized (dead) population. The proportions of these populations in the products tested differed.

(i) Orthiflorplus. According to the supplier, Orthiflorplus powder contains at least 3×10^9 germs per sachet (i.e., 3×10^7 cells per ml of suspension). The measured number of CFU was somewhat lower, 9.15×10^6 cells per ml. This might have been due to sticking of cells or to loss of viability during production and storage. The total cell number was 4.29×10^7 cells ml^{-1}; approximately 21% of these cells were culturable, 27% were intact and active but not culturable, and 52% were permeabilized.

(ii) Yakult. According to the supplier, Yakult drink contains 6.5×10^9 *L. casei* Shirota bacteria per 65-ml bottle (i.e., 1×10^8 bacteria per ml). The measured number of CFU was somewhat higher, 4.76×10^8 CFU per ml. The total cell number was 1.83×10^9 cells per ml. Most cells were stained by cF, and only a small population was stained by TOTO-1. The average fluorescence signal of the cF-stained cells was, however, quite low,and the cF population was not separated from the background. Thus, intact cells could not be enumerated directly by cF staining. The difference between the total number of cells determined with a heat-treated sample and the number of TOTO-1-stained cells in an untreated sample was used to calculate the number of intact cells. This was done for all samples of probiotics and starters, but only for Yakult was the number of intact cells calculated by this method substantially higher than the count of the cF-stained region for an untreated sample. The proportions of culturable, intact and active but not culturable, and permeabilized cells were 21, 76, and 3%, respectively.

(iii) Mona Vifit. According to the supplier, Mona Vifit yogurt drink contains three probiotic bacteria, *L. acidophilus*, *B. bifidum*, and *L. rhamnosus* Goldin and Gorbach. Since it is a yogurt, it should also contain at least 10^7 yogurt bacteria (*L. delbruecki* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*) per ml. Only for the probiotic *L. rhamnosus* Goldin and Gorbach is an amount mentioned on the package; there should be approximately 2×10^7 bacteria per ml. Our assays do not distinguish between species, so we do not know in what proportions the two yogurt species and the three probiotic species occur in Mona Vifit. The number of CFU in extracted samples was 1.93×10^7 CFU per ml. FCM counted a total of 1.13×10^9 cells per ml. The number of background events was high, but the populations of cF-stained and TOTO-1-stained cells were easily detectable. The fluorescence intensities of the cF-stained cells were homogeneous and high. Approximately 2% of the cells were culturable, 85% were intact and active but not culturable, and 10% were permeabilized.

DISCUSSION

In a previous study we developed an FCM viability assay with the fluorescent probes cFDA and TOTO-1 (6). This assay was used with various LAB species, which were harvested in the exponential growth phase and suspended in buffer. The plate counts were in agreement with the relative FCM counts, and the assay was validated for cells stressed by exposure to acid or bile. Assessment of viability by FCM has many possible applications. In the present study we show the usefulness of the method for bacterial suspensions in milk, fermentation starters, and probiotic products.

Bacteria are small compared to mammalian cells, and in FCM analysis the signal-to-noise ratio is of greater concern for bacterial cell samples than for mammalian cell samples, especially in a medium such as milk. Milk contains a very high concentration of colloidal and other particles that are in the size range of bacteria. Furthermore, milk might cause clogging of the flow system and turbulence of the flow. Analysis of low concentrations of bacteria in milk is not possible because a high dilution factor is needed for direct FCM of milk samples. A procedure that reduces the milk background but does not affect the bacteria is needed. To do this, we chose a method based on a commercially available clearing solution, which was used before by McClelland and Pinder for immunofluorescent detection of *Salmonella* in milk (20). The clearing solution contains a nonionic detergent, a chelating agent, and polystyrene beads (23). We optimized the method by using a clearing solution free of polystyrene beads and by removing cream that remained on the wall of the tube with a cotton-tipped stick. The optimized method cleared milk very well. The counts of particles that were similar in size to or larger than bacteria were decreased enormously. The extraction yield of bacteria was high, and the culturability and membrane integrity were not affected by the clearing step.

FCM indicated that the bacterial background in the pasteurized milk was, at most, 10^5 bacteria per ml and that all of the bacteria were dead. This is a normal result for milk, as determined by plate counting, total FCM counting, and direct epifluorescence filter technique viability counting just before pasteurization (13, 26). FCM counts for *L. plantarum* added to milk at concentrations of 5×10^5 cells per ml or more were highly accurate, especially with cFDA and TOTO-1 double staining. FCM counts for permeable cells were also accurate, since these cells were extracted by the clearing procedure with the same yield as intact cells.

This work is a step in the development of FCM methods for rapid monitoring of fermentation processes and for other applications of bacterial measurement in milk suspensions. A complete FCM assay, including extraction and labeling, can be done in 1 h, because no long incubation times are required. This makes FCM more useful than plate counting, which requires incubation for 2 days or more before a result can be obtained. Furthermore, FCM can be used for total cell counting, for specific detection of strains, and for measurement of various aspects of cell viability with individual cells.

FCM has been used before for bacteria in milk, but it has

been used mostly for pathogens and for bacteriological quality control (10, 13, 20, 24, 29, 34). FCM can also be used to measure fermentation starter bacteria. Here we used FCM with cFDA and TOTO-1 to investigate the viability of dairy fermentation starters. All samples were cleared, although this was not strictly necessary for the concentrated mother starters since they contained very high numbers of cells ($>10^{10}$ cells per ml). The results showed that approximately 50% of the cells in the mother starters were permeabilized. This must have occurred mainly during fermentation and not during freezing, since the freshly produced BOS bulk starter contained approximately the same proportion of permeable cells. The other 50% of the cells were intact and active. The numbers of intact cells in the starters were higher than the numbers of colonies on plates. Between 40 and 65% of the intact cells were culturable. Cells which are not culturable might well contribute to fermentation processes (21, 33). The enzymes present may still be active, and cells may even still have the capacity to synthesize enzymes. Hence, metabolic routes may still be functional, and these cells may be involved in fermentation.

We also used cFDA and TOTO-1 to investigate probiotic products with FCM. Three different types of probiotic products were selected: a powder (Orthiflorplus), a drink (Yakult), and a yogurt (Mona Vifit yogurt drink). Probiotics have been defined as living microorganisms which, upon ingestion in certain numbers, have health effects beyond inherent basic nutrition effects (12). A number of criteria have been defined for probiotic microorganisms, including human origin, resistance to technological processes, resistance to gastric acid and bile, modulation of the immune response, influence on metabolic activities, and persistence in the gastrointestinal tract (11, 27). In vitro and in vivo studies are important to elucidate effects of stress factors on the performance of probiotics and the effects of probiotics on human health. It is difficult to say in what numbers probiotic microorganisms should be ingested, because knowledge about survival, activity, and dose response is limited. The minimum therapeutic dose has been suggested to be 10^8 to 10^9 living microorganisms per day, and a criterion of a minimum of 10^6 CFU of probiotic bacteria per ml at the expiry date has been suggested (17, 30). However, 10^6 CFU ml^{-1} might not be enough, considering that only some of the bacteria reach the intestines alive. On the basis of the results of in vitro survival studies, a daily dose of 10^9 to 10^{10} CFU is assumed to be needed for probiotic effects (28). Our plate counts indicated that the numbers of CFU per recommended portion were approximately 10^9 CFU for Orthiflorplus (per sachet), 3×10^{10} CFU for Yakult (per 65-ml bottle), and 5×10^9 CFU for Mona Vifit (per 200-ml portion). These numbers are high enough to be effective probiotic doses according to the criterion of 10^9 to 10^{10} CFU per daily portion.

Most studies on probiotics use only plate counting to assess the viability of the microorganisms. However, cell viability is more complex than whether cells are culturable or not. Only for prolonged persistence would dividing cells be a prerequisite. Cells that are active but not culturable might contribute to many of the proposed health effects. For actions such as lactose conversion, production of antibacterial compounds, assimilation of cholesterol, and antioxidant effects, the microorganisms need to be active but not necessarily culturable. For some probiotic effects the microorganisms do not even have to perform metabolic activities. It has been reported that nonviable forms of probiotic bacteria can adhere to intestinal mucus and have immunomodulatory effects (22, 25). Because of the complexity of the possible contributions of different cell populations for proposed probiotic effects, FCM is a very appropriate tool to study probiotics. FCM can detect all individual cells and can distinguish between various populations.

The FCM viability assay with cFDA and TOTO-1 gave the total cell count and distinguished permeable cells from intact cells. In combination with plate counting three cell populations were revealed: culturable cells, cells that are intact and metabolically active but not culturable, and permeabilized cells. The proportions of these populations in the products tested differed.

The FCM viability assay could be used for various other purposes. For example, it could be used to study the effect of prolonged storage on probiotic products. It has been shown that the decrease in the number of CFU depends on factors such as pH, temperature, the level of inoculum, and the type of product (17, 28, 30). It would be interesting to measure the effects on the total number of cells and the number of active cells after prolonged storage. Furthermore, FCM could be used to investigate the effect of gastrointestinal tract passage on probiotic microorganisms and in strain selection. A combination of viability probes and probes for specific detection of a species would be needed to study one probiotic strain in a mixed culture, such as that in Orthiflorplus or Mona Vifit yogurt. This may also be done by FCM.

In conclusion, this study demonstrates the usefulness of FCM for analysis of the viability of bacterial suspensions in milk, dairy fermentation starters, and various types of probiotic products. The FCM assay with cFDA and TOTO-1 is very accurate and highly sensitive and provides tools to assess the functionality of different populations in fermentation starters and probiotic products.

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