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Article

High-Throughput Viability Testing of Microbial Communities in a Probiotic Product Using Flow Cytometry

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Abstract: There is growing scientific and commercial interest in multi-species probiotic products due to their potential benefits in maintaining gut health. Determining the viability of probiotic microorganisms in these products is essential to ensure that they confer maximal health benefits. The gold standard for enumerating probiotic viability is the plate count method. However, this may be inaccurate for enumerating mixed probiotic populations, with recognised limitations including difficulty measuring metabolically active yet unculturable, very slow growing microbes, microencapsulated, enteric coated microbes, or multi-strain formulations that require differing growth media. Here, we developed a flow-cytometry-based approach using SYTOX^{TM} Green dye to assess the viability of probiotic microorganisms in a multi-species, fibre-containing probiotic product and compared this to the traditional plate count method. This method was suitable for enumerating both total bacterial cells and the viable cell fraction in the complete product mixture, and could also be used to assess how stressors, such as gastric digestion and exposure to bile acids, affect bacterial cell viability. Flow cytometry measurements routinely detected higher viable cell counts than plate counting. This work demonstrates that flow cytometry assays can be established as a suitable method for rapid enumeration of viable cells in complex, multi-species probiotics.

Keywords: probiotics; multi-species; flow cytometry; SYTOX^{TM} Green; plate count; gastrointestinal tract digestion

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1. Introduction

The gut microbiome plays a crucial role in human metabolism and in maintaining the integrity of the gut mucosal layer and immune homeostasis [1]. Specific alterations in the composition and functions of the gut microbiome have been associated with various diseases [1]. Therapeutic modulation of the gut microbiome offers promising strategies to improve and maintain host health through prevention and treatment of diseases. Probiotics are studied and marketed for their ability to alter the gut microbiome and potential health-promoting benefits [2]. Consequently, the global market for probiotics has grown rapidly in the past few years [3,4]. Probiotics are defined as "live microorganisms, which, when administered in adequate amounts, confer a health benefit to the host" [5]. These probiotic microorganisms are generally consumed either in the form of capsules, powder, or tablets [6], or are contained within whole foods such as yogurt and milk [7,8], with the latter accounting for the largest share of the market [9].

Currently available probiotic products are diverse in their formulations and in the microbial strains they contain. Some products contain microorganisms belonging to a single genus, such as yogurt containing only *Lactobacillus* species [10], while other products contain mixed microbial populations including representatives of more than one genus, e.g., *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* [2]. Lactic acid bacteria belonging to *Lactobacillus* and *Bifidobacterium* genera represent groups commonly included in probiotics [11]. In addition to bacterial strains, some probiotic products also contain yeasts such as *Saccharomyces cerevisiae* and *Saccharomyces cerevisiae* var. *boulardii* which have been shown to improve human gastrointestinal health [12,13]. Probiotic products can additionally contain certain additives that are included to support viability and help stimulate the growth of probiotic bacteria in the gut. Among the most common additives are prebiotics, which are non-digestible food ingredients that are utilised as substrates by selective intestinal microflora, including externally applied probiotics [2,14]. Examples of prebiotic additives include oligosaccharides, such as lactulose, oligofructose, galacto-oligosaccharides, and polysaccharides, such as inulin [2,15].

For probiotic microbes to confer their beneficial effects, they need to be viable and have the capability to survive and maintain their properties during manufacturing processes and storage conditions [15,16]. Ideally, once ingested, these viable cells will reach the colon, adhere to the intestinal mucosa, colonise the gut [17,18], and become a crucial effector of host–microbial interactions [19]. There are also site-specific interactions where probiotics can confer a benefit at a certain point of the gastrointestinal (GI) tract, and this benefit can differ if the probiotic is viable, dead, or degraded entirely [20]. Therefore, the viability of microorganisms is an important property of probiotic products and accurate enumeration is paramount to assessing and predicting the efficacy of products [21]. The current industry standard for enumerating probiotic viability is the traditional culture-based plate count method, where the product is spread onto solid microbiological media in petri dishes, then incubated at growth-permissive temperatures for some period of time, following which visible colony forming units are counted. However, this method is limited in its abilities to accurately enumerate viable cells, particularly from products that contain multiple species. Firstly, plate counting is reliant on the ability of bacterial cells to form colonies on the specific microbiological media under the set incubation conditions [22] and is recognised to underestimate viable microbes by excluding viable but non-culturable cells (VBNCs) [23]. Bacteria in the VBNC state exhibit metabolic activity and are considered to be live but are unable to grow on culture medium [24]. They enter this state to resist stress and it has been suggested that stress associated with probiotic product processing and formulation may result in some proportion of cells entering the VBNC state [25]. Another limitation of the plate count method is the assumption that one microbial cell will give rise to a single colony [25]; however, there is a risk of clumping in probiotic dry powders where multiple cells may give rise to a single colony, resulting in an underestimation of viable cells in products. Multi-species probiotic products containing bacterial and yeast strains require selective media [25] and additional additives (cysteine, antibiotics, etc.) to enumerate different sets of microorganisms within the mixture [19,25]. Furthermore, competition among mixed microbial populations for nutrients can favour the growth of certain microorganisms over others, with those that are slow growing most likely to be under-considered by culture-based enumeration techniques [25].

Non-culture-based techniques such as PCR-based methods (qPCR and digital PCR) and flow cytometry have been used previously to assess viable microbial cells from culture isolates and simple microbial mixtures [26,27]. Application of qPCR-based methods for mixed-species products have some drawbacks as routine enumeration techniques requiring validated primer sets for different members of the mixture and are limited in their capacity to determine absolute rather than relative abundances without the use of spiked-in synthetic standards [28]. For studies involving cross-domain mixtures, such approaches also must consider two different taxonomic marker genes, running separate reactions to amplify both the 16S rRNA gene in prokaryotes and the ITS regions in eukaryotes [28]. Furthermore,

there are chances of PCR-related biases being introduced, for example, primer biases, and the complications of data interpretation due to varying 16S rRNA copy numbers in different bacteria. Flow-cytometric-based techniques have been shown to overcome many of the issues encountered with plate counting and those associated with PCR-based enumeration and have been successfully used to assess probiotic viability in products containing relatively simple mixes of bacteria [23,29,30].

Flow cytometry is a multi-parametric single cell analysis technique that identifies and enumerates cells from both homogeneous and heterogenous populations [22] based on the shape and size of cells [19]. Flow cytometry can be used to enumerate viable microbial cells by applying this technique in tandem with cell staining using established viability dyes such as SYTOX^{TM} Green, a nucleic acid stain that indicates bacterial cell membrane integrity status, to help discriminate live cells from dead cells [23,29–31]. Other nucleic acid stains, such as propidium iodide (PI), SYTO, and combinations of nucleic acid dyes including SYTO 9/PI [4,23], and dyes that can discriminate based on metabolic activity such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), are also used to assess the viability of bacterial cells using flow cytometry [32]. In some instances, flow cytometry is already used as an alternative for probiotic viability assessment [33]. The International Organization for Standardization and the International Dairy Federation have suggested the use of flow cytometry as the standard method for the enumeration of active lactic acid bacteria and other probiotic microorganisms from dairy products [30,33]. However, uptake of flow cytometry for product assessment is still limited within the broader probiotic industry. Furthermore, studies involving single probiotics species comparing flow cytometry and traditional plate counting have reported a strong agreement between the numbers of dead/live cells enumerated using both methods [34,35]. In contrast, examinations of probiotic bacterial blends containing both *Lactobacillus* spp. and *Bifidobacterium* spp. have reported a higher number of viable cells when assessed using flow cytometry compared to plate counting [23,29], demonstrating a discrepancy in viable cell counts observed between the two methods when more than one bacterial species or genera are enumerated. Given the increasing numbers of probiotic products with multiple species of bacteria, yeast, and other active ingredients such as prebiotic fibres, there is a clear and timely need to develop protocols to better utilise high-throughput techniques, such as flow cytometry, to enumerate the viable cell counts of probiotic products.

Here, we developed a flow cytometric approach using a single viability dye to accurately and rapidly analyse and quantify viable microbial cells from heterogenous bacterial and yeast populations from a complex commercial probiotic product (Factors Group Australia). This product contains multiple bacterial strains including representatives of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* genera, as well as strains of the yeast *Saccharomyces cerevisiae*, prebiotics, and other plant-based ingredients. As a proof-of-principle, we then applied this protocol to enumerate viable cells following simulated GI tract digestion of the probiotic product, comparing the results obtained from flow cytometry with those derived from standard plate count methods.

2. Materials and Methods

2.1. Factors Group Australia Multi-Species Probiotic Product

The multi-species probiotic product was provided by the manufacturer Factors Group Australia. Each capsule (transparent hard shell) contained a freeze-dried mixture of the live bacterial strains *Bifidobacterium longum* subsp. *infantis*, *Lactobacillus acidophilus*, *Lacticaseibacillus casei* (formerly *Lactobacillus casei*), *Lactobacillus gasseri*, *Lacticaseibacillus paracasei* subsp. *paracasei* (formerly *Lactobacillus paracasei*), *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarium*), *Lactobacillus reuteri*, *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*), *Ligilactobacillus salivarius* subsp. *salivarius* (formerly *Lactobacillus salivarius*), and *Streptococcus thermophilus*. Additionally, the capsules contained yeast strains including *Saccharomyces cerevisiae* and *Saccharomyces cerevisiae* (*boulardii*), as well as plant and fungal

extracts. Probiotic capsules containing only the lyophilised bacterial mixture without addition of yeast or other active ingredients were also provided.

2.2. Sample Processing

Each capsule was processed by dissolving capsule contents in 50 mL of 0.20 µm filtered phosphate buffered saline (PBS, Oxoid, Sydney, Australia), applying vortexing to ensure a homogenous mixture was generated. For flow cytometry assays where prebiotic fibre removal was necessary, the above-described protocol was carried out and the resulting solution was then passed through a 100 µm cell strainer (FalconA®, In Vitro Technologies, Sydney, Australia) to filter out larger particles such as plant material. The samples were then 1:10 serially diluted in PBS and the technique appropriate dilutions were used for enumeration via plate count and flow cytometry.

2.3. Enumeration and Sequencing of Viable Cells by the Plate Count Method

Bacterial cells within the Factors Group Australia probiotic product were enumerated by spread plating serially diluted capsule contents onto de Man, Rogosa and Sharpe (MRS, Merck, Sydney, Australia), MRS L-cysteine agar and *Streptococcus thermophilus* agar solid culture media. For each assay, three separate capsules (as biological replicates) were independently prepared as detailed in Section 2.2 and appropriate dilutions were spread plated onto agar plates in triplicate. The MRS and *Streptococcus thermophilus* agar plates were incubated aerobically at 37 °C for 72 h, while MRS L-cysteine agar plates were incubated anaerobically at 37 °C for 72 h. The plate count results were expressed as colony forming units/capsule (CFU/capsule). Visibly identifiable colonies were enumerated only from plates containing 30–300 colonies per plate. The 16S rRNA gene (using 27F 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R 5′-TACGGYTACCTTGTTACGACTT-3′ primers) of individual colony forming units presented on MRS L-cysteine agar and *Streptococcus thermophilus* agar plates were sequenced at Macrogen, Korea. The resulting nucleotide sequences were analysed using the basic local alignment search tool nucleotide (blastn) against the National Library of Medicine (NLM) NCBI database, considering the percent identify score when determining the taxonomy assignment.

2.4. Generation of Dead Cell Control Populations for Flow Cytometric Analysis

To locate the position of dead cells in the product on flow cytometric plots, a membrane compromised (dead) cell population was generated by exposing an aliquot of the diluted samples to heat at 70 °C for 30 min (water bath incubator, Thermo Fisher Scientific, Sydney, Australia). The resulting cell suspension served as the cell membrane compromised (dead) control population in subsequent viability assessment assays run with the SYTOX™ Green stain [36,37].

2.5. Cell Viability Staining for Flow Cytometric Analysis

Bacterial cell viability assays were carried out using SYTOX™ Green nucleic acid dye (Thermo Fisher Scientific, Sydney, Australia) which stains only membrane compromised cells [32], thus distinguishing membrane compromised (dead) from membrane intact cells (viable cells). Briefly, the diluted probiotic suspension samples along with the dead cell control population and filtered PBS negative controls were stained with SYTOX™ Green at a final concentration of 1 µM. These were incubated in the dark for 15 min at room temperature. The filtered PBS control was used to detect and measure any background non-specific fluorescence. All flow cytometry analyses were performed on three separate capsules with and without SYTOX ™ Green stain (stained cells and unstained cells), with each biological replicate analysed using three technical replicates.

2.6. Flow Cytometric Data Acquisition and Analysis

The samples were analysed on a CytoFLEX S (Beckman Coulter, Brea, CA, USA) flow cytometer using the CytExpert software (Beckman Coulter, Brea, CA, USA) for data

analysis. The following settings were used: gain for forward scatter (FSC)—100, side scatter (SSC)—100, and FITC (green fluorescence detector)—40, while the threshold for FSC was set to 1500 and that for SSC to 500 (the flow rate of the cells was set at the slow setting). The total cell counts were determined by applying gates based on the dot-plot SSC versus FSC (SSC-A vs. FSC-A). The positioning of the dead cell control population was used to set gates to separate the membrane compromised cells from the membrane intact cells. These gates were applied on the dot-plot SSC vs. FITC (SSC-A vs. FITC-A); both plots were set on $\log_{10}$-scale. The SYTOX green fluorescence intensity peak (indicative of membrane compromised cells) was also observed via a histogram plot of FITC vs. count, with FITC-A set on log scale and count set on linear scale. To identify and gate the bacterial population separately from the yeast population present in the multi-species probiotic product, probiotic capsules containing only the lyophilised lactic acid bacterial component of the product mixture were analysed. To account for the possible presence of instrument background noise, filtered PBS-only (blank) controls, which were prepared the same way as for the samples, were analysed on the flow cytometer and events were recorded at the beginning of each flow cytometry assay. To determine the total cell counts, any events detected in PBS controls were subtracted from the total events detected as the proportion of events attributable to background noise. The number of viable cells of the product was expressed as cells/capsule.

2.7. Tolerance to Conditions Simulating Gastrointestinal (GI) Tract Passage

The multi-species probiotic capsules were exposed to gastric digestion as per the protocol described by Shehata et al., with slight modifications [38]. Briefly, simulated gastric juice was prepared by suspending 0.3% (*w*/*v*) pepsin (Merck, Sydney, Australia) in PBS at pH 2.0. The simulated gastric juice and 0.5% NaCl were added to nine independent 15 mL sterile tubes, each containing a single probiotic capsule. The tubes were incubated at 37 °C with agitation at 100 rpm. At 0, 1.5, and 3 h of incubation, three tubes were removed for processing. Tube contents were vortexed and centrifuged at 7800 rpm at 4 °C for 10 min, followed by PBS washes of the cells until the pH values of the samples were 7.0. The resulting cell suspensions were then processed as described in Section 2.2. Tolerance to gastric digestion was assessed by determining viable counts at each time point using both plate counts and flow cytometric analyses in parallel by splitting the contents of each tube into two for use in each technique. The percent survivability was calculated by comparing the treatment samples (1.5 and 3 h) to the samples collected at 0 h.

For assessing resistance to bile salts, capsule contents were dispensed into tubes containing 0.3% (*w*/*v*) of bile salts (Merck, Sydney, Australia) suspended in PBS (pH 7.0). The samples were incubated at 37 °C with agitation at 100 rpm for 3 h. Samples were collected at intervals of 0, 1.5, and 3 h and processed as described above. Three technical replicates of each of the nine samples were diluted and assessed for the viable cell counts and % survivability by plate count method and flow cytometry as described above.

2.8. Statistical Analysis

Data were analysed using GraphPad Prism (version 9.4.1, GraphPad Software Inc., San Diego, CA, USA). Data is displayed as mean ± standard deviation (SD). The CFU/capsule and cells/capsule counts were transformed to Log_{10} values and for all experiments normality tests were performed using the D'Agostino and Pearson omnibus test to assess the distribution of data. Statistical analysis was performed using Student *t*-tests to compare the mean differences between two groups, while one-way ANOVA tests were used to compare the mean differences of more than two groups, with $p \leq 0.05$ used as the significance level cut-off for both.

3. Results

3.1. Determining Viable Cell Number Variability for Factors Group Australia Probiotic Capsules

The standard plate count technique using MRS culture media was used to count colony forming units and assess the cell count variability between three capsules of the multi-species probiotic product. Viable cell numbers between capsules were determined to be consistent (Table 1), with no significant differences recorded (one way ANOVA, $p = 0.59$).

Table 1. The viable cell counts of three multi-species probiotic capsules determined using the standard plate count method.

Samples	(Mean ± SD)	
	CFU/Capsule	**Log_{10} CFU/Capsule**
Capsule 1	$3.4 \pm 0.5 \times 10^9$	9.5 ± 0.09
Capsule 2	$3.2 \pm 0.1 \times 10^9$	9.5 ± 0.01
Capsule 3	$3.1 \pm 0.2 \times 10^9$	9.5 ± 0.02

Shown as mean ± SD; each capsule analysis was carried out in technical triplicate.

Serial dilutions of the complete product with mixed bacteria were plated on MRS L-cysteine agar and *Streptococcus thermophilus* agar plates. While colony forming units were seen on both media, sequencing of these colonies revealed taxonomic assignments to *Lactobacillus* spp. that are present in the product. Therefore, colony forming units obtained from MRS L-cysteine agar and *Streptococcus thermophilus* agar plates were not included in any of the plate counting results presented in this study.

3.2. Impact of Filtering out Prebiotic Fibres on the Viable Cell Counts of the Multi-Species Probiotic Product

While flow cytometry is a relatively versatile technique, to be suitable for analysis using this technique, any large particles or clumps must first be removed to ensure flow conditions are maintained and prevent clogs in the fluidic path. For this reason, prior to processing probiotic capsule contents, it was necessary to perform a pre-filtering step to remove particles over 100 µm in size. We performed a set of initial experiments to examine how such filtering impacts the flow-cytometry-derived viable cell counts in the multi-species probiotic product. The plate counting method was used to determine viable cell numbers for both the filtrate and the residue retained on the filter to determine the proportion of the viable cell population that was removed by the filtration process. There was a 4.3% reduction in the viable cell numbers in the filtrate compared to the viable cell numbers in capsules not subjected to filtration (Table 2), suggesting that filtration has a minimal impact on the viable cell counts of the product. As the difference in viable cell counts for filtered and unfiltered capsule suspensions was found not to be significant (Student *t*-test, $p = 0.39$), and removing the larger particles was necessary to enable reliable flow cytometry analysis of multi-component fibre-containing products, this filtering step was applied in all subsequent work.

Table 2. Plate-count-based assessment of the effect of filtration (100 µm) on viable cells counts for the multi-species probiotic product suspension.

	Viable Cell Counts (Mean ± SD)	
	CFU/Capsule	**Log_{10} CFU/Capsule**
Filtrate cell count	$2.2 \pm 0.1 \times 10^9$	9.3 ± 0.02
Capsule cell count (unfiltered)	$2.3 \pm 0.1 \times 10^9$	9.4 ± 0.02

Shown as mean ± SD; analyses were carried out in technical triplicate. CFU—colony forming units.

3.3. Enumeration of Viable Cells in the Multi-Species Probiotic Product

Following preliminary experiments to establish that individual capsules contained relatively consistent numbers of viable cells and that separation of viable cells from fibre was possible, we worked to develop a routine protocol for flow-cytometry-based viable cell enumeration. In addition to multiple strains of bacteria, the tested product also contained yeast. Therefore, to enable the positioning of separate gates for bacterial and yeast populations on the flow cytometric plots, we analysed capsules containing only the lyophilised bacterial mixture alongside standard capsules. The gating for yeast was consistent with the expected trend for larger and more internally complex cells (brown-coloured gate, Figure 1). To discriminate membrane compromised cells from membrane intact viable cells, flow cytometry assays were performed on cells stained with SYTOX^{TM} Green dye. To manually gate the membrane compromised bacterial and yeast cell populations in the product using flow cytometry, the cytograph positioning of heat-killed control populations (green- and brown-coloured gates, respectively, Figure 1A–C) was used. Analyses of the complete product with flow cytometry was then performed to allow both the viable and non-viable (membrane compromised) bacterial populations to be enumerated (representative results shown in Figure 1D–F). Due to the relatively small size of the bacterial cells in the product, it was not possible to set a flow cytometry threshold capable of completely removing all background noise, so PBS-only (blank) control mean cell counts were subtracted from the total bacterial cell counts to more accurately determine viable bacterial cell counts.

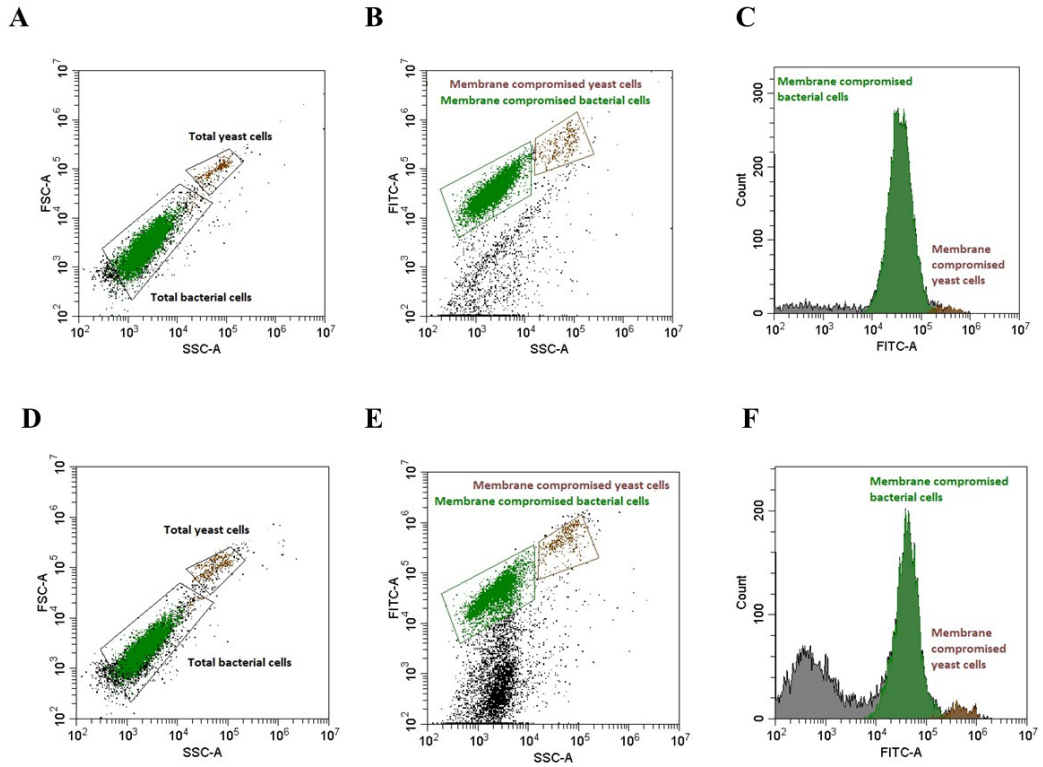

Figure 1. Representative flow cytometric plots of cells populations for heat-killed control populations (**A**–**C**) and the complete probiotic product (**D**–**F**), all analysed following SYTOX^{TM} Green viability dye staining. In panels (**A**,**D**), gating of total bacterial and yeast cells is shown. In plots (**B**,**E**), the green gates indicate membrane comprised bacterial cells and the brown gates indicate membrane compromised yeast cells. The coloured histogram peaks in (**C**,**F**) show that cells located within the "membrane compromised" gates had the expected high FITC-A intensity and are distinct populations, separate from the membrane intact populations (grey peak in (**F**), absent in the dead control in plot (**C**)).

In order to compare the flow cytometry-derived viable counts for this mixed-species probiotic with that obtained by traditional plate count methods, the standard capsule

analyses were performed in parallel. Comparing the culture-based CFU per capsule count to the viable cell counts obtained via flow cytometry, we found the latter showed significantly higher (Student *t*-test, $p < 0.001$) counts (Table 3).

Table 3. The average viable cell counts per capsule of the multi-species probiotic product determined by flow cytometry and plate count methods.

	Viable Cell Counts (Mean ± SD)	
Flow cytometry	$6.4 \pm 0.4 \times 10^9$ cells/capsule	9.8 ± 0.03 Log_{10} cells/capsule
Plate count	$2.0 \pm 0.1 \times 10^9$ CFU/capsule	9.3 ± 0.03 Log_{10} CFU/capsule

Shown as mean ± SD; analyses were carried out on three capsules (as biological replicates), each in technical triplicate. CFU—colony forming unit.

3.4. Testing the Reproducibility of the Flow Cytometry Protocol

To examine whether results obtained by flow cytometry and plate count methods are reproducible, testing was performed with six additional capsules using both techniques. Similar to our previous observations, higher viable cell counts were obtained using flow cytometry compared with the plate count method (Table 4). Notably, flow cytometry detected consistent numbers of viable cells across the six biological replicates, while for plate count assays there was greater variability, with fold change differences in the CFU/capsule count determined for capsules 1–3 compared to capsules 4–6 (Table 4). The results suggested a higher reproducibility of flow-cytometry-based enumeration of viable cells compared to traditional plate counting using MRS media.

Table 4. The viable cell counts of six individual capsules of the multi-species probiotic product obtained by flow cytometry and plate count methods.

Samples	Viable Cell Counts (Mean ± SD)			
	Flow Cytometry		Plate Count	
	Cells/Capsule	Log_{10} Cells/Capsule	CFU/Capsule	Log_{10} CFU/Capsule
Capsule 1	$6.8 \pm 0.2 \times 10^9$	9.8 ± 0.01	$4.5 \pm 0.5 \times 10^7$	7.6 ± 0.05
Capsule 2	$6.1 \pm 0.2 \times 10^9$	9.8 ± 0.01	$5.1 \pm 0.2 \times 10^7$	7.7 ± 0.02
Capsule 3	$5.6 \pm 0.1 \times 10^9$	9.7 ± 0.01	$3.9 \pm 0.3 \times 10^7$	7.6 ± 0.03
Capsule 4	$7.1 \pm 0.4 \times 10^9$	9.9 ± 0.02	$4.3 \pm 0.4 \times 10^8$	8.6 ± 0.04
Capsule 5	$6.6 \pm 0.1 \times 10^9$	9.8 ± 0.01	$6.3 \pm 0.2 \times 10^8$	8.8 ± 0.02
Capsule 6	$6.9 \pm 0.2 \times 10^9$	9.8 ± 0.01	$5.4 \pm 0.5 \times 10^8$	8.7 ± 0.04

Shown as mean ± SD; analyses were carried out in technical triplicate for each of the six capsules. CFU—colony forming unit.

3.5. Comparison of Flow Cytometry and Plate Counting Methods for Assessing Viability following Simulated GI Tract Digestion

To ascertain how each enumeration approach compared when used to determine cell survivability following a challenge, we performed assays examining the impact of exposure to gastric juice (0.3% pepsin at pH 2.0) on bacterial viability for the complete probiotic product. Both techniques showed that sizable populations of viable bacterial cells remained after each of the tested exposure periods (Table 5). However, flow cytometry assay-based enumeration indicated considerably higher proportions of bacteria remained viable at both 1.5 and 3 h than what was indicated by plate counting at each time point (Table 5).

Table 5. Impact of simulated gastric juice on the viable cells of multi-species probiotic capsules, determined by flow cytometry and plate count methods.

Exposure Time (Hours)	0.3% Pepsin (pH 2.0) (Mean ± SD)			
	Flow Cytometry		Plate Count	
	Cells/Capsule	Log_{10} Cells/Capsule	CFU/Capsule	Log_{10} CFU/Capsule
0	$8.5 \pm 0.2 \times 10^9$	9.9 ± 0.01	$9.7 \pm 0.3 \times 10^8$	8.8 ± 0.02
1.5	$3.9 \pm 0.2 \times 10^9$	9.6 ± 0.03	$9.3 \pm 0.3 \times 10^7$	7.9 ± 0.01
3	$3.9 \pm 0.5 \times 10^9$	9.6 ± 0.06	$9.1 \pm 0.8 \times 10^7$	7.9 ± 0.04

Shown as mean ± SD; each time point was carried out with three biological replicates, and each in technical triplicate.

Following the stomach digestion mimicking assays, we performed standard in vitro bile salt exposure assays, again examining viable cell survivability following different exposure times. Similar trends were observed in this second assay, with flow cytometry indicating substantially higher viable counts after both 1.5 and 3 h bile salt exposure than plate count assays (Table 6).

Table 6. Impact of exposure to bile salts on the viable cells of multi-species probiotic product, determined by flow cytometry and plate count methods.

Exposure Time (Hours)	0.3% Bile Salts (pH 7.0) (Mean ± SD)			
	Flow Cytometry		Plate Count	
	Cells/Capsule	Log_{10} Cells/Capsule	CFU/Capsule	Log_{10} CFU/Capsule
0	$8.1 \pm 0.3 \times 10^9$	9.9 ± 0.02	$4.4 \pm 0.6 \times 10^8$	8.6 ± 0.07
1.5	$4.6 \pm 0.3 \times 10^9$	9.7 ± 0.02	$1.9 \pm 0.2 \times 10^7$	7.8 ± 0.06
3	$4.8 \pm 0.2 \times 10^9$	9.7 ± 0.02	$1.6 \pm 0.1 \times 10^7$	7.7 ± 0.03

Shown as mean ± SD; each time point was carried out with three biological replicates, and each in technical triplicate.

4. Discussion

Here, we developed a flow-cytometry-based technique using a commercial viability dye to enumerate viable bacterial cells in a multi-species probiotic product containing bacteria, yeast, and other components. In this work we observed that viable cell counts based on flow enumeration were higher than those derived from plate counting method. This finding is consistent with what has been reported in a number of previous studies on probiotic blends containing *Lactobacillus* and *Bifidobacterium* spp. [23,29,39] and on multi-species probiotic products Lacidofil®, Protecflor®, and ProbioKid® (Mirabel, QC, Canada) [40]. It should be noted that the flow cytometry and plate count methods presented in our study are not measuring precisely the same thing, with flow cytometry providing estimates of membrane intact cells per capsule and plate count determining the numbers of colony forming units per capsule under the specific culture conditions applied (MRS medium, incubated aerobically at 37 °C for 72 h). These culture media and conditions used for plate counting typically favour growth of *Lactobacillus* spp., especially if they are present in a multi-species population. The product tested in this work, whilst comprised predominantly of strains of *Lactobacillus* spp., did also contain *Bifidobacterium longum* subsp. *infantis* and *Streptococcus thermophilus*. MRS L-cysteine agar and *Streptococcus thermophilus* agar, which are known to favour the growth of *Bifidobacterium* and *Streptococcus thermophilus*, respectively, were used to account for the different genera, but only *Lactobacillus* spp. were recovered on them. Similarly, work by Sohrabvandi et al., isolating lactic acid bacteria from yogurt, showed *Lactobacillus* spp. consistently outgrew *Bifidobacterium* spp., despite providing anaerobic conditions which are considered to be tolerated by both groups [41]. It

is possible that competition amongst these multiple species and genera, when co-plated as present in the product, contributed to the lack of viable *Bifidobacterium* and *Streptococcus* spp. on the MRS L-cysteine and *Streptococcus thermophilus* agar plates.

We consistently observed a lower count of colony forming units and higher variation in results when plate counting was used compared to the number of viable cells detected through flow cytometry. This could also be due to the competition between bacterial species and genera induced due to co-plating the mixed population of bacteria present in the tested product. Furthermore, the ability of flow cytometry to detect injured yet membrane intact cells [25], including cells in the VBNC state, may also have contributed to the higher number of cells detected by flow cytometry. The ability of flow cytometry to detect VBNCs and dormant cells, which are generally not detected by plate counting, may have been the reason that the stress tolerance assays resulted in particularly large discrepancies in viable cell counts between flow cytometry and plate counting, as these assays may have resulted in cells within this product entering into a VBNC or dormant state [25], in addition to the competition between the multiple species of bacteria for growth conditions as described above. Furthermore, these limitations associated with plate counting may have exacerbated any minor variation in the proportion of different bacteria present in the product, leading to a greater variation in colony forming units detected. It is also worth noting the technical limitations in enumerating multiple species and genera of bacteria present in products using plate counting due to the different growth conditions required to culture them. This is a significant limitation in assessing the viability of probiotics in complete products containing mixed populations of bacteria, which can be minimised using flow cytometry as we demonstrate here.

The ability of probiotic microorganisms to tolerate GI transit is considered to be an important feature of an effective probiotic product [42] and is therefore assessed at the initial stages of identifying microorganisms with probiotic characteristics. Probiotic survival through the gastrointestinal tract is very much dependent on the bacterial strain and the delivery mechanism of the bacteria (e.g., capsule or powder) [19]. Characteristically, when assessed in a simulated GI tract, probiotic cultures show a decrease in concentration in the stomach followed by an increase in concentration in the intestine, with factors such as the time in the stomach and the composition of foods in the stomach at the time influencing probiotic concentrations throughout the process [43]. Here, we applied flow cytometric enumeration of viable cells to assess the response of a mixed bacterial population in a probiotic product to conditions mimicking independent sections of GI transit. While such testing is routine, it is generally performed on pure cultures during single strain selection [16], instead of on the final probiotic product, and there are limited evaluations of products after freeze drying and formulation [44]. The stage at which such testing is conducted is important, as the survival after exposure to gastric acid and bile can differ significantly, sometimes by several log units depending on formulation, freeze-drying, and storage conditions [45]. One reason for this is that during production, dried lyophilised cells generally undergo rehydration which is challenging to the cell as it moves from a gel-like state to a crystalline liquid state [44]. In this work, lower survivability observed in plate growth assays may have been due to cells entering a transition phase where they could maintain their metabolic activity but were unable to form colonies on the culture medium. Studies that have compared the GI tolerance of the unencapsulated probiotics with the encapsulated probiotics (different encapsulation materials) have shown higher tolerance rates when probiotics are encapsulated [46–48]; however, this literature involved capsules that dissolved in bile salt assays, i.e., simulated intestinal conditions rather than in the stomach-mimicking conditions [46–48]. In our case, the capsule dissolved in the gastric juice with gentle agitation, suggesting that further work comparing effects of different capsule formulations on viable cell concentrations at different stages of continuous GI transit, transit time variation, and GI transit survivability across multiple complex product formulations would be of interest.

Both of the stressors examined can lead to cell injury, loss in viability, and death. However, the pH buffering capacity, product composition, transit time, and volume of gastric juice are also affected by recent food consumption and composition [49]. Different strains also have differences in bile tolerance due to the individual species' ability to express the bile salt hydrolase enzyme [50]. LAB strains that express more bile salt hydrolase can survive longer in bile salts. Hydrolysed bile salts are a key feature of probiotic bacteria; however, not all probiotics possess this capability to the same degree [5,51]. LAB strains can also possess other abilities to cope with bile exposure, such as active efflux of bile salts and changes in the cell wall and cell membrane composition, which may have downstream effects when assessing cell viability, a question which is worthy of further investigation.

In this work, flow cytometry was used to count the proportion of viable bacterial cells remaining following two different challenges. As the ability to tolerate each of these stressors is likely to differ to some extent between strains with the mixture [38], it would be useful to be able to determine the strain level composition of the surviving population. This could be achieved by using cell sorting to separate viable and non-viable populations following stress assays followed by 16S rRNA amplicon sequencing, which would aid in identification of strains that were more or less tolerant of exposure to such stressors. While the in vitro method used here to simulate stomach digestion and intestinal challenges conform with standard assays used for probiotic research [38,47,48], as discussed above, they have inherent limitations, offering only limited similarity to the complex GI transit of the human body [19,52]. The human digestive system is a dynamic process where there is continuous flow of fluids and enzymes, and varying concentrations of pH and bile salts, throughout the GI tract, which is generally difficult to maintain in an in vitro system [19,52]. Since the in vitro method is not a true representation of the human GI tract system, it is possible that the impact was either milder or more detrimental than what may occur in vivo.

There are many commercial cell viability dyes suitable for flow cytometry applications, with dyes that indicate membrane integrity, such as the SYTOX^{TM} Green dye used in our study, among the most common methods of viability assessment. SYTOX^{TM} Green dye stains nucleic acids in membrane compromised cells, while combinations of other nucleic acid dyes such as SYTO 9/PI allow the detection of total cells (SYTO 9) and enhance the discrimination of membrane compromised cells using PI [53]. Yet, these nucleic acid dyes do not distinguish between metabolically active and dormant cells or damaged cells with intact membranes. While the staining approach used here does not directly identify damaged cells, histogram plotting of the intensity of the fluorescence signal from the SYTOX dye when applied to the probiotic product (Figure 1F) revealed some continuity between the main peaks corresponding to membrane intact and membrane compromised cells, which might indicate the presence of membrane damaged cells. Further flow cytometric examination using additional dyes could be employed to explore the additional information on the physiological status of mixed-species probiotics that can be determined. Additional combinations of fluorescent dyes compatible with flow cytometry [30,54], could be trialled to gain more information about the physiological and metabolic state of cells in probiotic mixtures. Dyes such as CTC, which when reduced by dehydrogenases form fluorescent membrane-impermeant formazan, have been considered useful in providing an indication of actively respiring cells, as absence of formazan production indicates a lack of esterases or dehydrogenases, which could be indicative of dead or damaged cells [32]. It may also be possible to fluorescently tag antibodies specific to particular bacterial strains with viability stains to enable specific enumeration of viable cell numbers of each strain within a mixed population [40]. However, for complex probiotic products, establishing such assays would require considerable set up time and cost, which are likely to make this a less convenient approach for routine testing by probiotic industries.

Probiotic products are increasing in complexity and there are a range of products on the market that combine live probiotic bacteria with other ingredients, including prebiotic fibre and other active but non-living ingredients. For these, flow cytometry enumeration

requires an additional processing step to remove large particles to prevent clogging of flow cytometer tubing. Here, we showed that filtration (using 100 µm cell strainers) was successful in preparing such a product for flow cytometry, having a minimal impact on viable cell counts. Such processing is likely to be a necessary step for flow cytometric cell enumeration of similar products containing prebiotic fibres, but independent validation for different products using plate-count-based comparisons is required to ensure there is not a higher proportion of fibre-adherent cells in some products.

Flow cytometry provides various advantages over the standard plate count method. We have shown that flow cytometry can differentiate yeast and bacterial populations and rapidly enumerate viable cell numbers within complex mixtures in a matter of hours, while the traditional plate count method requires 48–72 h to grow visible colonies, providing counts of only culturable cells [40]. Culture-based approaches may also be more variable in assessing stress tolerance depending on recovery times used in assays prior to plating, as stressed cells may transfer to different non-culturable states, possibly resulting in the measured CFU differing to varying degrees from the actual viable cell count [19]. Furthermore, competition among mixed bacterial populations for nutrient sources can lead to outgrowth of certain microorganisms over others, likely leading to undercounting of slower growing or condition-sensitive organisms [25]. In addition to successfully overcoming known plate count limitations, flow cytometry may provide information on various aspects of cell physiology. The ability of flow cytometry to enumerate cells in probiotic mixtures at any stage in the product formulation process has the potential to provide valuable information on the impact of different manufacturing steps or compare alternative techniques for use in product preparation, such as spray drying and freeze drying, on the viability of probiotic microorganisms.

It should be noted that a known issue with flow cytometry is the presence of background noise, which can be especially problematic when analysing relatively small bacterial cells or complex mixtures [27]. While background noise can in some cases be excluded via threshold setting or gating, this is not always possible as the noise may overlap with populations of interest. In such cases, subtracting the average counts for repeated blank measurements from the cell counts of interest, as carried out in this study, has been considered an acceptable approach to overcome this issue [55]. Another option may be to use two fluorescent dyes in combination, which has the potential to reduce the amount of background noise that is inseparable from the signal indicative of stained cells. Additionally, while performing established assays with this technique is much faster than plate counting, it should be acknowledged that the design of reliable flow cytometric assays can take time. These initial steps require use of appropriate controls to optimise instrument settings and enable appropriate gates to be established to count subpopulations of interest.

While successful use of flow cytometry requires one to first establish reproducible protocols and optimise instrument settings for a particular probiotic product, once this has been achieved, the same protocol can subsequently be implemented as a routine, rapid enumeration technique. This has numerous possible applications, such as assessing viable cell counts across different batches of a product or to determine how different stressors impact the product. While some members of the probiotic industry are trialling flow cytometry [30,36], this technique has not yet been adopted by the regulatory bodies in most of countries, including Australia, for their product assessment activities. However, this may change in the future, as this technique shows considerable potential for providing more accurate measurements of viable cells in probiotic products and is already a regulated requirement for other industries such as the dairy industry [27].

5. Conclusions

Flow cytometry analysis has the potential to provide very detailed information about all cells within both simple and complex samples. However, certain knowledge of the cell population is required to be able to achieve an experimental design that will yield reliable information and reproducible results [56]. Here, we undertook to compare plate counting

and flow cytometry approaches for enumeration of viable cells in a probiotic product that contains a complex mixture of bacterial strains, yeast strains, and other active ingredients. For this, we developed a methodology for processing the probiotic capsule to allow flow cytometric analyses, along with instrument protocols and gating strategies to separate out subpopulations and enumerate viable cell fractions. This protocol has the potential to be applied to investigate how a range of different factors affect bacterial viability, from the impact of different manufacturing steps through to exploring batch variability, or shelf life, as product viability and composition can change within a probiotic product over time [57]. This could also be used to examine other factors of high interest to the probiotic industry, such as site-specific activity and lysis along the GI tract.

Flow cytometry was found to have advantages over plate counting in terms of speed, reproducibility, and sensitivity, likely due to the capacity to detect cells in the VBNC state. There are some acknowledged limitations, however, with flow cytometry requiring large particulates in samples, such as probiotic fibres, to be removed prior to analysis. This may, in some instances, affect viable cell counts, either due to high rates of fibre attachment or the potential that removal of additional active ingredients alters viability, possibly due to the protective effects of these additional components of the formulation [57]. Despite such issues, we conclude that flow cytometry has the potential to be used as a routine enumeration technique for rapid and accurate viability assessment for probiotics, especially for mixed-species probiotic products and, as such, is likely to be increasingly used in the coming years.

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