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Review

Flow cytometry as a potential method of measuring bacterial viability in probiotic products: A review

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1. Introduction

Bacterial viability currently refers to ability of a cell to grow and reproduce itself under a set of defined environmental conditions (Kennedy, Cronin, & Wilkinson, 2011). The characteristics of viable cells include the presence and functioning of a range of structural, metabolic, physiological and genetic properties. However, this traditional definition requires that a single "viable" cell must grow and subsequently generate a colony of cells which can be measured optically either in liquid or solid media, thus forming the basis of Koch's principles (Muller & Nebe-von-Caron, 2010). Hence, viability is generally equated with the ability to reproduce and subsequently by cultivability. However, it is clear that not all bacterial cells obey this relationship and indeed, while they may lack the ability to reproduce and grow under certain conditions, they may possess many of the properties of fully functioning viable cells. Hence, we arrive at another classification based on the term "vitality" or the degree to which a cell can perform various aspects of metabolic, physiological and genetic functionality and the extent of structural and morphological integrity (Kramer & Thielman, 2016). In this case, cell vitality is not exclusively related to reproducibility or cultivability and in certain aspects the cell can be described as being in a viable-but-non-culturable state (VBNC) as reviewed by Zhao, Zhong, Wei, Lin, and Ding (2017). A truly "dead" cell may therefore lack the minimal structural integrity and ability to carry out basic cell functionality such as control and activity of physiology, metabolism and genetic material and possess neither vitality or viability characteristics (Davis, 2014).

Based on these various states, enumeration of bacterial cells has evolved into two general methodologies; (1) culture dependent and (2) culture independent (Davis, 2014). In the former technique, a liquid sample is plated onto solid media, incubated under defined conditions of time and temperature with data expressed as numbers of colony forming units per gram of original material (cfu/g) (Davey, 2011). This traditional methodology is still the "gold standard" and is backed by legal status for use in routine enumeration and identification of live/viable microorganisms in food samples (FAO/WHO (2002); ISO (2002); Ministero della Salute (2013); Hill et al., 2014; Sanders et al. (2016)). This methodology is a classic example of where viability depends exclusively on the ability of the cell to reproduce with visual inspection of the colony formed from the original single cell. Culture-independent methodologies for bacterial enumeration involve DNA based technologies such as polymerase chain reaction (PCR) or quantitative PCR (qPCR) whereby a specific gene fragment within a DNA sample is amplified and the fluorescent signal directly related to the number of cells in the original sample. This technology is highly useful but may not always distinguish DNA originating from viable or non-viable cells. However, reagents such as ethidium monoazide (EMA) or propidium monoazide (PMA) can be included in the assay to prevent amplification of DNA from dead cells (Martinon, Cronin, Quealy, Stapleton, & Wilkinson, 2012). Another emerging rapid culture-independent technique for bacterial enumeration is flow cytometry, which utilises a combination of light scattering and emitted fluorescence to detect cells having various structural, physiological and genetic states (Cronin & Wilkinson, 2010).

2. Flow cytometry and microbiology

Flow cytometry (FCM) involves generating a liquid suspension of

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bacteria from a food sample which is then moved within a liquid stream known as sheath fluid. As each cell passes a point within the flow path it is interrogated by a laser which scatters light in two major directions known as Forward angle light scatter (Forward scatter or FSC) or Side angle light scatter (Side scatter or SSC). Both FSC and SSC can, in a general way, discriminate cells based on their size and granularity and hence is used to locate cells on a profile known as a cytograph. This is the first step in bacterial detection and enumeration and allows cells to be distinguished from inert matrix particles in a sample e.g dairy foods. The second, and more specific, cell detection and enumeration method involves collection of fluorescence signals emitted at differing wavelengths from labelled/stained cells as they pass lasers. Data is collected from individual stained cells and the degree of uptake of a particular stain allows discrimination of cells into discrete sub-populations. Thus, FCM data may reflect differing properties such as the extent of cell membrane integrity, functionality of membrane potential, presence of intracellular enzyme activity and DNA base composition (Egli & Kotzsch, 2015; Kennedy & Wilkinson, 2017; Overton, 2015; Wilkinson, 2016). In this way, additional data can be obtained on individual cell physiology and structure by staining of cells with specific fluorescent dyes. Combinations of dyes are frequently used to generate multiparametric data from individual cells and sub-populations. The main advantages of FCM for microbiology are: rapid assay times and data generation (1–2 min), high numbers of cells that can be analysed per sample (10,000 and upwards), minimal sample volume (from 5 μl), potential high throughput, multiplicity of stains available to examine various aspects of cell viability, structure and/or metabolism (multiparametric), and less labour and space required compared with conventional plating techniques (Wilkinson, 2016). Flow cytometry is used within microbiology in an expanding range of applications including; medical diagnostics (Aebisher, Bartusik, & Tabarkiewicz, 2017; Diaz, Herrero, Garcia, & Quiros, 2010), water treatment and supply quality monitoring (De Roy, Clement, Thas, Wang, & Boon, 2012; Egli & Kotzsch, 2015; Hammes et al., 2008), brewing fermentation monitoring (Achilles, Harms, & Müller, 2006; Bühligen et al., 2014; Overton, 2015), pharmaceutical, environmental testing (Herrero & Diaz, 2015), the dairy industry (Doolan, Nongonierma, Kilcawley, & Wilkinson, 2014; Pane, 2013; Sheehan, O'Loughlin, O'Cuinn, FitzGerald, & Wilkinson, 2005; Yanachkina, McCarthy, Guinee, & Wilkinson, 2016) and for manufacturers of probiotic cultures and products (Davis, 2014; Raymond & Champagne, 2015). The general industrial applications of FCM include rapid detection of pathogens, estimation of cell damage following processing steps such as heating, spray drying and freeze drying, and monitoring of bioprocesses (Diaz et al., 2010; Herrero & Diaz, 2015).

2.1. Stains and viability

In order to make an assessment of the viability or vitality of individual bacterial cells in foods, a range of fluorescent stains, also referred to as probes or dyes, have been developed for use in FCM based assays (Longin, Petitgonnet, Guilloux-Benatier, Rousseaux, & Alexandre, 2017). It is beyond the scope of this article to individually assess the properties of each stain, however, it is clear from the literature that an extensive range are now available (Davey & Winson, 2003; Diaz et al., 2010; Egli & Kotzsch, 2015; Overton, 2015; Strauber & Muller, 2010). The more relevant ones for microbial enumeration relate to those stains purporting to reflect viability or "Live" or "Dead" states. The most common stains used to assess viability are the Propidium Iodide (PI) and SYTO 9 combination. In this protocol, SYTO 9, a membrane permeant stain, binds to all bacterial DNA or RNA within a sample to generate a green fluorescent signal. On FCM analysis, detection of the SYTO 9 positive population should therefore represent a total count of the cells or events present. Exposure of the same bacterial population to a counterstain of PI, a membrane impermeant stain, which labels DNA and RNA but which displaces SYTO 9, should then yield a population comprised of live, damaged or dead cells. This estimation of viability is based on the assumption that intact (PI negative) cells are always live/viable while PI positive cells are always dead/damaged. However, most FCM profiles will yield a double positive population (PI and SYTO 9 positive) which may actually make up a very significant proportion of cells in sample e.g cheese during ripening (Sheehan et al., 2005; Yanachkina et al., 2016). The latter cells may have differing metabolic activities and may well reflect a VBNC state. Shi et al. (2007) highlighted the limits of PI as an indicator of the viability of two bacterial species originating from soil, *Sphingomonas* sp (Gram negative) and *Mycobacterium fredericksbergense* (Gram positive). These workers examined the effect of carbon source and stage of growth on stain uptake and viability on agar plates after cell sorting. During early exponential growth phase, temporary high levels of PI positive cells (8–40% of the population) were evident which differed depending on substrate but which subsequently reverted to lower levels (2–5% of the population). Cell sorting of PI positive ("dead") cells from both exponential and stationary phases of growth revealed species-specific responses to staining by PI. Significantly, the authors noted that high levels (50–70%) of PI positive cells were actually culturable after sorting but decreased substantially during stationary phase. In contrast, only 4% of *E. coli* cells labelled with PI were culturable and hence PI appeared to better reflect non-viability for this species. This study clearly indicated that PI staining is not always a reliable indicator of cell death and indeed certain bacterial species possess the ability to take up significant levels of PI during growth and yet remain culturable. A number of workers have used the stain c FDA (carboxyfluorescein diacetate) as a measure of viability of a population. This stain can passively diffuse into the cell interior where it is hydrolysed to the fluorescent CF derivative by intracellular esterases in actively metabolising cells to generate a green fluorescent signal. The latter stain is frequently combined with a membrane impermeant counter stain such as PI or TOTO-1 to yield viability data regarding the total culturable and non-viable/non-culturable populations. Hansen et al. (2015) examined the effect of various staining regimes on the physiology and culturability of three *Lactococcus lactis* strains. Exposure of the lactococcal strains to varying concentrations of the viability stains, c FDA, c FDA-SE, or the non-viability stains PI or TOTO-1 was simultaneously compared with culturability on agar plates and by monitoring of other physiological functions such as growth and acidification activity. Generally, exposure of lactococcal strains to either single or double staining procedures negatively affected growth and resulted in reductions in plate counts compared with unstained controls. A greater cytotoxic effect with reduced plate counts and elongated lag phase was noted for TOTO-1 compared with PI, while c FDA-SE also appeared to exert a greater negative effect on plate counts compared with c FDA. Therefore, the c FDA/PI combination would seem be more suitable for viability assessment compared with the c FDA-SE/TOTO-1 dual staining. This study highlighted the necessity for all FCM assays under development to include adequate examination of the effects of individual and combinations of stains on cell viability of cultures.

2.2. Direct correlations of bacterial enumeration using FCM and plate counting in foods

Gunasekera et al. (2000) reported a rapid FCM-based enumeration method for bacteria in UHT-treated or raw milks. These workers recognised the importance of first removing any interfering protein and lipid matrix particles prior to FCM analysis. Milks were cleared of particulates using a combination of a proteinase, lipase and the addition of NaCl and a detergent. UHT-treated milk samples were then spiked with viable cell populations of either *Escherichia coli* or *Staphylococcus aureus* in the range 10^3 to 10^8/ml. FCM data was compared with fluorescence microscopy and selective agar plating on Chromocult or Baird-Parker agars, respectively. FCM involved dual live/dead staining of pelleted cultures with SYTO BC and PI. In the case of *E coli*, plate

count data did not significantly differ ($P < 0.05$) from that obtained by FCM over the range 10^4 to 10^8/ml. Comparison of *S. aureus* plate counts and FCM data also indicated a high degree of agreement ($P < 0.05$) over the range 10^3 to 10^8 bacteria per ml. Evaluation of the method using 38 fresh raw milk samples or 21 samples stored for 48 h at 4C further indicated a good correlation between plate counts and FCM data ($r = 0.91$) which involved single staining with SYTO BC. The authors suggested that the sensitivity of the FCM method for raw milk analysis was $< 10^4$ bacterial cells per ml with a significant improvement in time to result (TTR) compared with standard plate counting techniques. A later study by these authors evaluated the potential of flow cytometry for enumeration of bacteria and somatic cells in both raw and UHT-treated milk (Gunasakera, Veal, & Attfield, 2003). A Peptide Nucleic Acid probe (PNA) in a fluorescent *in-situ* hybridisation FCM (FISH-FCM) assay was used to label and distinguish *E. coli* cells from matrix particles. Data from the FISH-FCM assay was compared with FCM using SYTO BC staining and with plate counts. At populations $> 10^4$ cells/ml, good agreement was obtained with plate counts for SYTO BC-FCM. In contrast, at populations $> 10^4$ cells/ml milk, FISH-FCM significantly underestimated numbers compared with plate counts. Interestingly, at 10^3/ml, FISH-FCM showed a good correlation with plate counts while at 10^2 cells/ml it overestimated cell numbers. Using sample clearing steps previously reported, the authors introduced varying levels of live and heat killed *S. aureus* or *Pseudomonas fluorescens* to a total population of 10^7/ml. FCM was carried out to distinguish live from dead cell sub-populations by staining of live cells with CTC (5-cyanol-2,3-ditolyl tetrazolium chloride) generating a red fluorescent signal in the presence of dehydrogenase activity. Dead cells were identified by use of a counterstain of Bis-1,3-dibutylbarbituric acid trimethine oxonol (OXN) which generated green fluorescence following a loss in cellular membrane potential. Unfortunately, data regarding any correlation between plate counts and CTC populations was not reported.

A comprehensive study of FCM and plate counting was undertaken by Khan, Pyle, and Camper (2010) aimed at developing rapid FCM differential staining methodology to enumerate culturable and viable-but-non-culturable (VBNC) populations of four Gram negative bacteria, *E. coli* O157:H7, *Pseudomonas aeruginosa*, *Pseudomas syringae* and *Salmonella enterica* serovar Typhimurium. A range of SYTO dyes were evaluated for enumeration of viable cells including SYTO9, SYTO13, SYTO17 and SYTO 40. Non-viable cell populations were stained using PI. Stain optimisation protocols were performed for each bacterial strain based on achievement of stable total (viable and non-culturable) populations following exposure to SYTO based stains for various durations. The authors recommended a 60 min incubation period for all strains when using the particular dyes chosen for population enumeration. SYTO9 and SYTO 17 were optimal for *E. coli* O157:H7, while SYTO 9 and SYTO 13 were optimal for the other three bacterial strains. Statistical data analysis was limited to plotting of plate count data in cfu/ml against total cell numbers per ml as determined by FCM using the optimised stains. Overall, counts determined using FCM appeared to be consistently higher than plate count data for all bacterial strains except for *P. syringae*. Despite discrepancies between data, the authors suggested that FCM count data appeared to be more repeatable with a greater precision, based on a narrower range of distribution of true mean values. However, FCM data appeared dependent on stain used and indeed the choice of stain affected the ratio of viable, culturable cells to VBNCs. An examination of the numbers of PI positive cells (dead or damaged/permeabilised) with plate count data of culturable cells following exposure of strains to heating at 72 °C for up to 15 min showed some interesting trends. Generally, increasing the duration of exposure to heating of cells resulted in a reduction in viability by plate counting, with the greatest reduction (> 2 log) noted after an initial 5 min heating step. However, trends noted by plate counting were not reflected in the numbers of PI positive cells detected by FCM and the treatments which resulted in highest levels of PI populations did not produce the greatest reduction in viable plate counts e.g for *P. aeruginosa*. This data indicates the complexity of the relationship between cell membrane damage, individual strain and resultant cultivability. Another factor in the above relationship is the influence of media type, while Khan et al. (2010) plated cells onto standard nutrient agar, Kennedy et al. (2011), noted differences in the percentage recovery of stressed cells following cell sorting onto either nutrient or selective agars. In terms of method development, Khan et al. (2010) established protocols for staining, detection and analysis of viable and VBNC populations using FCM and recommended a cell concentration of 10^4/ml as being optimal for this analysis. However, as FCM analysis of bacteria was not carried out within more complex food matrices it would be interesting to determine whether these cell concentrations are still feasible to analyse by FCM with particle interference. Khan et al. (2010) provided a useful insight into the magnitude of the VBNC populations present when cells were cultured in a non-nutrient limiting broth medium, showing that > 30% of cells were in the VBNC state while up to ~5% could be classified as dead. The effects of cell damage on *E. coli* and *S. aureus* strains induced by various ultrasound treatments were evaluated by Li et al. (2016). These authors compared plate counts with FCM following dual staining with PI and c FDA, the latter stain may reflect cell viability and/or cell vitality. Significant killing effects were noted for ultrasound treatments of up to 20 min duration and were reflected in decreased viable plate counts. The response of *E. coli* to ultrasound with time indicated an increase in PI populations compared with *S. aureus* and reflected plate count data. However, a statistical correlation was not presented regarding the extent of agreement for data obtained by plate counts and FCM.

Bunthof, Bloemen, Breeuwer, Rombouts, and Abee (2001) evaluated the fluorescent probes c FDA, PI and TOTO-1 to examine the viability of number of LAB strains following stressor treatments. Interestingly, the authors noted strain-related differences in the degree of fluorescence intensity following staining by c FDA, despite using a similar protocol for all strains. Differential labelling intensity of the various strains following heat killing was also noted for PI. Based on the response of heat killed or control cells to these stains, TOTO-1 was selected as a better indicator of non-viability than PI, while c FDA was considered as reflecting viability. Thereafter, FCM assays based on single staining with PI and c FDA were compared with viability by plate counting for *Lactococcus lactis*, *Leuconostoc lactis* and *Pediococcus acidilacti* grown in broth and exposed to stressors including heat, acids and bile salt concentrations. Single strain cultures were harvested from broth as concentrated cell suspensions and exposed to increasing concentrations of bile salts and decreasing pH values. This resulted in significant changes in FCM profiles (increased PI fluorescence and reduced c FDA fluorescence) and were reflected by decreased plate counts. Dual PI and c FDA staining indicated the ability of FCM to measure changes in sub-populations in response to stressor treatments. Cell sorting onto agar plates was then used to verify whether the sub-populations stained by either c FDA or PI reflected live/culturable or dead/non-culturable populations. However, while this study was very useful as regards method development and demonstration of the application of FCM to the study of population heterogeneity, statistical correlations were not determined for the nature of the relationship between FCM and plate count data. Rather, it appeared that the trends for FCM were in many cases reflected by a loss in culturability using plate count data.

An FCM method for analysis and enumeration of a single strain probiotic *Lactobacillus plantarum* culture and mixed strain cheese or yogurt starter cultures containing *Lactococcus*, *Leuconostoc*, *Streptococcus thermophilus* and *Lactobacillus* species was reported by Bunthof and Abee (2002). Difficulties were encountered with matrix particle interference with higher than threshold levels of FSC in milk samples. However, a clearing solution, containing enzymes and detergent, enabled satisfactory FCM analysis to be undertaken. Bacteria recovered from dairy products and milk samples were initially stained with SYTO 9 to obtain a total cell count. A second dual staining protocol was undertaken using cFDA (to detect live cells) and the membrane-

impermeant stain TOTO-1 to detect dead or damaged cells. Total counts were determined by FCM and involved calculations based on the difference in numbers obtained from staining by TOTO-1 of cells grown in milk heat treated at 70° C or in untreated milks. FCM data was correlated with plate count data from MRS agar. In the case of *L. plantarum* cells added to milk at levels of 10^5 to 10^9 cfu/ml, comparison of data indicated better replication for FCM due to the inherent ability of the FCM method to analyse 3,000 to 20,000 events, thus generating a considerably lower Coefficient of Variation (CV) compared with plate counts. A regression (r) value of ~0.999 was noted between plate count (expressed as Log plate counts/ml) and FCM data (expressed as Log FCM counts per ml) for SYTO 9 and for CFDA and TOTO-1 staining. A considerable loss of cells (~29%) was noted during the cell extraction procedure required for FCM analysis. The effects of mixing unheated milk, with milk treated at 70 °C, both containing *L. plantarum*, indicated the emergence of a significant TOTO-1 positive population, reflecting a degree of cell damage, with a high degree of correlation of FCM data with plate count data ($r > 0.99$). Analysis of mixed strain dairy starter preparations, either grown in milk or diluted from commercial cell concentrate preparations, was undertaken using FCM and plate counts, again samples were heat treated and compared with untreated controls. Generally, FCM counts using the dual staining procedure gave population data which was three times higher than that determined on agar plates, and, in the case of one starter preparation was five times higher. Analysis of commercial probiotic products were also undertaken, which required a clearing procedure for one sample, while two other products were not cleared prior to FCM analysis. Staining revealed the presence of three sub-populations in these products namely: culturable cells, intact cells with metabolic activity as measured by c FDA fluorescence, and permeabilised/dead cells. However, good agreement was not found between plate count data and total counts by FCM, with the latter method overestimating total counts by 1–2 log. In all three products analysed by FCM, variable levels of the three different sub-populations were present, including significant numbers of intact but non-culturable cells. The authors highlighted the usefulness and potential of this particular FCM assay for the profiling of cell sub-populations in dairy and probiotic products. However, correlations between FCM enumeration and plate count data were statistically robust only under certain controlled growth conditions and when using a single strain culture. This degree of correlation was not evident when FCM involved analysis of mixed strains in commercial products which may have been manufactured and stored under differing conditions. Further development and validation of an FCM based assay for total viable count enumeration of bacteria in milk and dairy products was reported by Flint et al. (2006) using a commercial flow cytometry based system, D-Count from Chemunex S.A, Paris, France. The method involved adding a defined amount of sample (1 g) to a broth and incubating for 90 min at ~63 °C to enable outgrowth of thermophilic spores. The microbiological sample was added to a buffer, mixed and centrifuged. The recovered pellet was stained using a proprietary fluorogenic esterase substrate hydrolysed by metabolically active intact viable cells to generate a fluorescent signal on the cytometer. Using this method, a validation trial of 178 milk powder samples was undertaken and compared with data from Milk Plate Count Agar incubated aerobically at 30 °C and 55 °C. For both methods, triplicate analyses were undertaken on each sample. Statistical analysis indicated a high level of agreement between the plate counts and FCM methods with a correlation coefficient of ~0.76. Additional samples were obtained during the manufacture of powder and concentrates with acceptable correlation coefficients e.g $r = 0.88$, for samples taken during processing of whole milk powder. This FCM-based assay was claimed to be capable of analysing 50 samples per hour and to be accurate from $\sim 1 \times 10^2$ to 1×10^5 cfu/g in milk and whey powder samples. In the case of liquid milk and whey samples, accurate counting of cells was given as being within the range 1×10^3 to 1×10^6 cfu/ml. This method did not enable any further differentiation of sub-populations such as permeabilised or dead cells and similar to most FCM methods was unable to detect specific microbial groups such as pathogens or spoilage bacteria.

The successful use of the red fluorescent single stain TO-PRO-3 for enumeration of *S. aureus*, *E. coli* and *B. subtilis* was reported by Kerstens et al. (2014). FCM analysis was based on binding of this stain to double stranded DNA in cells having compromised membranes, thereby identifying the non-viable cell sub-population within a culture. A direct comparison between viable plate counts and FCM was undertaken on overnight broth cultures diluted to give a range of cell concentrations. Stain optimisation was performed for the three cultures to ensure clear discrimination of a mixture of viable and non-viable (heat killed) cells. A high degree of correlation was determined between FCM and plate counts ($r^2 > 0.99$) over the range 6×10^3–6×10^7 viable cells/ml for the three strains. Similar to other reports, the precision of FCM data was higher than for manual plate counting which had a coefficient of variation (CV) of ~15–32% compared with FCM of ~3–7%. These workers proposed that FCM methodology may have further applications in rapid enumeration of bacteria in other more complex matrices such as food and environmental samples. Overall, data from published studies indicated that analysis of bacterial cultures with high viability, such as those used in spiking studies, resulted in a strong statistical correlation between plate counts and FCM. In contrast, cultures with reduced viability, as affected by storage time or other variables (Tripathi and Giri (2014)), did not generate the same degree of statistical correlation between methodologies. A generalised relationship between plate count data and FCM data from these studies is given in Fig. 1.

2.3. Immuno-FCM for differentiation of bacterial species and plate count comparisons

While many reports indicate differential uptake of probes by differing bacterial strains or species, the ability to enumerate specific bacterial strains within a mixture of strains is extremely difficult using only the fluorescent probe approach. Hickey, Fallico, Wilkinson, and Sheehan (2018) reported clear differentiation of FCM bi-plots of *Lb. helveticus* and *S. thermophilus* starter cultures in cheese extracts based on the differential uptake of SYTO 9 and PI stains by rods or cocci. However, an alternative method of species differentiation is to combine viability staining with specific antibody labelling to differentiate within mixed strains. Clarke and Pinder (1998) reported the specific labelling and enumeration of a viable *Salmonella typhimurium* population in the presence of an *E. coli* population. This study was carried out in buffer systems using a combination of a red fluorescent conjugated monoclonal antibody and a green fluorescent viability stain, which required intracellular bacterial enzymatic cleavage for fluorescence generation. Initial assay development work, using only the viability stain, indicated a strong correlation between FCM counts and plate counts over the range 10^2 to 10^7 cells/ml, while viable cells could be detected at $\sim 1 \times 10^2$/ml in the presence of $\sim 3 \times 10^7$ dead *S. typhimurium* cells. In a mixed population of *S. typhimurium* and *E. coli*, immuno-FCM was compared with plate counts which indicated a strong relationship between FCM data and plate counts over the range 10^2 to 10^7 cells/ml. Interestingly, statistical data analysis using the Student t-test indicated a difference between FCM viability data and plate counts, with FCM enumeration being lower than plate counts. This conclusion was not in agreement with other studies which generally reported a more consistent trend with FCM overestimating viable populations compared with plate counts. However, Clarke and Pinder (1998) recognised that the viability stain may not have been taken up by all of the population capable of growth, and that some resuscitation of damaged cells may also have occurred on agar media. Geng, Chiron, and Combrisson (2014) reported a proof of concept study using immuno-FCM to enumerate viable probiotic Bifidobacteria in dairy foods containing mixed cultures. The basis of the immuno-FCM assay involved labelling using a primary polyclonal antibody specific for *B. lactis* and *B. animalis* species.

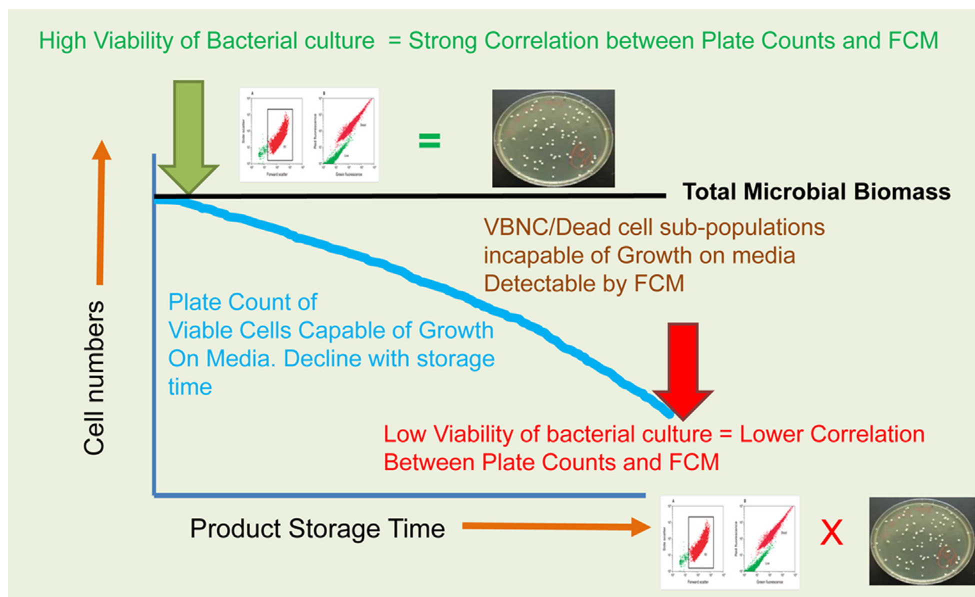

Fig. 1. Generalised relationship between viability of probiotic bacteria during product storage as measured by Plate Counts and Flow Cytometry data.

Thereafter, a secondary anti-rabbit IgG antibody conjugated to a red fluorescent tag was bound to the primary antibody-attached cell complex. This complex was then stained using the green fluorescent dye SYTO 24 which labelled all cells present in a prepared broth culture, at a concentration of 10^6 cfu/ml, to yield total cell counts. For analysis of Bifidobacteria in commercial probiotic dairy products viability was determined using c FDA labelling and FCM data was compared with an ISO method for plate counting. To minimise cell losses, a single step labelling of bacteria was carried out using a pre-formed primary-secondary antibody complex to reduce the two washing steps normally used for separate addition of primary and secondary antibodies. Total counts in commercial fermented milks were determined by c FDA staining measuring green fluorescence (FL1) versus Forward scatter (FSC), while specific counts of Bifidobacteria were determined by measuring the red fluorescence of the antibody-complex (FL4) and by cFDA staining (FL1), with a minimum of 10,000 events collected per sample. Statistical analysis of data indicated a high degree of correlation between FCM data and the ISO plate count method (0.954) with a time to result, TTR, of 2 h against 72 h for plate count data. No indication was given regarding limits of detection (LOD) or limit of quantification (LOQ) and populations measured ranged from 10^7 to 10^8/g.

Holm, Mathiasen, and Jespersen (2004) reported the development of an FCM method which enumerated total bacterial counts in raw bulk tank milk, and, within the same assay, also provided information on the nature of the contaminating species. Milk samples (1 ml) were pretreated using 9 ml of a clearing solution, bacteria were removed following a gradient centrifugation step and stained. FCM involved dual staining of cells using Wheat Germ agglutinin (WGA) conjugated to Oregon Green and a Hexidium Iodide (HI) stain. The latter generated red fluorescence and labelled all bacterial DNA in a sample following a cell permeabilisation step using EDTA and heating to 50° C for 15 min. The WGA conjugate stained the cell wall of Gram-positive bacteria within a sample. Initially, a comparison was made between total counts obtained by FCM with plate counts from (n = 75) raw milk samples using a standard International Dairy Federation (IDF) method. As noted in other plate counts, a significant difference was found between methods ($P < 0.05$). A correlation coefficient of ($r = 0.71$) was found between the two methods with data collected over the range 10^{5}–10^{7} cells per ml. In this study, 7 different bacterial species commonly isolated from (n = 75) bulk raw milk samples were spiked at 10^{7} cells/ml into raw milk having a background microflora of $< 10^{4}$ cells per ml, stained and analysed as before. Based on profiles of red and green fluorescence and forward scatter (FSC) and side scatter (SSC) patterns, treatment of data enabled differentiation of samples contaminated by bacteria of differing origins. A feature of the resolving power of the FCM method was related to data treatment whereby the natural log (Ln) of FSC/SSC was plotted against the Ln of green/red fluorescence to give three clearly defined regions which were related both to the origin and nature of the bacterial contamination in the milk sample. Clustering of *Bacillus cereus*, *Lactococcus lactis* and *E. coli* were noted in one area of the FCM profile with another cluster found for *P. fluorescens* and *P. putida*. In particular, milks could be differentiated based on clear separation of profiles obtained from *Pseudomonas fluorescens* and *Streptococcus uberis*. It would be interesting to ascertain the ability of the method to analyse milk samples which may contain variable populations of a more highly diverse microflora beyond the seven test isolates used and also over a seasonal milk supply.

2.4. Direct comparisons of bacterial enumeration by FCM and PCR

The use of quantitative Polymerase Chain Reaction (qPCR) and FCM assays for enumeration of bacteria have been reported in a number of studies. One of the main technical issues with PCR is the difficulty encountered in discriminating between genetic material originating from viable or non-viable cells. In part, this has been overcome through use of DNA-intercalating agents such as Propidium Monoazide (PMA) which bind to DNA in cells with damaged membranes to prevent the amplification reaction (Martinon et al., 2012). Kramer, Obermajer, Matijasic, Rogelj, and Kmetec (2009) obtained data by real time qPCR with PMA treatment of cells prior to analysis and compared it with plate counts for the probiotic strains *Lactobacillus acidophilus* and *Bifidobacterium animalis* in pure cultures or in lyophilised probiotic products. For PCR, standard curves were plotted for plate counts in cfu/ml against Ct values for the species-specific primers, and good agreement was noted over the range 10^4 to 10^7 cfu/ml using DNA from prepared cell standards or DNA from diluted product samples. FCM analysis of cell pellets recovered from the probiotic product indicated satisfactory discrimination of viable and non-viable cells in mixtures of alcohol-treated (dead cells) and live cells using SYTO 9 and PI, for viable and non-viable cell staining, respectively. Data from products stored up to 90 days indicated an increase in the permeabilised cell population from

~10 to 15% to 20–26%. However, a direct comparison between PCR and FCM enumeration data was not reported in this study. Friedrich and Lenke (2006) utilised multiplex qPCR and a combination of FCM with fluorescent *in situ* hybridisation (FLOW-FISH) to study the composition and populations of complex undefined mixed-strain commercial starter culture products comprising of *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Leuconostoc mesenteroides* subsp. *lactis*. The FLOW-FISH assay consisted of 16srRNA probes conjugated to Oregon Green fluorescent stain and the relative populations were determined based on numbers of FISH probe labelled cells against PI positive events. Additionally, SYTO 9/PI FCM analysis was carried out on strains or mixtures of strains. Overall, the authors reported a strong correlation between plate count data and that obtained by the FCM staining with SYTO 9 and PI and also with qPCR. In terms of population percentages within a commercial blend of starter strains, a high degree of correlation was determined for predicted mixing of percentages of the three component starters (based on plate counts in CFU/ml) with SYTO9/PI cytometric data for total cells ($r = 0.986$; $P < 0.001$). Interestingly, based on numbers of viable cells as enumerated by FCM, the relationship with plate counts decreased somewhat ($r = 0.936$; $P < 0.001$). This was ascribed by the authors to the effects of chain length of starter strains in the blend. Correlations between q PCR and FLOW-FISH data for the analysis of 20 different commercial mixed starter strain blends indicated a highly significant relationship between both nucleic-acid techniques for strain composition data ($r > 0.83$; $P < 0.001$). Additionally both q PCR and FLOW-FISH data correlated well with plate count data (on X-Gal-calcium citrate agar) for the proportions of *L. lactis* subsp. *cremoris* and *Leuconostoc* spp. within a particular starter blend. This excellent study provided a very comprehensive examination of the ability and performance of both q PCR and FLOW-FISH methodologies to differentiate and enumerate complex starter culture blends and concluded that both offer potential alternatives and advantages compared with traditional plate count enumeration of starter cultures.

2.5. Immuno-FCM and bacterial enumeration

Immunomagnetic Separation or IMS involves the use of magnetic beads coated with antibodies against target cells which are then specifically labelled within a liquidised sample with their subsequent capture on exposure to a magnetic field. This enables concentration of the target cells from a mixed microflora, followed by FCM detection of the antibody-target-stain complex. Hibi et al. (2006) reported the separation of viable *Listeria monocytogenes* from mixed cultures of pathogens using IMS followed by FCM analysis over range of 10^4–10^8 CFU/ml. This assay was applied to analysis of cultures inoculated into seawater and involved use of a FITC-labelled primary antibody against *L. monocytogenes* followed by secondary antibody detection using a tetrameric antibody complex combined with magnetic beads. Enumeration by FCM was based on counting of labelled events, which were also monitored by enumeration of *L. monocytogenes* on selective agar. A strong correlation ($R^2 = 0.97$) was noted between both methods over the range 8×10^2- 8×10^7 cells/ml. Improved IMS-FCM detection was reported as being possible by increasing sample volume. This assay represented a significantly improved TTR (~2 h) compared with plate counting, however, it must be recognised that samples were substantially free of particulates and matrix interference normally associated with food product analysis. In general, antibody capture of target cells such as *E. coli* O157:H7 or *Listeria monocytogenes* requires further enrichment to allow cell numbers to reach detectable levels using FCM (generally $> 10^4$ cfu/ml). Wilkes, Tucker, Montgomery, and Cooper (2012), Buzatu et al. (2014) and Williams et al. (2015), reported the development of an immuno-FCM assay for rapid qualitative detection of *E. coli* O157:H7 in spinach. This assay used two proprietary reagents, A or B, containing either FITC-conjugated polyclonal antibodies against the pathogen, detergents and conditioning chemicals for improved epitope presentation and PI staining for dead cell identification. Issues with particle interference, matrix colour interference and subsequent recovery and detection of pathogen were resolved and thereafter followed by a 4 h enrichment step. Buoyant gradient centrifugation enabled recovery of cells, which were then removed, washed and filtered (using a 5 µm filter) prior to antibody labelling and FCM analysis. A specialised cytometer having a wide cross section flow cell with a 130 nm resolution was claimed as being superior for analysis of samples containing particulates likely to interfere with bacterial analysis. FCM analysis involved the creation of a series of multi-dimensional gates starting out from FSC and SSC plots, with subsequent exclusion of PI positive dead cells and matrix particles such that the final gate represented only live labelled *E. coli* O157:H7. This qualitative immuno-FCM assay had a TTR of < 4.5 h, with an LOD of 1 viable cell in 25 g of sample but no comparative data was reported with plate counting. Williams et al. (2015) described a Level 2 FDA approved validation process for detection of *E. coli* O157:H7 in raw spinach. The procedure involved 20 spiked and 20 non-spiked samples for analysis with a comparison of data obtained by immuno-FCM with an FDA approved qPCR test. Levels of 1–4 viable cells per 100 µl were inoculated into test samples with a 17.5 h aging period prior to sampling. Subsequent preparation steps included addition of a photobleaching agent phloxine B, to reduce matrix colour interference, and a 5 h incubation to increase cell numbers to detectable levels. FCM analysis was carried out as reported by Wilkes et al. (2012). FCM compared favourably with the approved qPCR method and sensitivity was similar between methods at 2–4 cells per 100 µl, TTR for the FCM method was ~9 h while that for qPCR was ~51 h. The number of false negatives for the immuno-FCM method was 4 out of 10 and 5 out of 10 for the PCR method. However, no data was provided for comparison with traditional plate counts, most likely as it was beyond the scope of the study. Subires, Yuste, and Capellas (2014) reported on a method to directly detect *E. coli* O157:H7 in pasta salad. Preparatory steps included pulsification in 63 µm filter bags and centrifugal filtration. To evaluate the potential for detection and establish assay parameters, 10^3-10^7 cfu/g of live cells were inoculated into unpasteurised salads and subsequently stored at 4 °C for 2 weeks. Detection of the target pathogen utilised a polyclonal antibody conjugated to R-phycoerythrin (R-PE, yellow-orange fluorescence) with live/dead staining carried out on the labelled complex using SYBR GREEN I and PI. An enrichment step was omitted with direct labelling of a filtered and re-suspended cell pellet undertaken. A correlation was undertaken with conventional plate counting of samples which indicated a strong relationship between plate counts and FCM ($r = 0.9935$) at 0.015 µg/ml of antibody while at a higher antibody concentration (1,000 µg/ml) this correlation decreased ($r = 0.8961$). Careful selection of gates, with an extensive range of controls, enabled discrimination of cells from food particles and between live or dead/damaged cells. The assay data indicated an LOD of 10^5 cfu/g, which reflected significant residual matrix interference. Additional information generated during product storage by multi-parametric FCM using SYBR Green I and PI revealed that initially most cells had sustained membrane damage but appeared to have recovered by day 14. Buzatu et al. (2014) reported a rapid, direct and integrated immuno-FCM assay for pathogens such as *E. coli* O157:H7, denoted as the RAPID-B system. A series of programmed gate logic stages enabled presentation of a final cytograph of live and antibody labelled cells for the FCM operator. In addition, an FCM-based total count assay was incorporated using a Thiazole Orange (TO) and PI staining combination. Data for the *E. coli* O157:H7 assay was compared with plate counting as previously reported by Wilkes et al. (2012). However, Buzatu et al. (2014) outlined data from an extensive comparison of the RAPID immuno-FCM assays and that obtained from Plate Count Agar (PCA) and Sorbitol-McConkey Agar (SMAC). Data analysis revealed increased FCM counts (10–50%) over populations enumerated using either PCA or SMAC agars. Essentially, FCM delivered parallel but higher data with a linearity having comparable R^2 values (~0.99) for all three methods (FCM, PCA, SMAC). Performance of the assay was

reported as being capable of detection of a single cell in 25 g of a food product and involved a 6 h incubation period. The physiological insight obtainable from FCM data lies well beyond the capability of plate counting, and indeed some other techniques, but it can allow a greater understanding of the fate of stressed cells in foods and enable estimation of their potential for posing a latent threat to consumer safety e.g from convenience foods. Using the above studies to compare and contrast the ability of immuno-FCM to overcome the current issues with cell specific recovery and labelling, it is evident that good progress has been made towards the development of sensitive and rapid qualitative detection of particular pathogens in a limited range of foods. However, the direct species-specific enumeration of pathogens using reliable, sensitive, quantitative immuno-FCM assays is still some way off and will rely heavily on the further development of novel methods to recover cells from foods in concentrations which can be detected by sensitive antibodies and may also involve using cytometers specially adapted for sensitivity at low event detection limits.

2.6. Monitoring of bacterial viability during storage of food products using FCM and plate counting

Flow cytometry has been applied to the study of changes in viability, and physiological status of bacterial sub-populations in a number of food products. Monitoring of changes in sub-populations of Lactic Acid Bacteria (LAB) starters and probiotic strains in fermented dairy products has been undertaken in a number of studies. The application of FCM assays using multiple stain combinations in the monitoring of pathogen survival in other food types (Cronin & Wilkinson, 2009; Kennedy & Wilkinson, 2017) has also provided fascinating insights into the degree of heterogeneity which develops in microbial populations during storage under various conditions. However, in many of the published studies few direct comparisons have been carried out between data obtained by FCM enumeration and that obtained by traditional plate counting. Sheehan et al. (2005) noted strain related differences in the evolution of live, dead and permeabilised starter LAB strains in Cheddar cheese during ripening. FCM methodology involved the use of a SYTO9/PI stain combination with comparison of data from gates previously generated using a number of control populations. This experimental approach involved parallel monitoring of intracellular enzyme release which enabled clear trends in cell physiology from FCM data to be related to single strain-related differences in viability and permeabilisation properties during ripening. Other such studies on the fate of starter LAB and their intracellular enzymes during Cheddar cheese manufacture and ripening include those of Kilcawley, Nongonierma, Hannon, Doolan, and Wilkinson (2012), Rulikowska et al. (2013) Doolan et al. (2014), Yanachkina et al. (2016). While no direct statistical correlation between plate count data in cheese and FCM numbers were reported by these authors, a general decline in viability during ripening was reflected by plate counts and by FCM data for non-viable populations. The ability of FCM to give a unique insight into the nature of the heterogeneity of viable and non-viable LAB sub-populations was evident in all of the above studies. Hickey et al. (2018) utilised FCM to study the effects of differing salt concentrations on single or paired starter cultures during ripening of a brine-salted cheese. FCM staining by a SYTO9/PI combination was used for viability assessment, while the extent of oxidative stress associated with various salt levels in cheese was evaluated by staining of samples with 0.5 mM CellROX® Green (CRG) probe. Interestingly, *Lactobacillus helveticus* could be clearly distinguished from *S. thermophilus* in cheese samples based on fluorescence intensity of SYTO9/PI which was attributed to increased uptake of the stain by the larger *Lb. helveticus* cells. The authors converted FCM data from events counted in the gated profiles of live, dead or damaged cells obtained from a 10 g sample of cheese into log cfu/g of cheese. This calculation assumed that all events detected by FCM were actual bacterial cells. In parallel, plate count data was obtained from cheeses using standard counting methods and viability followed during ripening for 26 days. A non-statistically based comparison of plate count data with FCM indicated that the latter methodology generated higher *Lb. helveticus* counts. However, in the case of *S. thermophilus*, FCM viability data appeared more closely matched to that obtained by plate count methodology. Data from FCM profiles using the permeable CRG stain to detect the presence of reactive oxygen species (ROS) indicated the influence of manufacturing procedure as a significant cell stressor with highest fluorescence noted prior to the brining step of the process. Additionally, the authors noted a distinct alteration of FSC profiles due to the influence of varying salt levels which may have induced cell shrinkage/plasmolytic effects.

3. Bacterial enumeration using flow cytometry and cell sorting

Fluorescent activated cell sorting (FACS) or "cell sorting" involves an enhanced level of FCM-based analytical resolving power for use in food microbiology. Cell sorters are cytometers with the ability to physically sample cells originating from a sub-population of interest (Nebe-von-Caron, Stephens, Hewitt, Powell, & Bradley, 2000). A cell sorter has the ability to encapsulate a single cell within a single droplet which is then given either a positive or negative charge and deflected through a high voltage electrical field (~5000 V) to be deposited into various receptacles e.g agar plate, test tube, or a microtitre plate well (Kennedy et al., 2011; Muller & Nebe-von-Caron, 2010). Hence, stained or stained/antibody-labelled cells belonging to a sub-population e.g live, dead, damaged can be examined to verify and correlate their cytometric profile to various criteria including viability, possible VBNC status, and resuscitation potential on various agar media. For example, cell sorting could potentially take a food sample and using appropriate controls provide rapid and comparative data to examine the equivalence of FCM methodology with plate counting for particular bacterial species. Ben Amor et al. (2002) studied the effects of exposure to bile salts on the physiology of two probiotic Bifidobacteria strains (*B. lactis* and *B. adolescentis*). These workers evaluated the use of single staining using $DiBAC_4(3)$, PI, and cFDA which reflected alterations in membrane potential, membrane permeability, and intracellular esterase activity, respectively. FCM was undertaken using controls of heat killed and untreated cells and resultant data from stressor exposure compared with trends noted for plate counts. Populations of PI-and $DiBAC_4(3)$-stained bacteria were highly correlated, but inversely related to decreased plate counts used to indicate percentage cell survival. Trends for cFDA stained cells were similar to plate counts but appeared to overestimate viability of both Bifidobacteria strains by a margin of 20–30%. Subsequently, dual staining by PI/cFDA identified three main sub-populations, one of which was a double positive PI/cFDA, potentially indicative of damaged cells, which was sorted by FACS onto solid agar media. This particular sub-population was then shown to contain a substantial percentage of cells (40%) capable of re-growth on agar demonstrating the survival and resuscitation of strains after exposure to bile salt-induced stress. Doherty et al. (2010) examined the use of FACS to determine the viability of stationary phase-derived cells of the probiotic strain *Lactobacillus rhamnosus* GG encapsulated within various food matrices. Additionally, the effect of cell stressor treatments such as heating, porcine gastric incubation conditions and storage for up to 28 days at 37 °C were examined. Cells were released from encapsulated matrices following homogenisation and/or proteinase hydrolysis and thereafter FACS analysis of cell viability by staining with Thiazole Orange (TO) and PI was undertaken. Statistical comparison of data from FACS and plate counting indicated a high degree of correlation (r = 0.92–0.95) between both methods over the range 10^2 to 10^{10} cfu/ml. In certain cases (exposure to acidic porcine gastric), FACS appeared to overestimate cell numbers compared with plate counts which may have reflected the ability of FACS (and FCM in general) to detect and enumerate potential VBNC cells. The robustness of this particular methodology was attributed to the stringent sample preparation protocol used, which appeared to minimise any matrix particle interference

on subsequent cytometric analysis. Kennedy et al. (2011) examined the responses of the food pathogens *E. coli*, *Listeria monocytogenes* and *Staphylococcus aureus* when subjected to the effects of various stressors typically encountered by pathogens during food processing. The strains were analysed by FCM for viability using SYTO9/PI or for the presence/absence of a functioning membrane potential by staining with $DiOC_2$ (3). Using FACS sorting of various sub-populations, these workers demonstrated that extensively damaged cells (as per staining and FCM profiles) sorted onto various solid media were still capable of growth. Indeed, differing overall recovery rates were noted for the various pathogens on either selective and non-selective media with recoveries also influenced by differing stressor treatments. This study clearly showed the potential for survival and outgrowth of damaged cells, it also re-illustrated the heterogenous nature of sub-populations generated within a culture following exposure to stressors, and demonstrated the potential for cell sorting in gaining a novel profound insight into cell physiology and potential pathogenicity arising from persistence of damaged cells within foods. Sibanda and Buys (2017) reported the application of FACS to examine the resuscitation characteristics of various *L. monocytogenes* strains following acid, osmotic and heat induced stress. Cells were stained using a TO/PI combination and representatives of the potentially damaged cell sub-population (TO + /PI +) were sorted onto various media and growth kinetics examined by plate counting. Data indicated strain and treatment-related effects of various stressors and the nature of cell damage. Overall, cell sorting remains an expensive cytometry option requiring a high degree of technical skill, and to date, has not found widespread end use applications within the routine food microbiology laboratory. In the medium term, the development of more user friendly, less expensive cell sorters may enable wider adoption of this specialised analytical technique for microbiological food analysis.

4. Development of FCM as a routine enumeration methodology

A number of interesting developments have recently occurred towards acceptance of FCM as a method for bacterial enumeration. All probiotic products must contain a specified viable population, typically 10^7- 10^9 cfu/g, at point of sale (Corona-Hernandez et al., 2013; Sanders et al., 2016). This legal regulation has required the probiotics industry to undertake regular viability monitoring of probiotic cultures following their production (as freeze-dried/lyophilised or frozen-concentrate formats) and follow their survival in the product during storage. Viability enumeration traditionally involved sample dilution and subsequent plate counting on solid agar media following incubation at designated temperatures, with a time to result (TTR) measured in 1–4 days. To overcome the disadvantages of plate counting, the probiotics industry undertook the development and validation of an alternative FCM based method for rapid enumeration of cells using three staining protocols (PI/cFDA, SYTO 24/PI and $DiOC_2$). PI/cFDA, is a measurement of both membrane damage (dead and damaged cells) and the presence of intracellular esterase activity, SYTO 24/PI is a measurement of viable, dead and damaged cells, based on entry of membrane-impermeant PI into dead or damaged cells, while $DiOC_2$ enables cells containing a functioning membrane-potential to be quantified. These three staining protocols formed the basis of a standard method for flow cytometry-based enumeration of lactic acid bacteria in starter culture preparations, probiotics and fermented milk products published as an International Standards Organisation (ISO) and International Dairy Federation (IDF) standard ISO19344; IDF 232 (2015). Cytometric data is collected from all three staining procedures following dilution of the sample to an optimum level of $\sim 10^6$ cfu/ml. The minimum number of events collected is 1,000 in 10 µl of sample, while the maximum number is 50,000 in 100 µl of sample, with data generated under 1 min following passage through the cytometer. Data obtained is expressed as either; (a) AFU/ml, active fluorescent units (corresponding to viable cells), (b) n-AFU/ml, non-active fluorescent units (corresponding to dead or damaged cells), (c) total fluorescent units, TFU/ml, (sum of values for AFU plus n-AFU), and, (d) the percentage ratio of AFU to TFU. A collaborative study was undertaken by 15 laboratories in 5 countries with 1800 samples analysed using the FCM method (Casani, Hansen, & Chartier, 2015). Statistical analysis of data allowed examination of the equivalence of the three staining protocols along with evaluation of repeatability and reproducibility. Data was obtained mainly from analysis of commercial frozen and freeze-dried starter preparations and a single commercial yogurt product. No significant differences were found for AFU/g obtained between the three protocols for the samples analysed, indicating a high degree of equivalence for data obtained for the different staining protocols. In the case of AFU/g obtained for culture preparations, repeatability and reproducibility values were determined as being 0.06 and 0.45 $\log_{10}$ while for TFU/g these values were 0.07 and 0.38, respectively. Interestingly, values for yoghurt were somewhat higher due to analytical difficulties with matrix particle interference proving problematic for gating for TFU. The development and validation of an alternative FCM-based analytical method for quantification of LAB in dairy systems did not set out to replace the existing plate counting method, or indeed to compare data obtained by FCM with standard plate count methods. Instead, this industry-led initiative looked at the issue of viability through the new perspective of FCM analysis, whereby both viability and non-viability data can be rapidly obtained along with physiological status (vitality) of individual cells. Its' main premise is that for use of this FCM test, the absolute requirement for enumeration of cell viability as being indicated by growth of a colony on a plate (culturability) is not the determining factor in data collection. Rather, the end user accepts that multiplexed FCM-based data from this type of assay generates a series of individual morphological and physiological cell measurements which reflect both the concepts of cell viability and cell vitality and shows the complexity and heterogeneity that exists within a microbial population.

In microbiological water analysis, FCM has been developed as an actual direct replacement for the traditional standard methods for quality and production monitoring of microbiological water quality analysis. This major advancement is based on research undertaken at Eawag in Switzerland where FCM was clearly demonstrated as being capable of replacing a traditional agar-based heterotrophic plate count (HPC). Egli and Kotzsch (2015) outlined the evolution, widespread uptake and final acceptance by national regulatory bodies in Switzerland of an FCM based assay using SYBR Green I staining which rapidly enumerates total cell count (TCC). In addition to TCC, valuable information regarding the ratio of High Nucleic Acid (HNA) to Low Nucleic Acid (LNA) content of cells within a sample can be simultaneously gained by collecting SSC and Green fluorescence intensity data. Variation in the HNA/LNA ratio can indicate a degree of cell re-growth in response to environmental factors such as temperature shifts in the water treatment systems. An on-line FCM monitoring system is now commercially available as “BactoSense TCC” for rapid and continuous quality control of drinking water which represents a major step forward in the use of FCM as a real-time microbiological quality control methodology.

5. Conclusions

The investigation of FCM as a methodology either as direct replacement for, or as an alternative to, traditional plate counting for bacterial enumeration is an active research issue for the food industry. However, it is clear from the literature that only a limited number of studies have directly compared both FCM and plate count data for bacterial enumeration. From this data it would appear that FCM enumeration can rapidly generate a high degree of statistically based agreement with plate count data under a defined set of conditions. These conditions generally involved analysis of freshly grown cultures in exponential phase or which reached early stationary phase. In the case of the probiotics industry, the production of cell preparations for

addition to foods and supplements involves management of variables during fermentation such as pH, the degree of cellular oxygen incorporation along with exposure to downstream processing operations such as heat treatments. These factors impact the viability and cell survival characteristics of probiotic culture preparations and subsequently affect the relationship between FCM analytical data and plate counts. Optimal agreement with plate counts was obtained for cells grown in media free of particulates or through use of a clearing solution or preparation step(s) which enabled interference to be minimised. The application of FCM assays to the enumeration of highly concentrated cultures such as frozen or freeze-dried probiotic or starter concentrates is also facilitated as the dilution steps to achieve optimum analyte populations of $\sim 10^5$-10^6 cell per ml for FCM enabled subsequent removal of interfering matrix particulates. The issue of stain choice is of great importance and requires the end user to incorporate a sufficient panel of controls to eliminate the possibility of toxicity and to determine whether cells actively undertake an efflux of the stains chosen. Currently, an alternative validated ISO/IDF methodology exists for FCM-based enumeration of LAB cultures in dairy products. In addition, a direct replacement of plate counting by FCM has been validated for water quality analysis. However, a more complex scenario is presented for FCM analysis during storage of fermented dairy products including cheese and fermented milks as bacteria generally undergo a strain-related decline in viable populations. Hence, FCM enumeration of cultures in foods during storage can involve stain combinations which generate data on evolution of various sub-populations including, viable/intact, non-viable (permeabilised/dead) and cells having differential functionalities such as presence of cell membrane potential and intracellular enzyme activities. In this instance, the multi-parametric data generated by FCM provides a greatly enhanced insight into population heterogeneity than afforded by traditional viable plate counts. Hence, for the application of FCM enumeration during product storage the end user must recognise that the data generated by FCM reflects aspects of cell functionality (vitality) other than traditional culturability or simply the ability to grow and reproduce on an agar plate. Therefore, the traditional view of cell viability within a sample now becomes a matter of viewing FCM based data as being a broader quantitative technique for rapidly determining the presence of various cell functionalities which relate to both cell vitality and cell viability. What is now required is that both end users of FCM and regulatory agencies agree that a particular set of FCM based criteria can be measured within a validated assay such that the data satisfies the definitions of viability and vitality and which functions either as a direct replacement to plate counting or as an accepted alternative methodology. This is especially necessary for applications such as probiotic products requiring specific populations in the end product at point of sale. Overall, FCM enumeration is progressing towards being an accepted alternative to traditional plate count enumeration or as a direct replacement to plate counting.

Conflicts of interest

I was commissioned by the IPA as an Independent Expert to review all relevant published data and to give an unbiased opinion on the use of FCM as a means of assessing viability of probiotic strains in dairy products/supplements. In this regards, the opinions I formed and have expressed in the manuscript are entirely my own obtained after reviewing the Literature. The Scientific Committee of the IPA had a sight of the final document prior to journal submission and made some minor non-editorial comments some of which I took on board, if I agreed with them. However, throughout the process I have exercised full independent editorial control over the current manuscript and continue to do so.

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