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REVIEW

Flow cytometry applications in the food industry

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Abstract Flow cytometry has become a valuable tool in food microbiology. By analysing large numbers of cells individually using light-scattering and fluorescence measurements, this technique reveals both cellular characteristics and the levels of cellular components. Flow cytometry has been developed to rapidly enumerate microorganisms; to distinguish between viable, metabolically active and dead cells, which is of great importance in food development and food spoilage; and to detect specific pathogenic microorganisms by conjugating antibodies with fluorochromes, which is of great use in the food industry. In addition, high-speed multiparametric data acquisition, analysis and cell sorting, which allow other characteristics of individual cells to be studied, have increased the interest of food microbiologists in this technique. This mini-review gives an overview of the principles of flow cytometry and examples of the application of this technique in the food industry.

Keywords Flow cytometry · Food microbiology

Introduction

Flow cytometry is a technique for measuring the physical and chemical characteristics of cells or other biological particles. The data obtained can be used to understand and monitor biological processes and develop new methods and strategies for cell detection and quantification. Compared to other analytical tools, where a single value for each parameter is obtained for the whole population, flow cytometry provides data for every particle detected. As cells differ in their metabolic or physiological states, flow cytometry allows us not only to detect a particular cell type but also to find different subpopulations according to their structural or physiological parameters.

In flow cytometry, single cells or particles pass through a light beam in a directed fluid stream. The interaction of each individual cell with the beam can be recorded; the data obtained can be correlated with cell characteristics or components. In fluorescence-activated cell-sorting (FACS) instruments, detected particles can also be purified according to their characteristics.

Flow cytometry was first developed for medical and clinical applications such as haematology and oncology. These areas still account for the vast majority of publications on this technique, but during the past few years it has been used in other areas, such as bioprocess monitoring, pharmacology, toxicology, environmental sciences, bacteriology and virology. The use of commercially available instruments that are smaller and have fewer requirements along with new software for data acquisition and interpretation form the basis for the success of the technique. Here we review the principles of flow cytometry and its use in different areas in food microbiology.

Principles of flow cytometry

A flow cytometer consists of several systems integrated for cell detection. The optical system consists of one or more

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light sources (typically laser light is used, but a mercury lamp is provided in some instruments) and a series of lenses to focus the light beam. Optical filters combined with light detectors (photodiodes or photomultiplier tubes) allow sensitive detection of the signals of interest. A pneumatic system delivers the cell suspension into a laminar flow of sheath fluid; hydrodynamic focussing of the sample stream causes cells to pass, one after another, across the focussed light. The electronic system processes signals produced by the light detectors. Using modern electronics it is possible to detect up to 100,000 cells/s. Detected particles of interest can also be purified in cell sorters; most sorters use a droplet formation system, where the liquid sheath stream is broken regularly into droplets by the vibration of a piezoelectric crystal attached to the flow chamber. Droplets containing desired cells are charged and deflected as they pass a high-voltage electrical field, thus being recovered into suitable collection vessels.

Flow cytometric measurements

Due to differences between the refractive indices of cells and their surrounding media, light impinging upon the cells is scattered. The forward scatter light (FSC, light scattered at low angles) provides information on cell size, although there is no direct correlation between size and FSC [46]. Light scattered in an orthogonal or near-orthogonal direction can also be collected by a different detector (a side scatter or SSC detector), which provides information about granularity and cell morphology. Fluorescence produced by intracellular compounds with specific fluorescence [like nicotinamide adenosine dinucleotide phosphate (NADPH)] or specific compounds known as fluorochromes is detected by photomultiplier tubes, allowing certain cell components to be selectively assayed. Several compounds with high quantum efficiency are used in microbiological applications of flow cytometry, where low signals are expected due to the small size of the analysed cells. The scatter and fluorescence signals detected can be combined in various ways to allow the detection of subpopulations. The use of autofluorescence or fluorescent signals associated with cellular compounds or specific labelling can be used to distinguish cells in media in which nonbiological particles are also present.

Cytometric software usually presents data in the form of mono- or biparametric histograms, although multivariate data analysis methods have also been developed in order to obtain maximum information from multiple labelling. Software also allows data gating to focus on specific subpopulations, as only part of the data is represented on the histograms, facilitating detection of the cells of interest.

A combination of light-scattering and fluorescence measurements on stained or unstained cells allows the detection of multiple cellular parameters associated with cellular structures or functions. Depending on the dyes used, many of these measurements can be done simultaneously on the same cells; in multilaser commercial instruments it is quite common to find more than ten fluorescence detectors. Flow cytometry is used in microbiology for rapid counting, the study of heterogeneous bacterial populations, for strain improvement in industrial microbiology, and to sort bacteria for further molecular analyses [84]. Although many different measurements are possible, only those most relevant to microbiological applications will now be discussed.

Membrane integrity and enzyme activities

The staining of viable eukaryotic cells by fluorescent dyes and their detection by flow cytometry has been reported by several workers [77]. The use of a flow cytometer to detect viable bacteria was first reported by Resnick et al. [73], who described rhodamine 123 (Rh123) staining of *Mycobacterium smegmatis*. Diaper et al. [26] studied the ability of vital fluorescent stains in conjunction with FCM to rapidly detect and estimate the viability of a range of bacterial species.

Cell viability can be monitored by flow cytometry using the cell's capacity to maintain an effective barrier with external media, although this does not guarantee cell replication. Cells with a damaged membrane cannot sustain any electrochemical gradient and are normally classified as dead cells. Thus, loss of membrane integrity is considered indicative of cell death.

Membrane integrity can be detected by dye exclusion or dye retention. Exclusion dyes (like propidium iodide or ethidium bromide) stain nucleic acids in cells with permeabilized membranes (usually considered dead cells). Due to their bright fluorescence resulting from high quantum efficiency, new dyes—like the cell-impermeant SYTOX® family—have been used to detect nucleic acids in cells such as bacteria that have a lower DNA content.

The presence of DNA in all microorganisms makes this an ideal staining target. The combination of DNA staining with the test for a selectively permeable membrane surrounding and protecting the DNA is a robust approach, especially in media with high background levels.

The combination of a cell-permeant dye (like most of the SYTO family) and a cell-impermeant dye such as propidium iodide can be used together to distinguish between live and dead cells resuspended in particulated media. Thus, cells will be stained with one fluorochrome or the other depending on their physiological state, while noncellular particles will remain unstained. Commercial viability kits for bacteria can be found using this kind of

fluorochrome combination. Determination of antibiotic susceptibility or determination of stress in bacteria after the effects of osmotic shock has been tested using these dyes [21, 26, 70].

Dye retention methods use nonfluorescent cell-permeant esterase substrates (e.g. fluorescein diacetate), which are used by intracellular enzymes of live cells to produce fluorescent products. While substrate can be incorporated inside the cell due to its membrane permeability, the product of the enzymatic reaction is trapped inside the cell by its electrical charge and polarity. Even if residual esterase activity was found inside a dead cell, the diffusion of the product would allow clear differentiation due to its fluorescence from dead cells.

A number of esterase substrates have been tested on several organisms [22, 85]. Problems in detecting fluorescence may be due to dye loading or extrusion, or its dependence on pH. The ability of most flow cytometers to produce dual-parameter histograms of fluorescence versus time of acquisition provides a way to measure enzyme kinetics, as changes in fluorescence can be monitored during the time of acquisition.

Membrane potential

Fluorescent membrane potential probes are charged lipophilic compounds that can readily cross the cytoplasmatic membrane and accumulate inside the cell according to the cellular electrical charge. However, care must be taken, as fluorescence intensity depends not only on membrane potential but also on the number of lipophilic binding sites within the cell, the volume of dye concentration and the number of binding sites in solution. Other problems can be encountered when using these distributional probes due to active dye extrusion and the need to make outer cell envelopes permeable—i.e. the outer cell membranes of Gram-negative bacteria.

Cationic cyanin dyes were the first molecules used to measure cellular membrane potential [81]. A second class of dyes, the oxonols, are functionally similar to the cyanins but have opposite polarity as they are anionic. Cyanins are excluded from cells as they are depolarised, whereas oxonols are excluded from repolarised or hyperpolarised cells. Fluorochromes like rhodamine 123 and oxonols have been widely used in bacteria, either alone [54, 57] or combined with membrane integrity dyes such as propidium iodide [12, 55].

Pathogen detection

The identification of microbes and the direct detection of microbial components are the main tools used for the laboratory diagnosis of microbial contamination. Early detection is crucial in many cases; therefore, rapid detection methods like flow cytometry are of great interest.

Specific detection of pathogenic strains can be achieved by flow cytometry using immunofluorescence techniques, which allow microorganism detection at the single-cell level. It can be used for food samples, but requires prior isolation of the target organism to generate antibodies. The use of specific antibodies directed against pathogens such as certain strains of *Escherichia coli*, *Salmonella*, *Pseudomonas* and other cell types allowed their cytometric detection with sensitivity and specificity [1]. Magnetic separation can also be used to make the analysis faster and more specific, and to overcome the presence of noncellular particles [85].

The detection of resistant forms of microorganisms has been achieved cytometrically. Bacterial endospores can remain in a dormant state for long periods with little or no metabolic activity, being resistant to heat, radiation and toxic chemicals. Flow cytometry in combination with biochemical techniques has been used to reveal the heterogeneity of *Bacillus* cultures and to distinguish between live and dead endospores on the basis of their cytometric scatter alone [83] or combined with nucleic acid stains [23].

Respiratory activity

Metabolic activity in the form of respiration in aerobic bacteria can be detected using an indicator of oxidative metabolism such as a tetrazolium dye. The use of cyanoditolyl tetrazolium chloride (CTC) has been reported [48] in *Micrococcus luteus*; the reduction of CTC was detected by flow cytometry by means of its fluorescence combined with changes in scatter due to the intracellular accumulation of an insoluble fluorescent formazan. CTC has also been used to report the presence of metabolically active *Salmonella typhimurium* in drinking water.

Multiple labelling combined with sorting has been used by Nebe-von-Caron et al. [63] and Hewitt and Nebe-von-Caron [41] to distinguish between permeabilized cells, depolarised cells, de-energised cells and active pumping cells according to their different staining patterns when using $DiBAC_4(3)$, ethidium bromide and propidium iodide. Cellular characterization of intact cells was achieved by active exclusion of ethidium bromide (EB) (metabolically active cells), uptake of EB, but exclusion of bis-oxonol ($DiBAC_4(3)$) (de-energized but with a polarized cell membrane) and uptake of both dyes (depolarized). Permeabilized cells were identified by propidium iodide (PI) uptake.

Intracellular pH

The pH gradient can potentially provide information about viability. Most viable cells are able to maintain a pH

gradient when the extracellular pH is moderately decreased. Fluorescent probes such as carboxyfluorescein diacetate (CFDA) or biscarboxyethyl carboxyfluorescein (BCECF) are well suited to measuring the intracellular pH, as they are highly pH dependent and have a fast response time [40]. Molenaar et al. [62] described a method for measuring cytoplasmic pH in *Latococcus lactis*, which in brief consists of treating cells with acid in the presence of BCECF, resulting in accumulation of the probe in the cytoplasm. When no active extrusion mechanism is present, viable cells retain BCECF well. After correlation of the fluorescent signal with the cytoplasmic pH, the method may be applied to study pH regulation in bacteria or to elucidate the dependence of inactivation treatments on internal pH.

The intracellular pH of *Corynebacterium glutamicum* grown in batch or fed-batch cultures was measured by FCM using the fluorochrome c-SNARF1, which is more sensitive than BCECF. The fluorescence was proportional to the pH_i of the cells grown in fermenters between 6.6 and 8.3 [52]. In this study, pH_i was found to be a good indicator of the physiological state of the cells, and FCM a sensitive and rapid tool for measuring cytoplasmic pH.

Food microbiology applications of FCM

Flow cytometry has been widely used in research since early 1975 for yeast and bacterial cell analysis, but the routine application of FCM in industrial microbiology was nonexistent before 1988.

According to Fleet [33], the dynamics of the growth, survival and biochemical activity of microorganisms in food are the result of stress reactions in response to the changing physical and chemical conditions in the food microenvironment (e.g. the gradients of pH, oxygen, water activity, salt, and temperature), the ability to colonise the food matrix and to grow (e.g. microcolonies and biofilms), the presence of other microorganisms, and the in situ cell-to-cell ecological interactions that often happen in a solid phase. Ecological approaches to studying the evolution of microbial flora would be useful to better comprehend the microbiological processes involved in food processing and ripening, to improve microbiological safety by monitoring pathogenic bacteria in situ, and to study microbial populations in food fermentation [36].

Stress is a change in the genome, proteome or environment that imposes either reduced growth or survival potential. For any stress, the bacterial cell has a defined range within which the rate of increase of colony forming units (CFUs) is positive (growth), zero (survival) or negative (death). In microbial populations, viable cells are usually countable on both nonselective and selective media, whereas stressed cells are able to form colonies on nonselective media but are not countable on selective media. In foods, many adverse conditions such as nutrient depletion, low temperature and other stresses can sublethally damage microorganisms. Environmental stresses cause the bacteria to enter a viable but noncultivable (VBNC) state, inducing healthy, cultivable cells to enter a phase in which they are still capable of metabolic activity but do not produce colonies on media (both nonselective and selective) that normally support their growth [11].

The advantage of FCM is its ability to detect microorganisms at relatively low concentrations in a short time. Multiple labelling allows the detection of different organisms or different stages in the same sample, such as food matrices. Table 1 gives an overview of several fluorescent dyes used for specific staining in flow cytometry applications in food microbiology.

Viability and cell number

Food microbial analysis is routinely performed by colony counting using suitable media and conditions for incubation. However, plate-culturing techniques are time consuming and they only reveal a small proportion (i.e. those that are viable and cultivable) of the true microbial population. This can be largely explained by two ecological factors: (a) an inability to detect novel microorganisms that might not be cultivable with known media, and (b) an inability to recover known microorganisms that are either stressed or actively growing but which enter a noncultivable state [9, 18, 36, 49]. Several direct microscopic examination techniques using viability stains have been developed [15]. Direct counting of methylene-blue-stained yeasts is frequently used in oenology; however, it is highly subjective, the cells counted under the microscope are not necessarily representative of the entire population of yeast cells, and it is nonreproducible [14, 64]. Fluorescence microscopy has been used as a tool for estimating microbial population density and viability. It is based on the fact that acridine dyes absorb light of one wavelength and emit light of different wavelengths. The direct epifluorescent filter technique (DEFT) generally involves staining of the microorganisms with a fluorochrome dye, collection on a nonfluorescing membrane filter, and examination of the membrane using a fluorescent microscopy. DEFT has been used in milk [68, 75], yoghurt [76], meat and poultry [30, 78, 84], and wine [27, 35]. Although it is a relatively rapid and sensitive method, the dyes used react indiscriminately with organic material and interference from preservatives such as sorbic acid has been reported. Lonvaud-Funel and Joyeux [53] and Dowhanick and Russel [29] evaluated bioluminescence as a rapid technique to enumerate viable cells in wine samples, but they concluded that it is not adequate because its threshold level is too high and it cannot distinguish yeasts from bacteria.

Table 1 Selected fluorescent dyes for specific staining in flow cytometry applications in food microbiology

Fluorescent dye	Properties and actions	References
Ethidium bromide (EB)	Positively charged dye, intercalates RNA and DNA. Slow penetration of intact membranes.	[61, 66]
Hoechst 33342	Permeable DNA-stain, binds preferrentially to A–T base pairs. Discrimination between cells and background.	[61]
Propidium iodide (PI)	Properties like EB, but more membrane impermeable. Enter cells with compromised membranes. DNA stain.	[2, 3, 9, 13, 18–20, 30, 32, 38, 74, 79, 87, 89]
1′-(4,4,7,7-Tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro(benzo-1,3-oxazole)-2-methylidene]-1-(3′-trimethylammoniumpropyl)-pyridinium tetraiodide (TOTO-1)		
SYTOX®		
SYTO 9, SYTO 13, SYTO BC	Permeable nucleic acid stains. Discrimination between cell fractions and inorganic fractions.	[13, 20, 22, 30, 38, 79, 89]
ChemChrome V6 (CV6)	Permeable dye. Metabolic activity stain.	[71]
Oxonol Bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$)	Distributional membrane-potential probe, enters depolarized cells, binds to lipid-rich components. Viability stain.	[6, 9, 41, 63, 74]
Fluorescein diacetate (FDA)	Neutral esterase substrate. Crosses even intact membranes. Cleavage by esterases to polar fluorescent stain CF, retained by cells with intact membranes. Viability stain.	[2, 3, 6, 9, 18, 19, 50, 56, 74]
Carboxyfluorescein diacetate (CFDA)		
Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)		
Thiazole orange (TO)	Permeable nucleic acid-stain	[32]
Hexidium iodide	Permeable Gram-positive bacteria. Blocked by lipopolysaccharide layer of Gram-negative bacteria. Binds to DNA.	[57]
Oregon Green—conjugated wheat germ agglutinin (WGA)	Binds to *N*-acetylglucosamine in the peptidoglycan layer of the cell wall Gram stain of Gram-positive bacteria.	[42, 43]
Fluorescein isothiocyanate (FITC)	Protein stain. Reacts with amines and forms carboxyamines	[24, 28, 59, 86]
2′,7′-Biscarbpoxyehtyl-5,6-carboxyfluorescein (BCECF)	Intracellular pH. The amount of stain retained by cells depends on pH_i.	[40, 52, 62]
Carboxyfluprescein diacetate succinimidyl ester (CFDA-SE)		
Spiro[7H-benzo(c)xanthene-7,1′-(3H)-isobenzofuran]-ar′-carboxylic acid, 3-(acetyloxy)-10-(dimethylamino)-3′-oxo-(acetyloxy)methylester (SNARF1 AM)		

Although the technique is reportedly very reproducible, a major drawback with the method lies in translating ATP measurements into viable cell counts.

Flow cytometry is the science of measuring components (cells) and the properties of individual cells in liquid suspension. In essence, suspended cells are brought to a detector, one by one, by means of a flow channel. Fluidic devices under laminar flow define the trajectories and velocities that cells traverse across the detector, and fluorescence, absorbance, and light scattering are among the cell properties that can be detected. Flow sorting allows individual cells to be sorted on the basis of their measured properties, and one to three or more global properties of the cell can be measured [60]. Flow cytometers and cell sorters make use of one or more excitation sources and one or two fluorescent dyes to measure and characterise several thousands of cells per second. Flow cytometry gives objective and accurate results [19, 80], overcoming the problems with the analytical procedures described above.

The application of flow cytometry directly to food samples using a commercial flow cytometer for the multiparameter analysis of bacteria was first described by Patchett et al. [66]. Use of the Skatron Argus 100 flow cytometer enabled the counting of pure cultures of bacteria to $<10^3$ cfu/mL within a few minutes. Application of the technique to meat samples gave a good correlation ($r = 0.95$) with plate counts and enabled enumeration down to approximately 1×10^4 cells/mL of supernatant (equivalent to 1×10^5 cfu/g meat) within minutes. However, in samples of paté and milk inoculated with pure cultures of *E. coli*, sensitivity was reduced to 1×10^6 cfu/mL and 6×10^7 cfu/mL, respectively.

Molecular Probes Inc. (Leiden, The Netherlands) has developed a fluorescent stain, the LIVE/DEAD® *Bac*Light™ bacterial viability kit, which is composed of two nucleic acid-binding stains: SYTO9™ and propidium iodide (PI). SYTO9™ penetrates all bacterial membranes and stains cells green, while PI only penetrates cells with damaged membranes, and the combination of the two stains produces red-fluorescing cells. Both reagents are contained in a solution of anhydrous dimethylsulfoxide (DMSO). *Bac*Light staining has several advantages. It is a reliable, rapid and easy-to-use test, and yields both viable and total counts in one step. The preparations are easy to read because of the high degree of contrast between the green colour of the viable bacteria and the red colour of the dead cells, and background fluorescence is minimal. Images of the stained cells are differentiated using either epifluorescence microscopy, confocal laser scanning microscopy, or flow cytometry. There is considerable potential for the use of this stain to determine viable counts in a range of processed foods, including irradiated foods, and for measuring viable bacteria in drinking water [13, 30]. Zhang and Fang [89] have demonstrated the use of the *Bac*Light kit for the determination of yeast cell viability.

The application of flow cytometry to foods is restricted by the presence of high levels of suspended material. The use of reagents such as benzalkonium chloride may help to reduce background signals, possibly by disrupting lipids, but further improvements are necessary. Successful enumeration of bacteria has been achieved in samples containing fewer suspended particulates. However, correctly setting the discriminator gates to select bacterial counts from background signals is a critical task and could be a significant problem with food samples containing an unknown or variable microbiota.

For example, Gunasekera et al. [38] developed a rapid ($\leq$1 h) FCM assay based on enzymatic clearing of milk to determine total bacteria in milk. They applied enzymatic treatment with proteases to remove or modify proteins from the milk samples in concert with lipid removal by centrifugation. Their study demonstrated the ability of FCM to determine total and viable bacterial numbers after dual staining of bacteria with SYTO BC and propidium iodide (PI). The sensitivity of the FCM procedure was $\leq 10^4$ total bacteria/mL, which is below the level of detection required for raw milk to satisfy legislation in many countries [44, 72]. A milk clearance reagent from FOSS Analytical A/S and removal by gradient centrifugation were used by Holm et al. [43] for quantification and differentiation of bacteria in Danish bulk tank milk samples exceeding 3×10^4 cfu/mL prior to flow cytometry analysis in order to predict the main cause of elevated bacterial counts in routine samples.

The suitability of FCM for the brewing [14, 45] and oenology industries [6, 16, 32, 56] has been demonstrated. The fluorogenic substrates Fluorassure Substrate B.W. [45] and Yeast Viability Substrate [16] and the fluorescent dye oxonol [14] have been used as single viability stains. Fluorescent dyes that stain cells only if they have damaged membrane potential (oxonol), have increased membrane permeability (propidium iodide) or have active esterases (CFDA) were used by Attfield et al. [6] to analyse populations of rehydrated yeasts. The detection limit for yeasts was on the order of 10^3 cells/mL. Viable, dead and injured *Saccharomyces cerevisiae* cells were enumerated in high-sugar Chardonnay fermentations using thiazole orange (TO) and propidium iodide (PI) [32]. TO, a permeant DNA-reactive stain, enters live and dead cells, thus giving a total cell count. PI, an impermeant DNA-reactive stain, can only enter dead or injured cells, differentiating them from viable cells. Wine samples were washed twice by centrifugation in sheath fluid containing Triton X-100 to reduce sugar and phenolic compounds that may interfere with dye uptake. A combination of gating on fluorescence and scatter allows yeast cells to be distinguished from debris. Flow cytometry was also applied to simultaneously detect

malolactic bacteria and yeasts inoculated in wine. Fluorescein acetate (FDA) was suitable for staining viable yeasts and bacteria. However, the fluorescence intensity of labelled malolactic bacteria depended on the strain. The lowest bacterial concentration detected by FCM was 10^4 cells/mL [56]. The interference of natural debris present in wine was overcome by optimising sample preparation, i.e. centrifuging and washing the cells twice with PBS. We have studied the suitability of FCM for detecting lactic acid and acetic acid bacteria in yeast starter cultures and sparkling wines after dual microbial staining with SYTO 13, a permeant-DNA stain, and PI. The lowest bacterial concentration detected by FCM was 5×10^3 cells/mL. The presence of lactic acid bacteria and acetic acid bacteria in yeast starters can result in a poorer wine quality and in an economic loss in oenological industries. In our work, acetic acid and lactic acid bacteria were distinguished by analysing the light scatter properties of the viable cells (data not published).

Very recently, Quiros et al. [71] showed the applicability of FCM for the development of kinetic models, using pure culture fermentation experiments of *Lactobacillus hilgardii* and *Saccharomyces cerevisiae* growing in standard culture media (MRS and YPD). They found that a large number of cells were in a viable but noncultivable (VBNC) state, which resulted in a subpopulation much larger than the damaged-cell subpopulation. The determination of the evolution of viable, VBCN and dead cells allowed them to develop a segregated kinetic model to describe the population dynamics and glucose consumption of yeast and the bacteria in batch cultures. Their model describes the behaviour and the functionality of the cultures used more accurately, providing a deeper knowledge of the status and course of the bioprocess in real time.

Spoilage and pathogen microorganism contamination

Contamination of food products by bacteria, yeasts and moulds is a major concern of industries involved in food transformation. The presence of these microorganisms in products, even at low concentrations, can severely affect their quality.

The final microbiotic composition of food products is reflective of the normal biota of the raw ingredients and the hygienic procedures used during processing. A method differentiating Gram-positive and Gram-negative bacterial populations would provide information on the source of contamination. Several Gram-staining techniques for flow cytometry have been proposed. One combines the two fluorescent DNA-binding stains SYTO 13 and hexidium iodide (HI). SYTO 13 is a membrane-permeable stain, and HI is blocked by the lipopolysaccharide layer of Gram-negative bacteria and is thus only permeable to Gram-positive bacteria and Gram-negative bacteria with a destabilised lipopolysaccharide layer [58]. Another similar technique combines Oregon Green-conjugated wheat germ agglutinin (WGA) with HI. WGA binds to *N*-acetylglucosamine in the peptidoglycan layer of the cell wall of Gram-positive bacteria whereas HI binds to the DNA of all bacteria after permeabilization by EDTA and incubation at 50°C for 15 min [42]. Using WGA instead of SYTO 13 and treating the cells with EDTA allows the latter technique to be used directly in milk samples without precultivation of the sample [42, 43].

The best-known approach that utilises flow cytometry for the specific detection of microorganisms is the use of monoclonal or polyclonal antibodies conjugated to fluorochromes such as fluorescein isothiocyanate (FITC) or phycoerythrin [28, 59, 86]. Although this labelling method represents a direct technique for the detection of specific microorganisms, it suffers from significant limitations. The first impediment for specific microbial detection in a complex medium by FCM is the high sensitivity required, especially where pathogens are concerned. Regulations for food products stipulate, for example, the absence of *Salmonella* sp. in 25 g of product, which corresponds to a sterility test. Optoelectronic noise generated at the instrument settings needed to detect small particles such as immunofluorescent bacteria as well as interferences from particles contained in the product which produce the same fluorescence as labelled bacteria are both likely to prevent the direct detection of such low bacterial concentrations [69]. Thus, in these circumstances, amplification of bacterial concentrations by culture is likely to be necessary before FCM analysis.

Chemunex S.A. developed and marketed an instrument (which, together with the reagents, is called the ChemFlow System) to detect contaminating yeast in dairy and fruit products The procedure used by this system calls for incubation of the product for 16–20 h followed by centrifugation to separate and concentrate the cells. The stain is then added, and a sample is passed through the flow cytometer for analysis. Investigation of the ChemFlow system by Bankes et al. [7] utilised a range of dairy and fruit-based products inoculated with yeast. The results indicated that yeast levels as low as 1 cell/25 g could be detected in 24 h in dairy products. A similar sensitivity was reported in fruit juices; however, a 48-h period was required to ensure that this was achieved. The system was found to be reliable and easy to use [67].

Laplace-Builhé et al. [51] showed that yeasts and mould contamination can be rapidly detected using fluorogenic viability substrates (Chemunex SA), specific mainly for esterases, for FCM analysis. Levels of less than 5 spores/g for mould and less than 0.5 yeast/g were detected within 24 h. A sample is considered contaminated above 100 counts/g. High levels of contamination ($\geq$100 yeast/g) were detected within a shorter period. A comparison of

FCM results with shelf life was also carried out. There was a direct relationship between FCM counts after 24 h of incubation and the quality of the product.

Whole fixed cells can be identified and counted directly by fluorescent in situ hybridization (FISH), allowing different microbial species in mixed cultures to be distinguished [8, 39, 82]. Detection of specific food contaminants can be achieved by FISH coupled with FCM (FLOW–FISH). For example, rapid and sensitive FLOW–FISH methods have been used for detecting and enumerating carnobacteria and *Lactobacillus brevis* in seafood products [24] and lactic acid bacteria used as starter cultures [34]. In this latter work, FLOW–FISH was able to discriminate the two subspecies *Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*, and *Leuconostoc* spp. Quantitative information about the composition of starter cultures is very important to maintain or optimise the properties of the culture. In this regard, by identifying specific target sequences of the 16S rRNA of the taxa of interest and using live/dead dyes, FLOW–FISH can be used to calculate the percentages of the relevant taxa in probiotics and starters.

Starters

Starter culture strains are selected for their fermentation capacities and their flavour formation characteristics. Other important criteria are the robustness of the cells during processing and the maintenance of different strains in a mixed starter [17]. In the food industry, lactic acid bacteria (LAB) and yeasts are used for the production of fermented foods, such as cheese, yoghurt, wine, and fermented meat products.

The main starter strains used in the dairy industries are lactic acid bacteria. These strains play an essential role in the production of lactic acid and of volatile compounds such as diacetyl and acetaldehyde. All factors that may reduce or stop the normal process of acidification during fermentation (i.e. antibiotics, bacteriophage attack or low viability of the starter culture) can induce defective products and require costly clean-up procedures before new products can be produced. The first control carried out on the starter cultures is the evaluation of the number of viable bacteria. The use of FCM for cell counting and for the specific detection of strains has already been summarised in this review.

Some starters have a dual role during food manufacture. For example, in cheese production LAB are responsible for the rapid acidification of the milk. During a late stage of the process, intracellular enzymes of the starter bacteria are released through permeabilization or complete autolysis of the cell into the cheese, playing a crucial role in the generation of flavour components. By selecting rapidly lysing strains and process conditions that favour lysis, flavour development may be enhanced during ripening [25]. FCM has been proven useful for measuring the lysis of cheese bacteria. Using the LIVE/DEAD *Bac*Light bacterial viability kit, the lysis of *Lactococcus lactis* was monitored by counting the number of intact and permeable cells at different time points [20, 79]. FCM showed the presence of permeabilized cells at day 1 of cheese ripening. Throughout ripening, the permeabilized cell population cells increased as a proportion of the total cell population. Another complete FCM assay, with CFDA and TOTO-1, was developed for measuring the viability of dairy fermentation starters [18, 19]. 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. TOTO-1 is a nucleic acid dye that is excluded by intact cells and stains membrane-compromised cells yellow–green. Staining four dairy starters with CFDA and TOTO-1 revealed three populations: cultivable cells, cells that were intact and metabolically active but not cultivable, and permeabilized cells. The total FCM counts were three to five times higher than the respective plate counts. Approximately 50% of the mother starters were permeabilized. The other 50% of the cells were intact and active. The number of intact cells in the starters was higher than the number of colonies on plates. Cells that are not cultivable—between 35 and 60% of the intact cells—may still be metabolically active, and these cells may be involved in fermentation [18]. The FCM assay with dual staining is very accurate and highly sensitive, and provides tools to assess the functionalities of different populations in fermentation starters.

The infection of starter cultures by virulent bacteriophages is a serious problem for the dairy industry, leading to either complete loss of the fermentation batch or, if only one out of several strains is affected, an altered flavour. Michelsen et al. [61] have recently used FCM for fast and early detection of phage-infected *Lactococcus lactis*. Infected cells at the lysis phase were clearly separated from the uninfected cells by flow cytometry. Light scatter of the infected cells was dramatically reduced to 30–50% of that of normal cells due to a loss of mass from the cells. Fluorescence was used to monitor the DNA content, which decreased when cells were infected by phages that degraded the host DNA during infection. This method allows the detection of phage-infected bacteria, independent of the number of strains in the starter culture, if food debris is removed beforehand.

Probiotics

Oral probiotics are living microorganisms that, upon ingestion in certain numbers, exert health effects beyond inherent

basic nutrition. Probiotics may be consumed as either a food component or a non-food preparation [37]. In the development of probiotic foods intended for human consumption, strains of lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium* have been used most commonly. Some important issues in the development of probiotics are growth and survival during production and shelf life, resistance to gastric acid and bile salts, modulation of the immune response, and persistence in the gastrointestinal tract [31, 47]. Although knowledge regarding the survival, activity and dose response of probiotics is limited, a minimum daily dose of 10^8–10^9 living microorganisms has been suggested. The use of FCM to enumerate LAB was addressed in the previous section [9, 18, 50].

Assessment of viability is not the only trait to consider in the selection of probiotics. Cells that are active but not cultivable might contribute to many of the proposed health effects (e.g. lactose conversion). Furthermore, some probiotic effects are performed by nonviable cells of probiotic bacteria (e.g. adhesion to intestinal mucus and modulation of immune response) [65]. Because of the complexity of the possible contributions of different cell populations to the proposed probiotic effects, FCM could be a very useful tool to study probiotics. Bunthof and Abee [18] used FCM with CFDA and TOTO-1 to quantify intact cells and cells with damaged membranes in three probiotic products: a powder, a drink and a yoghurt. When the results of FCM and plate counting analyses were combined, three populations were evident: a cultivable population, the population of intact and metabolically active but not cultivable cells (FCM count with CFDA minus plate count), and the permeabilized (dead) population (FCM count with TOTO-1). The proportions of the populations differed in the products tested [10].

The evaluation of cell viabilities in stored probiotic foods is of vital importance economically and technologically, and it is also important for efficacy. The FCM viability assay could also be used to study the effect of prolonged storage on probiotic products. Lahtinen et al. [50] investigated the viability and intrinsic properties of three probiotic bacteria during six weeks of storage. Membrane integrity was assessed using a LIVE/DEAD *Bac*Light bacterial viability kit. Esterase activity was measured using CFDA-PI and cells incubated with succinimidyl ester of CFDA (CFDA-SE). The CFDA-SE-stained cells were also used to measure intracellular pH. The cells with an internal pH higher than the minimum intracellular pH required for cell growth were identified as viable. Although the strains studied showed a reduction in plate counts, indicating a loss of cell cultivability, the cells were able to maintain cytoplasmic membrane integrity, esterase activity, and pH gradient. Thus, FCM is a useful tool to detect so-called dormant cells.

Resistance to bile salts and persistence in the gastrointestinal tract are important characteristics of probiotic bacteria. Ben Amor et al. [9] used single staining with CFDA, oxonol and PI in combination with FCM to measure the esterase activity, membrane potential and membrane integrity, respectively, of two *Bifidobacterium* probiotic strains under bile salt stress conditions. These single stains reflected the responses of probiotic strains to the damaging effects of bile salts, revealing the degree of cell viability and the physiological status of stressed cells. Multiparameter FCM in combination with cell sorting confirmed that the bile salt-stressed cell populations contained a mixture of viable cells, dead cells, and an injured subpopulation. Moreover, this method revealed the physiological heterogeneity within the injured cell population. In conclusion, FCM with cell sorting may provide an ideal tool to analyse the activity and stability of probiotics under stress conditions [63].

Food technology and preservation

Although heat is still the treatment most commonly used to preserve foodstuffs, thermal processing has several undesirable effects on the nutritional and sensory attributes of foods. Alternative methods to conventional heat treatments for preservation purposes have received increasing attention in the food industry, e.g. high hydrostatic pressure, high-intensity ultrasound and pulsed electric field treatments.

The resistance of microorganisms to high pressure is variable, and the reduction in the microbial population is directly related to the amount of hydrostatic pressure applied [4]. Cell lysis and the viability of different populations of saprophytic, pathogenic and phytopathogenic microorganisms following pressurization treatments were measured by FCM. PI was used to measure membrane integrity. Flow cytometry has been proven to be a useful tool for establishing the efficacy of pressurisation by determining the percentages of dead, living and metabolically inactive cells [5]. Ritz et al. [74] characterised the morphological and physiological states of cells when high-pressure treatment was applied to a population of *Listeria monocytogenes*. Cellular morphology was studied by estimating the cellular volume measured by light-scattering flow cytometry. The fluorescent dyes PI, $DIBAC_4(3)$, and CFDA were used to assess the membrane integrity, membrane potential, and enzymatic activities, respectively, of cells by flow cytometry. FCM revealed that cellular morphology was not really affected; esterase activity was dramatically lowered, but not completely obliterated, after pressure treatment; and membrane integrity and membrane potential were not homogeneous across the cellular population. The effectiveness of flow cytometric multiparameter analysis in characterising the physiological state of cells after pressurisation

was validated by other analytical methods. Flow cytometry with CFDA and PI staining was also used to monitor pressure-induced changes in *Lactobacillus rhamnosus* [2]. Their results correlate well with those found in *L. monocytogenes*-pressurised cells [74]. Both studies showed that pressurised inactivated bacteria were still in possession of enzyme activity, and not completely membrane compromised. The heterogeneity of the treated cell population suggests that reversible damage may occur and that it should be taken into account in high-pressure treatment applications in the food preservation industry. The presence of metabolically active but not cultivable bacteria with a high degree of membrane intactness in food may be critical in terms of their potential for excreting toxic or food-spoiling metabolites.

The concept of ultrasound-assisted thermal processing (thermosonication), which is based on the synergy between ultrasound and heat for bacterial inactivation, is of potential interest in food preservation, especially for enhancing the detrimental effects of conventional thermal treatments on food quality and functionality [88]. Ananta et al. [3] studied the effect of high-intensity ultrasound on *L. rhamnosus* and *E. coli*. The Gram-positive species was more resistant to the lethal effect of ultrasound than the Gram-negative species. CFDA and PI staining in combination with flow cytometry demonstrated the rupture of the lipopolysaccharide layer of the outer membranes of Gram-negative bacteria by ultrasound. It is known that the presence of outer membrane disables the entry of many bacteriocins, reducing their bactericidal efficacy. This limitation could be overcome by ultrasound-assisted physical disruption of the outer membrane.

Pulsed electric field (PEF) treatment is the application of pulses with very high field strength for a short time (microseconds) to foods placed between two electrodes. Membrane permeabilization due to PEF treatment of *Lactobacillus* species was investigated by Wouters et al. [87] using PI uptake and single-cell analysis with flow cytometry. The electric field, the amount of energy input (i.e. the number of pulses), treatment time, treatment medium conductivity and pH, and the growth phase affected the membrane permeabilization of cells, as demonstrated by PI uptake. Cell size and shape in the *L. plantarum* population were estimated by measuring the forward and sideward scatter by the FCM method. Following sorting of the cells with the FCM technique, two populations were distinguished from each other on the basis of size distribution and cell shape. The small cells appeared to be less vulnerable to membrane permeabilization by the PEF treatment than the large cells. However, after increasing the time of treatment or the energy input of the PEF, the difference between the numbers of permeabilized cells in the small- and large-cell populations disappeared. The membrane permeabilization of two *Lactobacillus* strains, *L. plantarum* and *L. fermentum*, after PEF inactivation was compared. The *L. fermentum* strain was found to be more PEF resistant and showed less membrane permeabilization than the *L. plantarum* strain, which was the more PEF-sensitive strain. Thus, permeabilization of the membrane is involved in determining the inactivation of vegetative cells during PEF treatment. Therefore, many factors are involved in determining the inactivation of microorganisms by PEF treatment.

Conclusions

Flow cytometry can be applied in many areas of food microbiology. This technique can provide a rapid and sensitive method for the rapid enumeration of microorganisms. The success of the system depends on the development and use of suitable staining systems, and protocols for the separation of microorganisms from food debris that would otherwise interfere with the detection system. Multiparameter flow cytometry and single cell scatter allow the analysis of many parameters at once, thus considerably simplifying testing regimes. The possibility of simultaneously detecting the membrane integrity, membrane potential, intracellular pH, and the enzyme activity of a microorganism in culture makes flow cytometry valuable for understanding the effect of different treatments on microorganisms, and hence for evaluating the effectiveness of technological processing for microbial inactivation purposes.

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