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A novel pharmaceutical approach for the analytical validation of probiotic bacterial count by flow cytometry

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A B S T R A C T

Introduction: Flow cytometry is a powerful and sensitive technique able to characterize single cells within a heterogeneous population. Different fluorescent dyes can be combined and used together to analyze a great variety of parameters simultaneously. In particular, flow-cytometry allows to measure viability and vitality of probiotics measuring their metabolic activity, fermentation capacity, acidification potential or oxygen uptake ability (Hayouni et al., 2008). To now, plate counting is considered *the gold standard* in microbiological technique for probiotic enumeration. However, this approach is limited to the detection of only those viable cells which are able to proliferate and form colonies on a solid medium but is not able to recognize not cultivable bacteria and nonviable cells. *Aim*: The aim of the present study was to apply The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) parameters for the validation of new analytical methods in microbiology. ICH requirements, which are commonly employed for the analysis of drugs and chemical analytes, have been here applied to live cells for the comparison between a flow-cytometric assay and the traditional plate count method for the quantification of viable probiotics bacteria.

Methods and results: Combining specific viability dyes such as thiazole orange (TO) and propidium iodide (PI), probiotic counts of *Lactobacillus* and *Bifidobacterium* species were carried out using a FACS Verse (*BD Biosciences*) cytometer. Analyses were conducted in parallel with the traditional plate count, on specific media. Raw data were analyzed using the FACSuite software (*BD Biosciences*) and then elaborated with the statistical software Neolicy (*VWR International*). Results indicated that flow cytometry provides very similar results in cell counting if compared to classical microbiology approaches, showing better performances (ICH parameters) than the traditional plate count method.

Conclusions: This work demonstrated the analytical ICH validation of probiotic counts in food supplement products using a robust flow cytometric approach able to enumerate and to assess bacteria viability with stronger results in comparison to the traditional plate count.

List of abbreviations

ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
TO	Thiazole orange
PI	Propidium iodide
qPCR	real-time quantitative Polymerase Chain Reaction
ISO	International Standard Organization
IDF	International Dairy Federation
LOD	Limit of detection
LOQ	Limit of Quantification
FSC	Forward Scatter
SSC	Side Scatter
CFU	Colony Forming Units
FITC	Fluorescein IsoThioCyanate
FOS	Fructo-Oligosaccharides

1. Introduction

Flow cytometry is a technique based on the contemporary measure and analysis of particles' physical parameters, dispersed in a flow and irradiated by a light source. The power of flow cytometry is the opportunity to determine a wide range of single cell features in minimal time and with low costs. Using an optical system coupled with an electronic processor, it allows to measure single cell properties such as size, membrane and intracellular complexity, shape, density and fluorescence intensity, determined respectively by the scatter of incident light and by the fluorescence emission. This technique was initially developed for the study of eukaryotic cells but recently the use of flow cytometry has been extended to explore the physiological state of prokaryotic cells, using specific fluorescent probes (Alvarez-Barrientos et al., 2000;Raymond and Champagne, 2015; Robinson and Roederer, 2015; Tracy, 2008;Muller and Nebe-von-Caron, 2010). In this regard, flow-cytometry has been applied to numerous fields including pharmaceutical, food, water and environmental sectors (Alvarez-Barrientos, 2000; Weiss, 2002;Assuncao et al., 2007;Comas-Riu and Rius, 2009;Wilkes et al., 2012) Flow-cytometry is also used for the qualitative evaluation of raw materials intermediate and finished products both for their microbiological and stability tests and for the possibility to

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characterize their specific components (Holm et al., 2004; Laplace-Builhe et al., 1993; Ruszczynska et al., 2007;Novo et al., 2000).

At present, plate count is the traditional method used for quality assurance of probiotic products. (Garcia-Armesto et al., 1993) This assay consists in the capability of bacterial cells to grow and subsequently generate colonies, optically measured either in liquid media or solid agar plates (Charteris et al., 1997; Sincock et al., 2001; Kerstens et al., 2014; Chiron et al., 2017; Wilkinson et al., 2018;Chiron et al., 2018; Davey, 2002; Doherty et al., 2010; Want et al., 2011)

Lactobacillus and *Bifidobacterium* species are the most common probiotics used in dietary supplements and, according to current definitions, they have to be metabolically active to proliferate and to exert their benefic effects in the small intestine and colon surviving both to gastric acid and bile (Roy, 2001; Sohier et al., 2014). The physiological state of the cells is difficult to evaluate using traditional culture techniques as these provide only information on the microbial growth and ability of in vitro micro-organisms duplication activity. Damaged cells and quiescent cells, which are viable but non-cultivable, are not correctly detectable and may be caused quantification errors in plate count (Ashraf and Shah, 2011; Lahtinen, 2005, 2008; McHugh and Tucker, 2007).

To overcome these microbiology limitations, different non-culture-based methods have been developed in the last decades, to evaluate the physiological state of the cells. PCR-based techniques, such as real-time qPCR, (Nocker et al., 2006; Kramer et al., 2009;Gracias and McKillip, 2004) and flow-cytometry can distinguish between viable bacteria from total population (Nocker et al., 2006;Malacrino et al., 2001). Another way to gain more insight into the physiological state and metabolic activities of the cells, is the use of specific fluorescent stains, which detect viable, damaged, and dead bacterial cells. Typically, an approach including a combination of dyes able to discriminate between viable and apoptotic/necrotic cells is employed to obtain cell number counts. (Bunthof and Abee, 2002;Novo, 2000; Rault, 2007) In particular, this is a very important issue for probiotics because their metabolically activity brings benefits to the guest.

In recent years, the publication by the International Standard in according with the International Dairy Federation (IDF) of ISO 19344 (IDF 232), has suggested a new method for the selective enumeration of active lactic acid bacteria by flow cytometry to assess the quality of fermented products (ISO 19344 2015). High-throughput analysis using a flow-cytometric counter gives the advantages of a lower variation, a reduction of testing time, and the quantification of active cells on total cells. The alignment to ISO guidelines ensures optimal and harmonized procedures in analytical processes, provides safeguards to the equivalence of testing results, also among different instruments and labs.

The aim of the present study was the application of the ICH criteria for the validation of a flow-cytometric approach for probiotic count and the stability assessment in relative humidity conditions. ICH criteria are usually employed for drugs and chemical analytes, but not for live cells considered as active ingredient of the food supplement. In the last years, live cells have become increasingly important to be even mentioned by Pharmacopoeia, which usually norms analytical methods for drugs.

The method consists in a two-color approach (orange thiazole and propidium iodide) optimized to assess *Lactobacillus* and *Bifidobacterium* cell viability, based on their membrane integrity, as described in International Standard Organization (ISO) 19344|IDF 23220154|IDF 232 (ISO 19344; 2015) and the comparison of obtained results with the classical microbiology total microbial count (TBC) in solid media. This study evaluates the favorable impact of a novel analytical method based on a flow cytometric approach in a production company for the quantitative and qualitative characterization of probiotics, in the industry.

In the first part, the bases of both procedures are described, then in the second one the statistical analysis in terms of accuracy, linearity, selectivity, precision and Limit of detection (LOD)/ Limit of Quantification (LOQ) are outlined.

2. Materials and methods

2.1. Samples composition

The samples analyzed consisted in raw material powder containing probiotic bacteria, mixed into a matrix composed of commonly used excipients such as sugars as oligosaccharide (FOS), herbal extracts, maltodextrin, vitamins and minerals. Commercial selected species of probiotics such as *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus acidophilus*, were employed in order to design and optimize our new analytical approach. The final probiotic reconstituted samples employed for the validation were: one product containing excipients and *Bifidobacterium animalis* subsp. *lactis*, one product containing excipients and *Lactobacillus acidophilus*, one product containing excipients, *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus acidophilus*, one product with only excipients and without bacteria. Probiotic raw materials were also analyzed alone in order to define the best analytical parameters in terms of both morphological and fluorescent signals.

2.2. Samples preparation

10 Grams of probiotic samples were initially diluted with 90 mL of appropriate sterile diluent Maximum Recovery Diluent (MRD – *Biolife*) and then consecutive serial decimal dilutions, until appropriated, were performed. The appropriate chosen dilutions were inoculated on a petri plate containing de Man, Rogosa, Sharpe agar (MRS - *Biolife*), added with 0,05% L-cystein HCl (*Sigma Aldrich*). At least 2 plates for each dilution were prepared. Probiotic cultures were incubated in anaerobiosis, at 37 ± 2 °C for 48–72 h. The equivalent dilutions were used for the flow cytometry staining protocol.

2.2.1. Colony identification and count

Probiotic colonies, grown at least on two plates, for two consecutive dilutions, have been counted. After the confirmation of an acceptable proportionality between number of colonies, the calculation of *Kp* (coverage factor to establish measurement uncertainty, as described by ISO 13005) and G^2 (*chi square*-test), the average number of colonies, for each dilution considered, was computed using the following equation:

$$C_n = \frac{C_n1 + C_n2 + \ldots + C_nm}{m}$$

(n = considered dilution; m = number of plates retained)

Plate counts were performed ensuring proportionality between the different dilutions tested. In order to calculate the average numbers of colonies for n and $n + 1$ dilutions, the following equation was applied:

$$N = \frac{\sum C}{V \times (1 + 0,1) \times d}$$

(ΣC = sum of the average numbers obtained from the count of the colonies on the plates in the 2 consecutive dilutions considered; V = volume (mL) of the inoculated amount in each plate; d = dilution factor corresponding to the first dilution considered)

The results were expressed as Number (N) of colony forming units per gram, in scientific notation, with 1 or 2 decimal numbers. In absence of bacterial growth, data was expressed as $< 1/dilution$ micro-organism per g of product.

2.3. Flow cytometry staining protocol

Two color staining of probiotic cell suspension using the BD Cell Viability Kit (*BD Biosciences*) was performed to distinguish live and dead cells by flow-cytometry. Briefly, samples were incubated for 15 min in the dark with a combination of 42 μmol/L for Thiazole Orange (TO) which entered in all cells, and further 15 min with 4.3 mmol/L of Propidium Iodide (PI) specific for dead cells, following manufacturer's instructions. Properly conjugated isotype-matched dyes

and unstained bacterial sample were used as negative controls during the optimization steps. After the incubation, stained samples were analyzed employing a FACS Verse (*BD Biosciences*) flow cytometer. A total of 10,000 events were acquired per sample. Only live cells (positive for TO) were considered as the ones able to grow in colonies on agar plates.

2.3.1. Data acquisition and analysis

Initial settings of the flow-cytometer were: Threshold—FSC 200 arbitrary units (a.u).; logarithmic amplification; FL1, bi-exponential amplification and FL3, bi-exponential amplification. Automatic compensation was used. Acquired data were analyzed with the FACSuite™ (BD) software, in the "Acquisition-to-Analysis" mode. A FSC vs SSC plot with a physical gate was designed to identify the bacterial population of interest. Then, another specific region in the FL1 (TO) vs FL3 (PI) plot was gated to display the live/dead stain results. To determine the absolute count, expressed as UFC/g, the following equation was used:

$$N = n \times 1000 \times d$$

(n = number of events per μL; d = dilution factor corresponding to the dilution)

Before starting the validation process, the flow-cytometer was set to ensure optimal performance of the assay of both bacterial populations (*Bifidobacterium animalis*, *Lactobacillus acidophilus*, and mixed) in MRD medium. Finally, consecutive dilutions of the samples were evaluated after the staining procedure and the dilution 1,E-05 was chosen as the most suitable.

2.3.2. Data statistical analysis

The statistical parameters measured were the average, the mean standard deviation (Dev Std), the Student's *t*-test and the *F*-test, the correlation coefficient (R), the determination coefficient (R^2), the linearity regression and the rate of recovery. *F*-test and *t*-test were used to compare the two different methods; in particular, *F*-test was used to compare more than two sample averages and to evaluate if they had the same parametric mean, while t-test was used to determine if the means of two sets were significantly different. Correlation coefficient (R) and determination coefficient (R Amor et al., 2002) were used to evaluate the linearity. The two methods were evaluated with a confidence interval of 95% ($p\ value\ <\ .05$).

3. Results

3.1. Probiotic plate count

During the validation process, the traditional plate count was performed evaluating the typical morphology of *Bifidobacterium animalis* and *Lactobacillus acidophilus* colonies. Their peculiar morphology allows to differential count and correctly identify the probiotic bacteria, when both present.

The $\log_{10}$ of the CFU/g values, resulting from the analyses of the tested samples, are given in each specifics Tables of corresponding paragraphs (Tables from 1 to 13, see *Paragraph 3.3 "Statistical validation of data"*). Raw data are not shown.

3.2. Live/dead discrimination of bacteria by flow-cytometry

The combination of the TO and PI dyes allowed to distinguish between live, damaged and dead cells inside a heterogeneous bacteria population (Fig. 1). The injured population can often be observed intermediated between the live and dead populations (blue gate). Only live cell populations are able to proliferate and generate colonies on plates (green gate indicated as Live in TO_FITC vs Pi_PerCP_Cy5 plot). The number of proliferating cells has been used for the statistical evaluation and for the comparison with plate count method. All colonies' count, corrected for the dilution factor, were linear with flow cytometric results. For each experimental condition tested, a negative control was included in order to set the correct threshold value between noise and positive signal. The E + 05 dilution was chosen for the analysis.

3.3. Statistical validation of data: Comparison between flow cytometry and plate count

According to ICH procedures, both methods, plate count and flow cytometry, were validated for specificity, limit of detection/limit of quantification (LOD/LOQ), linearity, accuracy and precision (in terms of repeatability, system and intermediate precision).

3.3.1. Specificity

Specificity is the ability of a method to avoid placebo interferences with the active ingredient analysis. Tested samples included probiotic strains as *Lactobacillus acidophilus*, *Bifidobacterium animalis*, or both *Lactobacillus acidophilus* and *Bifidobacterium animalis*, raw materials, placebo and blank.

The enumeration of *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus acidophilus* probiotics in the reconstituted samples, using the classic plate method, can be considered specific; all parameters fulfil the specifications. In the same way, the analytical method for probiotic quantification in the reconstituted samples by flow cytometry, can be considered selective and specific because, in the same way, all parameters fulfil the specifications.

Regarding raw material results, the probiotic enumeration of each batch was compliant with the specification defined by the supplier and there was not significant difference between plate count and flow cytometer in bacteria quantification. Both counting methods gave comparable performance (Supplementary Tables 1A and 1B).

Placebo and blank samples correlate in both the analytical methods tested.

3.3.2. Linearity

To evaluate method's linearity, samples containing note concentrations of *Bifidobacterium animalis* or *Lactobacillus acidophilus* have been processed. In particular, three replicates of each theoretical concentration equal to 50%–80%–100%–120%–150% of the of finished product were tested; for each sample, 3 consecutive dilutions (from 1,E + 09 to 1,E + 11) were tested.

The method's linearity has been evaluated by calculating the regression line with the log value of the raw data results (Table 1).

There was a strong correlation ($R\ >\ 0.99960$) between flow cytometry and plate count results, in particular in the linear range from − 50% and + 150% of the theoretical specification. All correlation (R) and Determination (R^2) (Amor et al., 2002) coefficients agreed with ICH specifications (values ≥0.99).

All values obtained were close to each other, and for this reason the intercept was forced to 0.

3.3.3. Repeatability and intermediate precision

In order to study both repeatability and precision of the two tested methods, flow cytometry analysis and classical microbiology culture were performed in parallel to compare microbial enumeration. Matrices employed were finished product containing single probiotic bacterial strain of *Bifidobacterium animalis*, finished product containing single probiotic bacterial strain of *Lactobacillus acidophilus* and a multi-strain probiotic containing both *Bifidobacterium animalis* and *Lactobacillus acidophilus*. Each test was conducted by two different operators using the same flow cytometer, analyzing six replicates. From the results, no significant difference emerged between classical microbiology and flow cytometry quantification of *Bifidobacterium animalis* (Table 2) and *Lactobacillus acidophilus* (Table 3). Regarding inter-operator's differences, a good comparability (*t*-test < 2.12 and *F*-test < 5.05 for both *Bifidobacterium animalis* and *Lactobacillus acidophilus*) between the two

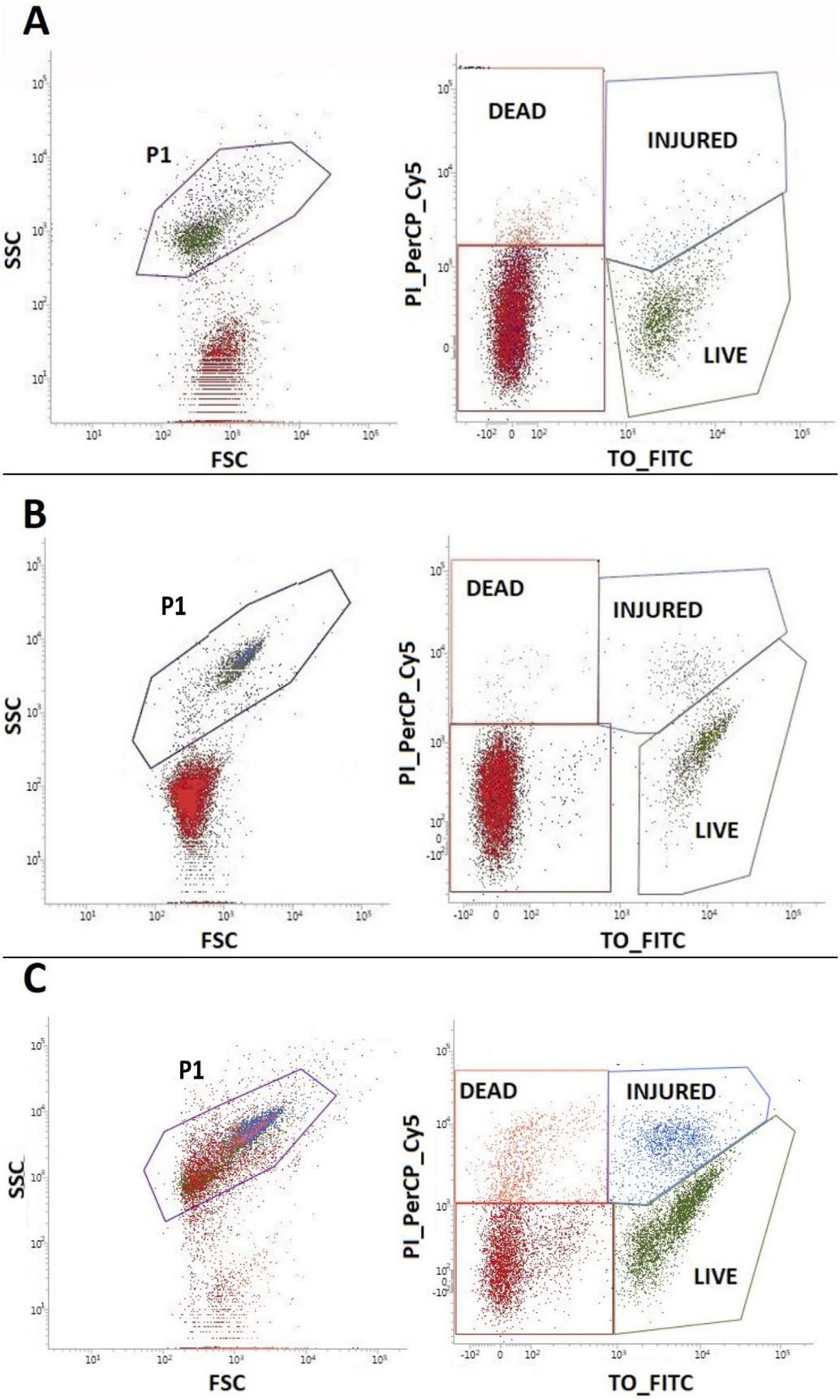

Fig. 1. Representative dot-plots of both morphological (SSC vs FSC) and fluorescence (PI_PerCP_Cy5.5 vs TO_FITC) probiotic parameters obtained from FACS analysis. Results are referred to live/dead staining used to evaluate probiotic count vitality. In the morphological plot gate P1 identify the size of the population of interest. In the plots on the right, three populations are distinguished: live cells positive only for TO staining (green), dead cells positive only for PI (orange) and a double positive population representing injured bacteria (blue). In particular, in Fig. 1A: *Bifidobacterium animalis* is reported, in Fig. 1B: *Lactobacillus acidophilus* and in Fig. 1C mixed population of *Bifidobacterium animalis* and *Lactobacillus acidophilus* are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Comparison between linearity values obtained from L. *acidophilus* and *B. animalis* count by plate method and flow cytometry.

	L. acidophilus	*L. acidophilus*	*B. animalis*	*B. animalis*
	Plate count	Flow cytometry	Plate count	Flow cytometry
Linearity regression	Y = –0.005 + 0.995×	Y = −0.003 + 0.991×	Y = −0.005 + 0.998×	Y = 0 + 0.973×
Correlation (R)	0.999641	0.999675	0.999672	0.99982
Determination (R^2)	0.9993	0.9993	0.9993	0.9996
Intercept	0.159937/−0.170070	0.15329/−0.15959	0.15385/−0.163139	0.11442/−0.114366

approaches were highlighted.

There was no significant difference in the multi-strain probiotic counts obtained both by classical microbiology and flow cytometry. In this case, the inter-operator variability gave an incomplete comparability (Table 4). The *F* calculated between compared mean values was 7.61, higher than the *F* tabulated of 2.85.

3.3.3.1. System precision. System precision was measured in the same condition and on the same products tested for repeatability and precision. There were no significant differences between all bacterial counts obtained by classical microbiology and flow cytometry. In particular, *Bifidobacterium animalis* results are reported in Table 5, *Lactobacillus acidophilus* in Table 6 and the multi-strain results in Table 7.

The statistical tests (*t-test* and *F-test*) confirmed the comparability between the two approaches under validation. For *Bifidobacterium animalis*, *F-test* was 2.78 and *t*-test was 0.0203, for *Lactobacillus acidophilus F-test* was 4.65 and *t-test* was 0.1414 while for the product with *Bifidobacterium animalis* and *Lactobacillus acidophilus F-test* was 4.60 and *t-test* was 0.1229 (for all, respectively lower than the tabulated values of 5.05 and 2.12).

3.3.4. Accuracy

As known, accuracy is the ability of tested method to give an analytical response as similar as possible to the real value. Its measurement can be performed by two different ways according to the matrix.

In our case, nine reconstituted samples in total, obtained by mixing placebo and the active ingredient in suitable proportion, have been evaluated. Test samples were prepared as blending containing 80%, 100% and 120% of the nominal level of active ingredients, 3 samples for each level, in order to asses three different level of concentration in presence of the same quantity of placebo. Because of the matrix complexity, all the reconstituted specimens must be homogenized before weighting samples.

The mean recovery rate reached for both the plate count and the flow cytometry method, were included within 95% and 105% of the theoretical value. The coefficient of variation % for each level was < 3%, while the coefficient of variation % of the total level was < 5% (Table 8, Table 9, Table 10).

The two methods can be considered strongly accurate within the range 80–120% of the theoretical value.

3.3.5. LOD/LOQ

To define both the Limit of Detection and the Limit of Quantification, experiments were conducted by a single operator using three replicates of 3 or 4 consecutive dilutions (for flow cytometry and for plate count respectively), chosen at upper limit of the specificity range. No significant differences emerged by classical microbiology and flow cytometry in terms of LOD and LOQ in the *Bifidobacterium animalis* and *Lactobacillus acidophilus* count results. *t-test* and *F-test* were not evaluated because not required by the ICH guidelines. Counts in flow cytometry at 1,E+07 have not been performed. (Table 11, 12 and 13)

4. Discussion

The validation of a novel method is an essential phase of the entire workflow in a quality control laboratory. A robust analytical assessment should be performed in order to respect pharmaceutical industry recommendations. At the moment, ICH guidelines found application mainly on drugs and chemical analytes such as vitamins, amino acids and other bio-molecules, but they had never been extended to live cells. In the last 20 years, food supplements and medical devices (prior to

Table 2
Comparison between repeatability and intermediate precision obtained from counting *Bifidobacterium animalis* colonies on plate method vs flow cytometer enumeration.

	Bifidobacterium animalis							
	Plate count				Flow cytometry			
	Operator 1		Operator 2		Operator 1		Operator 2	
Samples	Results (cfu/g)	Log_{10}	Results (cfu/g)	Log_{10}	Results (cfu/g)	Log_{10}	Results (cfu/g)	Log_{10}
1	1,62E+09	9,21	1,44E+09	9,16	1,80E+09	9,26	1,55E+09	9,19
2	1,54E+09	9,19	1,45E+09	9,16	1,27E+09	9,10	1,49E+09	9,17
3	1,98E+09	9,30	1,65E+09	9,22	1,89E+09	9,28	1,59E+09	9,20
4	1,68E+09	9,23	1,46E+09	9,17	1,62E+09	9,21	1,23E+09	9,09
5	1,84E+09	9,26	1,29E+09	9,11	1,44E+09	9,16	1,11E+09	9,05
6	1,66E+09	9,22	1,61E+09	9,21	1,32E+09	9,12	1,23E+09	9,09
Average	1,72E+09	9,23	1,48E+09	9,170	1,56E+09	9,188	1,37E+09	9,132
Dev std%	1,61E+08	0,040	1,30E+08	0,039	2,55E+08	0,071	2,01E+08	0,065
CV%	9,36	0,433	8,776	0,422	16,407	0,772	14,668	0,706
F-test	1.05 < *F* tab (5.05)				1.19 < *F* tab (5.05)			
t-test	0.2597 < *t* tab (2.12)				0.1309 < *t* tab (2.12)			
F-test plates method vs flow cytometry					2.01 < *F* tab (2.85)			
t-test plates method vs flow cytometry					0.1080 < *t* tab (2.2)			

Table 3
Repeatability and intermediate precision data resulted from the *Lactobacillus acidophilus* enumeration by plate method and flow cytometry.

	Lactobacillus acidophilus							
	Plate count				Flow cytometry			
	Operator 1		Operator 2		Operator 1		Operator 2	
Samples	Results (cfu/g)	Log_{10}	Results (cfu/g)	Log_{10}	Results (cfu/g)	Log_{10}	Results (cfu/g)	Log_{10}
1	2,56E + 09	9,41	1,68E + 09	9,23	2,44E + 09	9,39	2,05E + 09	9,31
2	2,84E + 09	9,45	2,14E + 09	9,33	1,88E + 09	9,27	1,91E + 09	9,28
3	1,62E + 09	9,21	2,16E + 09	9,34	1,55E + 09	9,19	1,99E + 09	9,30
4	2,88E + 09	9,46	2,16E + 09	9,34	1,67E + 09	9,22	1,73E + 09	9,24
5	2,97E + 09	9,47	2,32E + 09	9,37	1,53E + 09	9,18	1,66E + 09	9,22
6	2,42E + 09	9,38	2,45E + 09	9,39	1,46E + 09	9,16	1,53E + 09	9,18
Average	2,55E + 09	9,398	2,15E + 09	9,330	1,75E + 09	9,237	1,81E + 09	9,256
Dev std%	5,01E + 08	0,098	2,59E + 08	0,056	3,68E + 08	0,083	2,05E + 08	0,050
CV%	19,672	1,098	12,036	0,598	20,981	0,902	11,336	0,540
F-test	3.07 < F tab (5.05)				2.79 < *F* tab (5.05)			
t-test	0.1379 < *t* tab (2.12)				0.0428 < *t* tab (2.12)			
F-test plates method vs flow cytometry					1.57 < *F* tab (2.85)			
t-test plates method vs flow cytometry					0.2506 < *t* tab (2.2)			

Table 4
Repeatability and intermediate precision of Bifidobacterium animalis and Lactobacillus acidophilus data count derived from the plate method and flow cytometry.

	Bifidobacterium animalis + *Lacobacillus acidophilus*							
	Plate count method				Flow cytometry			
	Operator 1		Operator 2		Operator 1		Operator 2	
Samples	Results (cfu/g)	Log_{10}	Results (cfu/g)	Log_{10}	Results (cfu/g)	Log_{10}	Results (cfu/g)	Log_{10}
1	1,91E + 09	9,28	4,18E + 09	9,62	2,02E + 09	9,31	2,67E + 09	9,43
2	3,73E + 09	9,57	3,64E + 09	9,56	2,44E + 09	9,39	2,87E + 09	9,46
3	1,27E + 09	9,10	4,00E + 09	9,60	2,58E + 09	9,41	2,20E + 09	9,34
4	2,55E + 09	9,41	3,91E + 09	9,59	2,90E + 09	9,46	2,25E + 09	9,35
5	3,64E + 09	9,56	3,27E + 09	9,51	2,73E + 09	9,44	3,66E + 09	9,56
6	1,82E + 02	9,26	1,45E + 09	9,16	2,40E + 09	9,38	2,50E + 09	9,40
Average	2,48E + 09	9,364	3,41E + 09	9,509	2,51E + 09	9,397	2,69E + 09	9,423
Dev std%	1,01E + 09	0,184	1,01E + 09	0,174	3,04E + 08	0,054	5,38E + 08	0,081
CV%	40,718	1,960	29,587	1,826	12,098	0,578	20,002	0,865
F-test	1.15 < F tab (5.05)				2.23 < *F* tab (5.05)			
t-test	0.1327 < t tab (2.12)				0.0611 < *t* tab (2.12)			
F-test plates method vs flow cytometry					7.61 < *F* tab (2.85)			
t-test plates method vs flow cytometry					0.030 < *t* tab (2.2)			

Table 5
Results regarding system precision of *Bifidobacterium animalis* obtained by plate method and flow cytometry in parallel.

	Bifidobacterium animalis			
	Plate count		Flow cytometry	
	Results (cfu/g)	Log_{10}	Results (cfu/g)	Log_{10}
1	1,85E + 09	9,27	1,89E + 09	9,28
2	1,64E + 09	9,21	1,74E + 09	9,24
3	1,92E + 09	9,28	1,75E + 09	9,24
4	1,74E + 09	9,24	1,77E + 09	9,25
5	1,78E + 09	9,25	1,73E + 09	9,24
6	1,72E + 09	9,24	1,81E + 09	9,26
Average	1,77E + 09	9,248	1,78E + 09	9,251
Dev std%	1,01E + 08	0,025	6,17E + 07	0,015
CV%	5,682	0,266	3,461	0,160
F-test	2.78 < F tab (5.05)			
t-test	0.0203 < t tab (2.12)			

Table 6
Results regarding system precision of *Lactobacillus acidophilus* obtained by plate method and flow cytometry in parallel.

	Lactobacillus acidophilus			
	Plate count		Flow cytometry	
	Results (cfu/g)	Log_{10}	Results (cfu/g)	Log_{10}
1	2,25E + 09	9,35	2,43E + 09	9,39
2	2,39E + 09	9,38	2,36E + 09	9,37
3	2,55E + 09	9,41	2,39E + 09	9,38
4	2,61E + 09	9,42	2,29E + 09	9,36
5	2,45E + 09	9,39	2,35E + 09	9,37
6	2,39E + 09	9,38	2,29E + 09	9,36
Average	2,44E + 09	9,387	2,35E + 09	9,371
Dev std%	1,29E + 08	0,023	5,77E + 07	0,011
CV%	5,269	0,245	2,455	0,114
F-test	4.65 < *F* tab (5.05)			
t-test	0.1414 < *t* tab (2.12)			

Table 7
Results regarding system precision of multistrain samples derived from the plate method and flow cytometry in parallel.

	Bifidobacterium animalis + *Lactobacillus acidophilus*			
	Plate count		Flow cytometry	
	Results (cfu/g)	Log_{10}	Results (cfu/g)	Log_{10}
1	2,95E + 09	9,47	2,72E + 09	9,43
2	2,32E + 09	9,37	3,08E + 09	9,49
3	3,36E + 09	9,53	3,23E + 09	9,51
4	3,50E + 09	9,54	3,07E + 09	9,49
5	2,32E + 09	9,37	3,09E + 09	9,49
6	2,55E + 09	9,41	3,53E + 09	9,55
Average	2,83E + 09	9,446	3,12E + 09	9,493
Dev std%	5,20E + 08	0,079	2,66E + 08	0,037
CV%	18,366	0,839	8,523	0,391
F-test	4.60 < *F* tab (5.05)			
t-test	0.1229 < *t* tab (2.12)			

regulation EU2017/745) based on probiotic cells met with great interest consumers and markets. Only live cells ensure a direct and real activity and exert their benefit. With regards to medical devices, the new EU regulation 2017/745 does not apply in products containing viable organisms, as reported in 6.h of L 117/14 of the Regulation. Before that, the development of medical devices containing probiotic bacteria was focused on vaginal and small intestine dysbiosis; the main benefit of the probiotics in these settings consists in their high adhesive properties (Goldstein et al., 2015) that prevents and competes the adhesion of pathogens. In fact, adhesion of lactobacilli to the epithelium has been described as the first step in the formation of a barrier to prevent undesirable pathogen colonization by the formation of a bacterial film on the epithelium which may contribute to the exclusion of pathogens (Ortiz et al., 2014). Lactobacilli producing lactic acid also contribute to restore and maintain the well-being of the vaginal ecosystem, favoring a correct acidification, thereby inhibiting the proliferation of pathogens and favoring the adhesion of "good" bacteria.

In this context, flow-cytometry, a multiparameter technique, is able to identify viable cells in an entire population of bacteria (Nocker et al., 2006; Wilkinson et al., 2018; Gruden, 2004; Jozwa and Czaczyk, 2012;Chiron et al., 2018), analyzing the physiological state and the metabolic activities of the cells using fluorescent dyes (Kerstens et al., 2014; Chiron et al., 2017;Nebe-von-Caron et al., 2000; Lahtinen, 2005, 2008). Flow cytometry in microbiological examination is a novel approach not yet diffused; in fact, the *gold standard* for routine bacterial enumeration is still considered the plate count (Gracias and McKillip, 2004; Holm et al., 2004; Jozwa and Czaczyk, 2012). Extending the ICH principles on probiotic-based products and comparing classical plate count method with a new vitality assay by flow cytometer represents the innovative and powerful features of this work.

The composition of our tested sample was chosen in order to emulate both generic and complex food supplement. The presence of different vitamins, minerals, plant extract, maltodextrins and FOS, has been necessary to evaluate any possible interference by the substances present in commercial product. *Bifidobacterium* spp. and *Lactobacillus* spp. are the most common genres used in dietary supplements. Their physiological state is difficult to evaluate using traditional culture techniques, in fact damaged and quiescent cells, which are viable but non-cultivable, are not correctly detectable by culture and caused quantification errors in estimate the amount of real efficient cells (Sincock et al., 2001; Ashraf and Shah, 2011).

The flow-cytometer was set before validation, in order to exclude interferences derived from dyes, diluent media and placebo formulation. For each experiment, only live cells, positive for the TO staining which correspond to the ones able to grow in colonies on agar plates, were considered. Bacterial counts had been converted in log values, both for culture method and for flow cytometry, before the statistical calculations. Method specificity, limit of detection/limit of quantification (LOD/LOQ), linearity, accuracy, precision (in terms of repeatability, system and intermediate precision) have been measured, according the ICH chemical criteria.

All statistical values, except for *F-test* in intermediate precision of multistrain product, confirmed the comparability of viable cell counts between plate method and flow cytometry. Selectivity had been performed with both methods, analyzing diluent blank solution, placebo, one without *Bifidobacterium animalis*, one without *Lactobacillus acidophilus* and one without both of them.

Results obtained from finished products and reconstituted samples indicated a good correlation between plate counts and flow cytometry; moreover, data regarding the second one seemed to be more repeatable with a higher precision and stronger results. Data correlation between flow cytometer and plate count enumeration of single strain cultures were robust. When both species were considered, the count became underestimated by flow cytometry, probably due to the high number of cells per dilution. Consequently, higher dilutions of both species, resulted in a better precision. Considering that linearity is the ability of a method to have proportional response to active ingredient concentration, the correlation between our two tested methods was notable, considering the high values of the active principle, as probiotic cell

Table 8
Accuracy results of *Bifidobacterium animalis* obtain by plate method vs flow cytometer method.

Bifidobacterium animalis									
Plate count					Flow cytometry				
Levels	80%	100%	120%	Total	Levels	80%	100%	120%	Total
Recovery%	97,44	100,87	96,86	97,44	Recovery%	97,47	96,80	96,79	97,47
	95,17	96,77	97,36	95,17		95,65	96,91	97,66	95,65
	97,06	97,39	97,12	97,06		96,88	96,99	97,32	96,88
				100,87					96,80
				96,77					96,91
				97,39					96,99
				96,86					96,79
				97,36					97,66
				97,12					97,32
No determinations	3	3	3	9	No determinations	3	3	3	9
Freedom degrees	2	2	2	8	Freedom degrees	2	2	2	8
Average	96,56	98,34	97,11	97,34	Average	96,67	96,90	97,26	96,94
Dev std	1,214	2,208	0,255	1,494	Dev std	0,928	0,095	0,439	0,575
CV %	1,257	2,246	0,262	1,535	CV %	0,960	0,098	0,451	0,593

Table 9
Accuracy of *Lactobacillus acidophilus* of plate method vs flow cytometer method.

Lactobacillus acidophilus									
Plate count					Flow cytometry				
Levels	80%	100%	120%	Total	Levels	80%	100%	120%	Total
Recovery %	100,79	100,59	100,59	100,79	Recovery %	100,86	101,14	100,86	100,86
	100,97	100,27	100,46	100,97		100,95	100,74	100,95	100,95
	99,75	99,16	99,43	99,75		100,56	101,09	100,56	100,56
				100,59					101,14
				100,27					100,74
				99,16					101,09
				100,59					100,86
				100,46					100,95
				99,43					100,56
No determinations	3	3	3	9	No determinations	3	3	3	9
Freedom degrees	2	2	2	8	Freedom degrees	2	2	2	8
Average	100,50	100,01	100,16	100,22	Average	100,79	100,99	100,79	100,86
Dev std	0,662	0,754	0,632	0,632	Dev std	0,206	0,218	0,206	0,208
CV %	0,659	0,754	0,631	0,631	CV %	0,204	0,216	0,204	0,206

Table 10
Accuracy reached by the two methods in *Lactobacillus acidophilus* and *Bifidobacterium animalis* mixed samples detection.

Bifidobacterium animalis + *Lactobacillus acidophilus*									
Plate count					Flow cytometry				
Levels	80%	100%	120%	Total	Levels	80%	100%	120%	Total
Recovery %	97,99	98,72	99,23	97,99	Recovery %	98,15	98,82	99,56	98,15
	98,45	98,83	98,98	98,45		98,74	99,03	99,38	98,74
	97,99	98,26	98,80	97,99		98,29	98,76	98,86	98,29
				98,72					98,82
				98,83					99,03
				98,26					98,76
				99,23					99,56
				98,98					99,38
				98,80					98,86
No determinations	3	3	3	9	No determinations	3	3	3	9
Freedom degrees	2	2	2	8	Freedom degrees	2	2	2	8
Average	98,14	98,60	99,00	98,58	Average	98,39	98,87	99,26	98,84
Dev std	0,266	0,302	0,213	0,435	Dev std	0,307	0,144	0,362	0,452
CV %	0,271	0,306	0,215	0,442	CV %	0,312	0,145	0,365	0,457

Table 11
LOD/LOQ concerning *Bifidobacterium animalis* identification by plate method vs flow cytometry.

	Bifidobacterium animalis (theoretical value: 2,56E + 09 cfu/g)					
	Plate count			Flow cytometry		
Dilution	No colonies	Average	Result (cfu/g)	No events	Average	Result (cfu/g)
1,E + 07	198	258,3	2,58E + 09	N.A.	N.A.	N.A.
	291			N.A.		
	286			N.A.		
1,E + 08	16	17,7	1,77E + 09	20	17,3	1,73E + 09
	17			15,3		
	20			16,7		
1,E + 09	2	3,3	3,33E + 09	3,8	2,6	2,57E + 09
	3			3,4		
	5			0,5		
1,E + 10	0	0,0	0,00E + 00	0	0,0	0,00E + 00
	0			0		
	0			0,1		

Table 12
LOD/LOQ concerning *Lactobacillus acidophilus* identification by plate method vs flow cytometry.

	Lactobacillus acidophilus (theoretical value: 2,40E + 09 cfu/g)					
	Plate count			Flow cytometry		
Dilution	No colonies	Average	Result (cfu/g)	No events	Average	Result (cfu/g)
1,E + 07	197	193,3	1,93E + 09	N.A.	N.A.	N.A.
	198			N.A.		
	185			N.A.		
1,E + 08	15	15,0	1,50E + 09	15,1	15,5	1,55E + 09
	13			17,1		
	17			14,3		
1,E + 09	2	2,0	2,00E + 09	0,3	0,7	7,33E + 08
	2			0,5		
	2			1,4		
1,E + 10	0	0,0	0,00E + 00	0	0,0	0,00E + 00
	0			0		
	0			0		

counts.

Precision has been evaluated by three steps: method precision, intermediate precision and system precision. For each parameter, the calculation was performed both for plate count method and flow cytometry, applied to samples containing *Bifidobacterium animalis*, *Lactobacillus acidophilus* or both of them.

Regarding method precision there was no significant difference between bacterial counts obtained by classical microbiology and flow cytometry in sample containing *Bifidobacterium animalis*, *Lactobacillus acidophilus* and in sample with *Bifidobacterium animalis* plus *Lactobacillus acidophilus*.

Also the intermediate precision measurement gave no significant difference between the two approaches (in terms of *t-test* and *F-test*). In particular, the *F-test* was higher than *F* tabulated comparing the two methods, probably due to flow cytometry underestimation and to plate count method variability.

System precision, in terms of Coefficient of variation % of each single analysis, was in compliance with the acceptance criteria defined by ICH Q2(R1). The Coefficient of variation %, was < 1%. Furthermore, data achieved with flow cytometry are considerably lower when compared to plate count method.

Regarding accuracy, the mean recovery achieved by the plate count and by the flow cytometry methods was within 95–105% of the theoretical value. The Coefficient of variation % for each level was < 3%, while the Coefficient of variation % of the total level was < 5%. Both count methods could be considered accurate within the range 80–120% of the theoretical value, so they could be defined suitable accurate.

LOD and LOQ were evaluated performing analysis on the higher dilutions tested. This ensured the identification of the effectively viable cells by the flow cytometer, compared to traditional count.

Morphological and fluorescence gates used during flow cytometry acquisition were set in order to discriminate signal from the background noise. The parameters selected for the analysis were in line with the ICH's definition of LOD, defined as 3,3 times the background noise, in logarithmic scale.

In general, the average of the three measures for each dilution had confirmed the comparability between flow cytometry and plate count results, according with theoretical values for each probiotic strain tested.

Considering all these results together, the methodology optimized and validated for bacteria enumeration using flow cytometry in the respected of ICH recommendations, laid the bases for the application of chemical-pharmaceutical rules also in viable cell count for probiotic production.

5. Conclusions

Flow cytometry is an optimal alternative as a non-culture based method for enumerating live cells in Quality Control (QC) assessment, using the ICH system.

Results obtained in this study had shown that flow cytometry was applicable in the probiotic count assessment, allowing to identify viable and non-viable cells and providing additional information on the physiological state and the metabolic activity of the cells. Bacteria enumeration achieved by flow cytometry was comparable with the classical plate count considering all specie tested, *Lactobacillus acidophilus*,

Table 13
LOD/LOQ related to *Bifidobacterium animalis and Lactobacillus acidophilus* identification by plate method vs flow cytometry.

	Bifidobacterium animalis + *Lactobacillus acidophilus* (theoretical value: 4,96 E + 09 cfu/g)					
	Plate count			Flow cytometry		
Dilution	No colonies	Average	Result (cfu/g)	No events	Average	Result (cfu/g)
1,E + 07	431	431,7	4,32E + 09	N.A.	N.A.	N.A.
	450			N.A.		
	414			N.A.		
1,E + 08	54	51,3	5,13E + 09	36,4	46,9	4,69E + 09
	53			53,7		
	47			50,6		
1,E + 09	5	3,7	3,67E + 09	1,2	2,5	2,47E + 09
	3			2,9		
	3			3,3		
1,E + 10	0	0,0	0,00E + 00	0,2	0,1	0,00E + 00
	0			0		
	0			0		

Bifidobacterium animalis and multistrain samples.

Another important workflow improvement was related to the reduction of analysis time, 2 h versus 3 days for the traditional method.

In conclusion, the application of a chemical-pharmaceutical validation approach, using ICH on viable probiotic, ensures a higher and more robust quality control in flow cytometry compared to the standard method used.

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Author contributions

L.M. and E.N., conceptualization, data curation; L.M., methodology and formal analysis; E.N., methodology and interpretation of results; all authors, writing-review & editing.

Data and materials availability

Processed and calculated data associated with this study are present in the paper.

Declaration of Competing Interests

Authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2020.105834.

References

Alvarez-Barrientos, A., et al., 2000. Applications of flow cytometry to clinical microbiology. Clin. Microbiol. Rev. 13 (2), 167–195.
Amor, K.B., et al., 2002. Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead bifidobacterium cells during bile salt stress. Appl. Environ. Microbiol. 68 (11), 5209–5216.
Ashraf, R., Shah, N.P., 2011. Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt–a review. Int. J. Food Microbiol. 149 (3), 194–208.
Assuncao, P., et al., 2007. Detection of mycoplasmas in goat milk by flow cytometry. Cytometry A 71 (12), 1034–1038.
Bunthof, C.J., Abee, T., 2002. Development of a flow cytometric method to analyze subpopulations of bacteria in probiotic products and dairy starters. Appl. Environ. Microbiol. 68 (6), 2934–2942.
Charteris, W.P., et al., 1997. Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. Int. J. Food Microbiol. 35 (1), 1–27.
Chiron, C., et al., 2017. Flow cytometry: A versatile technology for specific quantification and viability assessment of micro-organisms in multistrain probiotic products. J. Appl. Microbiol 18 (124), 572–584.
Chiron, C., et al., 2018. Flow cytometry: A versatile technology for specific quantification and viability assessment of micro-organisms in multistrain probiotic products. J. Appl. Microbiol. 124 (2), 572–584.
Comas-Riu, J., Rius, N., 2009. Flow cytometry applications in the food industry. J. Ind. Microbiol. Biotechnol. 36 (8), 999–1011.
Davey, H.M., 2002. Flow cytometric techniques for the detection of microorganisms. Methods Cell Sci. 24 (1–3), 91–97.
Doherty, S.B., et al., 2010. Use of viability staining in combination with flow cytometry for rapid viability assessment of *Lactobacillus rhamnosus* GG in complex protein matrices. J. Microbiol. Methods 82 (3), 301–310.
Garcia-Armesto, M.R., et al., 1993. Modern microbiological methods for foods: colony count and direct count methods. A review. Microbiologia 9 (1), 1–13.
Goldstein, E.J., et al., 2015. *Lactobacillus* species: Taxonomic complexity and controversial susceptibilities. Clin. Infect. Dis. 60 (Suppl. 2), s98–107.
Gracias, K.S., McKillip, J.L., 2004. A review of conventional detection and enumeration methods for pathogenic bacteria in food. Can. J. Microbiol. 50 (11), 883–890.
Gruden, C., et al., 2004. Flow cytometry for microbial sensing in environmental sustainability applications: Current status and future prospects. FEMS Microbiol. Ecol. 49 (1), 37–49.
Holm, C., et al., 2004. A flow cytometric technique for quantification and differentiation of bacteria in bulk tank milk. J. Appl. Microbiol. 97 (5), 935–941.
Jozwa, W., Czaczyk, K., 2012. Flow cytometric analysis of microbial contamination in food industry technological lines–initial study. Acta Sci. Pol. Technol. Aliment. 11 (2), 110–119.
Kerstens, M., et al., 2014. Flow cytometric enumeration of bacteria using to-pro®-3 iodide as a single-stain viability dye. J. Lab. Automat. 19 (6), 555–561.
Kramer, M., et al., 2009. Quantification of live and dead probiotic bacteria in lyophilised product by real-time PCR and by flow cytometry. Appl. Microbiol. Biotechnol. 84 (6), 1137–1147.
Lahtinen, S.J., et al., 2005. Probiotic bacteria may become dormant during storage. Appl. Environ. Microbiol. 71 (3), 1662–1663.
Lahtinen, S.J., et al., 2008. Degradation of 16S rRNA and attributes of viability of viable but nonculturable probiotic bacteria. Lett. Appl. Microbiol. 46 (6), 693–698.
Laplace-Builhe, C., et al., 1993. Application of flow cytometry to rapid microbial analysis in food and drinks industries. Biol. Cell. 78 (1–2), 123–128.
Malacrino, P., et al., 2001. Rapid detection of viable yeasts and bacteria in wine by flow cytometry. J. Microbiol. Methods 45 (2), 127–134.
McHugh, I.O., Tucker, A.L., 2007. Flow cytometry for the rapid detection of bacteria in cell culture production medium. Cytometry A 71 (12), 1019–1026.
Muller, S., Nebe-von-Caron, G., 2010. Functional single-cell analyses: Flow cytometry and cell sorting of microbial populations and communities. FEMS Microbiol. Rev. 34 (4), 554–587.
Nebe-von-Caron, G., et al., 2000. Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. J. Microbiol. Methods 42 (1), 97–114.
Nocker, A., et al., 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Methods 67 (2), 310–320.
Novo, D.J., et al., 2000. Multiparameter flow cytometric analysis of antibiotic effects on membrane potential, membrane permeability, and bacterial counts of *Staphylococcus aureus* and *Micrococcus luteus*. Antimicrob. Agents Chemother. 44 (4), 827–834.
Ortiz, l, et al., 2014. Effect of two probiotic strains of lactobacillus on in vitro adherence of *Listeria monocytogenes*, *Streptococcus agalactiae*, and *Staphylococcus aureus* to vaginal epithelial cells. Curr. Microbial. 68 (6), 679–684.
Rault, A., et al., 2007. Multiparametric flow cytometry allows rapid assessment and comparison of lactic acid bacteria viability after freezing and during frozen storage. Cryobiology 55 (1), 35–43.
Raymond, Y., Champagne, C.P., 2015. The use of flow cytometry to accurately ascertain total and viable counts of *Lactobacillus rhamnosus* in chocolate. Food Microbiol. 46, 176–183.
Robinson, J.P., Roederer, M., 2015. History of science. Flow cytometry strikes gold. Science 350 (6262), 739–740.
Roy, D., 2001. Media for the isolation and enumeration of bifidobacteria in dairy products. Int. J. Food Microbiol. 69 (3), 167–182.
Ruszczynska, A., et al., 2007. Flow cytometry as a method for the evaluation of raw material, product and process in the dairy industry. Pol. J. Vet. Sci. 10 (2), 119–121.
Sincock, S.A., et al., 2001. Flow cytometric analysis of microorganisms. Methods Cell Biol. 64.
Sohier, D., et al., 2014. Evolution of microbiological analytical methods for dairy industry needs. Front. Microbiol. 5, 16.
Tracy, B.P., et al., 2008. Development and application of flow-cytometric techniques for analyzing and sorting endospore-forming clostridia. Appl. Environ. Microbiol. 74 (24), 7497–7506.
Want, A., et al., 2011. Multi-parameter flow cytometry and cell sorting reveal extensive physiological heterogeneity in *Bacillus cereus* batch cultures. Biotechnol. Lett. 33 (7), 1395–1405.
Weiss, D.J., 2002. Application of flow cytometric techniques to veterinary clinical hematology. Vet. Clin. Pathol. 31 (2), 72–82.
Wilkes, J.G., et al., 2012. Reduction of food matrix interference by a combination of sample preparation and multi-dimensional gating techniques to facilitate rapid, high sensitivity analysis for *Escherichia coli* serotype O157 by flow cytometry. Food Microbiol. 30 (1), 281–288.
Wilkinson, M.G., et al., 2018. Flow cytometry as a potential method of measuring bacterial viability in probiotic products: A review. Trends Food Sci. Technol. 78, 1–10.