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ORIGINAL ARTICLE

Flow cytometry: a versatile technology for specific quantification and viability assessment of micro-organisms in multistrain probiotic products

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Abstract

Aims: Classical microbiology techniques are the gold standard for probiotic enumeration. However, these techniques are limited by parameters of time, specificity and incapacity to detect viable but nonculturable (VBNC) micro-organisms and nonviable cells. The aim of the study was to evaluate flow cytometry as a novel method for the specific quantification of viable and nonviable probiotics in multistrain products.

Methods and Results: Custom polyclonal antibodies were produced against five probiotic strains from different species (*Bifidobacterium bifidum* R0071, *Bifidobacterium longum* ssp. *infantis* R0033, *Bifidobacterium longum* ssp. *longum* R0175, *Lactobacillus helveticus* R0052 and *Lactobacillus rhamnosus* R0011). Evaluation of specificity confirmed that all antibodies were specific at least at the subspecies level. A flow cytometry method combining specific antibodies and viability assessment with SYTO®24 and propidium iodide was applied to quantify these strains in three commercial products. Analyses were conducted on two flow cytometry instruments by two operators and compared with classical microbiology using selective media. Results indicated that flow cytometry provides higher cell counts than classical microbiology ($P < 0{\cdot}05$) in 73% of cases highlighting the possible presence of VBNC. Equivalent performances (repeatability and reproducibility) were obtained for both methods.

Conclusions: This study showed that flow cytometry methods can be applied to probiotic enumeration and viability assessment. Combination with polyclonal antibodies can achieve sufficient specificity to differentiate closely related strains.

Significance and Impact of the Study: Flow cytometry provides absolute and specific quantification of viable and nonviable probiotic strains in a very short time (<2 h) compared with classical techniques (>48 h), bringing efficient tools for research and development and quality control.

Introduction

Probiotics are defined as 'live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2001). Thus, probiotic functionality is impacted by the quantity and viability of micro-organisms being consumed. Probiotic manufacturers, therefore, need accurate analytical tools to determine the quantity of viable probiotic micro-organisms in a minimal time. The microbes most commonly used as dietary supplements belong to the *Lactobacillus* and *Bifidobacterium* genera and are often composed of a mix of different species and strains. Consequently, analytical methods have to be specific enough to differentiate between several species of lactobacilli and bifidobacteria that may be closely related.

Classical microbiology culture-based techniques remain the gold standard for the enumeration of micro-organisms, and results are presented in colony forming units (CFU). Selective growth can be achieved with the use of culture media with specific nutrients, pH and/or antibiotics to inhibit growth of nondesired micro-organisms (Charteris *et al.* 1997). As an example, a recent study published the development of a chromogenic culture medium to differentiate and enumerate several lactic acid bacteria (LAB) species (Galat *et al.* 2016). International Organization for Standardization/International Dairy Federation (ISO/IDF) standard methods are also available for the selective enumeration of *Lactobacillus acidophilus* (ISO 20128 2006) and *Bifidobacteria* spp. using antibiotics as selective agents (ISO 29981|IDF 220 2010). However, most culture-based methods are only capable of discriminating bacteria at the genus level and seldom at the species level (Ashraf and Shah 2011); to our knowledge, it is still difficult to reliably differentiate between species of *Bifidobacterium*. These methods are time consuming because probiotic bacteria need 1–5 days of culturing before delivering a result. Furthermore, classical microbiology techniques are limited to the detection of culturable micro-organisms only (cells in a replicative state having known growth conditions). Thus, these methods are inefficient and unsuitable for the enumeration of viable but nonculturable (VBNC) bacteria (Kell *et al.* 1998; Lahtinen *et al.* 2005; Oliver 2005). Thus, there is a need for novel analytical methods allowing specific enumeration and viability assessment of probiotics.

Nonculture-based methods have been extensively developed to overcome classical microbiology limitations. Molecular biology-based techniques, such as real-time quantitative PCR (qPCR) methods, have been developed for specific quantification of viable probiotics using Propidium MonoAzide (PMA) or related molecules (Nocker *et al.* 2006; Kramer *et al.* 2009). These methods distinguish viable bacteria from total population based on membrane integrity assessment (Nocker *et al.* 2006). Another way to quantify viable bacteria is to employ RNA-targeting techniques (Lahtinen *et al.* 2008). However, RNA manipulation is difficult due to the short half-life of microbial RNA (Sohier *et al.* 2014). Molecular biology protocols require several delicate steps (nucleic acid extraction and amplification) that can be tedious. Such methods are very useful for research or clinical purposes but are less suitable for quality control and industrial production environments. Flow cytometry is a technology used for the multiparametric analysis of cells based on light scattering and fluorescence at the single-cell level (Robinson and Roederer 2015). The use of flow cytometry in combination with fluorescent staining has increased within the microbiology field over the last 20 years (Comas-Riu and Rius 2009; Davis 2014; Sohier *et al.* 2014). Viability staining and flow cytometry (viable flow cytometry) has been extensively used to monitor bacteria physiological state (Joux and Lebaron 2000; Doherty *et al.* 2010), survival upon physical or chemical stresses (Amor *et al.* 2002; Rault *et al.* 2007; Chen *et al.* 2012), antibiotic susceptibility (Novo *et al.* 2000) and microbial communities study (Apajalahti *et al.* 2002; Muller and Nebe-von-Caron 2010; Koch *et al.* 2014). Viable flow cytometry techniques have also been employed for LAB and probiotic quantification on various products such as lyophilized powders (Kramer *et al.* 2009), fermented products (Doherty *et al.* 2010), dairy (Gunasekera *et al.* 2000), food (Raymond and Champagne 2015) and complex matrices, such as artificial gut microbiota (Grootaert *et al.* 2011). Moreover, an ISO/IDF method has been released in 2015 for the enumeration of viable and nonviable LAB in starter cultures and probiotics in fermented products by flow cytometry (ISO 19344 2015). Specific detection of microbes has been shown to be possible with the production of ligands (protein or oligonucleotide) targeting specific molecules on the bacteria cell surface (Joux and Lebaron 2000; Nebe-von-Caron *et al.* 2000). However, little information has been reported to date on the specific quantification of viable probiotics and LAB by flow cytometry.

The objective of this study was to develop flow cytometry methods for the specific quantification and viability assessment of freeze-dried probiotics in multistrain formulas for research and development (R&D) and quality control (QC) laboratories. A three-colour approach was developed using specific custom antibodies and DNA stains (SYTO®24 and propidium iodide) for viability assessment based on membrane integrity, as described in ISO 19344|IDF 232 20154|IDF 232 (ISO 19344 2015). First, custom polyclonal antibodies were produced against five probiotic strains of interest and specificity was evaluated on various probiotic species and genera. Second, multistrain probiotic products were analysed by flow cytometry and compared with classical microbiology. This study evaluates flow cytometry methods for quantitative and qualitative analysis of bacteria and substantiates application of such novel analytical methods in the industry.

Material and methods

Freeze-dried probiotic strains and multistrain products

All micro-organisms and products used in this study were obtained from Lallemand Health Solutions (LHS) Inc. (Mirabel, QC, Canada). Multistrain products used in the study are described in Table 1. Two production lots were used per product.

Table 1 Probiotic multistrain products used in the study

	Formula composition		Specification*	
Product name	Strain	%	(CFU per g)	(log CFU per g)
Lacidofil®	*Lactobacillus rhamnosus* R0011	95%	$1{\cdot}00 \times 10^{10}$	10·00
	Lactobacillus helveticus R0052	5%		
Protecflor®	*Lactobacillus rhamnosus* R0011	33%	$1{\cdot}25 \times 10^{10}$	10·10
	Lactobacillus helveticus R0052	33%		
	Bifidobacterium longum ssp. *longum* R0175	33%		
	Saccharomyces cerevisiae var. *boulardii* CNCM I-1079	–	$6{\cdot}25 \times 10^{9}$	9·80
ProbioKid®	*Lactobacillus helveticus* R0052	60%	$7{\cdot}50 \times 10^{9}$	9·88
	Bifidobacterium longum ssp. *infantis* R0033	20%		
	Bifidobacterium bifidum R0071	20%		

*Total concentration (CFU per g or log CFU per g) guaranteed at product shelf life (24 months at 25°C).

Polyclonal antibody production

Antigen preparation and immunization

Rabbit polyclonal antibodies (pAb) were produced in female New Zealand rabbits with support of the National Research Council of Canada (NRC) (Montreal, QC, Canada). This study was carried out in strict accordance with Canadian Council on Animal Care policy and guidelines. Animal protocol was reviewed by the Animal Care Committee of Biotechnology Research Institute at National Research Council of Canada (protocol no. 13-MAR-I-062). All efforts were made to minimize suffering. Four antibodies were produced against heat-killed freeze-dried bacteria (*B. bifidum* R0071, *B. longum* ssp. *infantis* R0033, *B. longum* ssp. *longum* R0175 and *L. rhamnosus* R0011) and one against surface-layer protein (SLP) extracted from *L. helveticus* R0052. Briefly, antigens composed of whole bacteria were prepared by dilution of freeze-dried bacteria in Dulbecco's phosphate-buffered saline (DPBS; Hyclone Laboratories Inc., Logan, UT, USA) to reach concentration of *c.* 10^{10} bacteria per ml (determined by flow cytometry). A heat treatment of 20 min at 80°C was performed to kill bacteria and keep cells intact. Samples were washed once in DPBS by centrifugation 5 min at 9000 × ***g*** to remove debris. Purified *L. helveticus* R0052 SLP was extracted using a 5 mol l^{-1} lithium chloride protocol adapted from previously established methods (Taverniti *et al.* 2013; MacPherson *et al.* 2017). Protein extracts were suspended in DPBS and stored at −20°C until use. Purity was determined on a SDS-PAGE gel. SLP extract of *L. helveticus* R0052 was concentrated to 2 g l^{-1} prior to injection.

A minimal dose of 1×10^{9} heat-killed bacteria was injected per animal. Bacteria concentration was determined by flow cytometry using SYTO®24 stain (Molecular Probes Inc., Eugene, OR, USA). For each antigen, two animals were immunized with antigen preparation containing 1:1 Freund's incomplete adjuvant. Sera were collected 80 days after initial immunization, and during this period, animals received three boosters (days 21, 43 and 70). Productions with *B. longum* ssp. *infantis* R0033 and *B. bifidum* R0071 antigens were done at a second NRC facility in Ottawa using a 70-day protocol including two boosters (days 28 and 56). Sera titration was performed at day 52 (or 46 for R0033 and R0071) by flow cytometry (antiwhole bacteria) or Enzyme Linked ImmunoSorbent Assay (ELISA) (anti-R0052-SLP).

Unless unequal titres, sera from both animals immunized with same antigen were mixed 1:1; from these combined sera, IgG were purified with ProSep-A (EMD Millipore Corp., Billerica, MA, USA) affinity-chromatography and suspended in DPBS. Purified IgG concentration was standardized to 1 g l^{-1}.

Antibody specificity evaluation

Antibody cross-reactivity was evaluated for 34 different probiotic strains (including *Lactobacillus, Bifidobacterium,* and yeast) from the Lallemand Bacteria Culture Collection (Table 2) by an ELISA-based test. Briefly, each bacterium was streaked on solid media and grown under optimal conditions to obtain isolated colonies (Table 2); 100 μl of primary IgG antibody 0·1 mg l^{-1} diluted was mixed with 1–2 colonies of bacteria or DPBS (negative control) and incubated for 1 h at room temperature. Unbound antibodies were removed by washing twice with 200 μl of washing buffer (DPBS-0·1% Tween 20-0·5% BSA) and centrifuged at 9000 × ***g*** for 5 min. Next, bacteria pellets were resuspended and incubated in same conditions with 100 μl of peroxidase-conjugated anti-rabbit antibody (Jackson Immunoresearch Labs., West Grove, PA, USA) 0·01 mg l^{-1}. Unbound antibodies were removed by washing twice as previously mentioned. Pellets were resuspended with 100 μl of TetraMethylBenzidine (TMB) substrate (Life Technologies, Carlsbad, CA, USA), and samples were transferred into a 96-well plate and incubated in the dark for 30 min. Peroxidase reacts

Table 2 List of micro-organisms used to evaluate antibody specificity

Genus	Species/subspecies	Strain	Culture conditions
Bacillus	*subtilis*	1	TSA, Aerobiosis 37°C, 24–48 h
Bifidobacterium	*breve*	2	RCM, Anaerobiosis
	animalis ssp. *lactis*	2	37°C, 48–72 h
	bifidum	1	
	longum ssp. *infantis*	1	
	longum ssp. *longum*	1	
Enterococcus	*faecium*	2	MRS, Anaerobiosis 37°C, 48–72 h
Lactobacillus	*acidophilus*	1	MRS, Anaerobiosis
	brevis	1	37°C, 48–72 h
	delbrueckii ssp. *bulgaricus*	2	
	farciminis	1	MRS, Aerobiosis
	fermentum	1	37°C, 48–72 h
	helveticus	2	MRS, Anaerobiosis
	paracasei ssp. *paracasei*	4	37°C 48–72 h
	plantarum	2	MRS, Aerobiosis 37°C, 48–72 h
	reuteri	1	MRS, Anaerobiosis
	rhamnosus	2	37°C, 48–72 h
	salivarius ssp. *salivarius*	1	MRS, Aerobiosis 37°C, 48–72 h
Lactococcus	*lactis* ssp. *lactis*	1	M17+lactose 10%, Aerobiosis 37°C, 48–72 h
Pediococcus	*acidilactici*	1	MRS, Anaerobiosis 37°C, 48–72 h
Propionibacterium	*freudenreichii* ssp. *shermanii*	1	Lactate agar, Anaerobiosis 37°C, 48–72 h
Saccharomyces	*cerevisiae* var. *boulardii*	1	YEPD, Aerobiosis 30°C, 24–48 h
Streptococcus	*thermophilus*	2	M17+lactose 10% Anaerobiosis 41°C, 48–72 h

TSA, tryptone soy agar (CM0131, Oxoid); RCM, reinforced clostridiales medium (CM0151, Oxoid); MRS, de mann, rogosa, sharpe agar (CM0361, Oxoid), M17 agar (CM0817 Oxoid); lactate agar (internal recipe); YEPD, yeast extract peptone dextrose agar (242720, Difco, BD).

with TMB substrate resulting in the formation of a blue-coloured product. Reaction was stopped by adding 100 μl 1N HCl solution forming a yellow colour. Absorbance was immediately read at 450 nm using Multiskan™ GO microplate spectrophotometer (ThermoFisher Scientific Inc., Waltham, MA, USA).

For each antibody, ELISA-positive strains were analysed by flow cytometry using a fluorescent staining described below to determine percentage of antibody reactivity.

Fluorescent staining

The antibody staining protocol was adapted from a publication (Nebe-von-Caron *et al.* 2000). Washing step between primary and secondary antibody was changed to a dilution step to allow absolute quantification of the sample. Probiotic samples were rehydrated and diluted in buffered peptone water (0·1% soy peptone, 2·5 mmol l^{-1} KH_2PO_4, 6·9 mM K_2HPO_4) to reach *c.* 5×10^6 bacteria per ml. Briefly, 1 μg of primary antibody (1 g l^{-1}) was used to stain 200 μl of bacteria suspension. Samples were vortexed vigorously and incubated for 30 min at room temperature. Following incubation with primary antibody, the suspension was diluted 1/5 in DPBS to limit aggregation of free primary with secondary antibodies. Next, 1 μg of Alexa Fluor® 647-conjugated $F(ab')_2$ secondary antibodies goat-anti-rabbit (Molecular Probes) (1 g l^{-1}) was used to stain bacteria suspension. Samples were mixed vigorously and incubated for 30 min at room temperature in the dark. Following antibody staining, samples were diluted 1/2 (Accuri™) or 1/10 (CyFlow®) in DPBS and stained with 0·1 μmol l^{-1} SYTO®24 and 0·2 μmol l^{-1} Propidium Iodide (PI) (Molecular Probes) for 15 min at 37°C as described in the ISO 19344|IDF 232 20154|IDF 232 (ISO 19344 2015). The combination of SYTO®24 and PI fluorescent dyes can determine bacteria viability based on membrane integrity. SYTO®24 is a cell-permeant green fluorescent nucleic-acid stain. PI is a cell-impermeant red fluorescent DNA intercalator, commonly used to stain nonviable bacteria because PI enters bacteria only if their membrane is compromised. As reported by ISO 19344, the number of viable cells is reported as 'active fluorescent units' (AFU) and nonviable cells are reported as 'nonactive fluorescent units' (n-AFU).

Flow cytometry analysis

Experiments were run on two flow cytometry instruments: the CyFlow® Space (Sysmex-Partec GmbH, Görlitz, Germany) and the Accuri™ C6 (BD BioSciences, San Jose, CA, USA) equipped with CSampler™ plate reader. Both instruments were equipped with two lasers (488 nm, 635 nm) and six parameters including FSC, SSC and four fluorescent channels (FL1-FL4). Acquisition and data analysis were performed with FloMax® (Sysmex-Partec) and CSampler software (BD). Optimal concentration for absolute quantification was between 10^4 and 10^5 events per ml, under speed rate 3 μl s^{-1} (CyFlow) or 'Medium speed' rate of 35 μl min^{-1} (Accuri). To avoid background noise, CyFlow trigger was set on total bacteria green fluorescence (FL1), and Accuri optimal threshold levels of 2500 and 1000 were set on

FCS and FL1, respectively. At least three independent analyses of each product were performed by two operators on each flow cytometry instrument.

Epifluorescence microscopy

Microscopy observations of probiotic product stained with antibodies were realized to visualize antibody staining location and fluorescence intensity. Protecflor® stained with antibodies (anti-*L. rhamnosus* R0011, anti-*B. longum* ssp. *longum* R0175 or anti-*L. helveticus* SLP-R0052) and SYTO®24 as described above was analysed with epifluorescence microscopy. Stained samples were concentrated by centrifugation 5 min at 9000 × ***g*** and resuspended in 10 μl of DPBS. Bacteria were mounted on slides using ProLong® Diamond (Molecular Probes) antifade mounting medium and dried overnight at room temperature. Phase contrast and epifluorescence pictures were taken using a Leica DMIRE2 inverted microscope (Leica Microsystems Canada Inc., Richmond, ON, Canada) equipped with a Hamamatsu cooled charge-coupled-device camera at 630× magnification. Green fluorescence (SYTO®24) was analysed with green fluorescent protein (GFP) narrow filter, and far-red fluorescence (AlexaFluor 647) was analysed with Cy5-4040A filter (Semrock Inc., Rochester, NY, USA). Data analysis was performed with ImageJ (Bethesda, MD, NIH, USA).

Classical microbiology

One gram of probiotic powder was dissolved into 9 ml of buffered peptone water and vortexed until the sample was completely dissolved. From this first dilution, 10-fold serial dilutions were prepared in buffered peptone water. Appropriate dilutions were cultured on MRS agar (de Mann, Rogosa and Sharpe; Oxoid, Nepean, ON, Canada) for total count of bacteria in Lacidofil® or RCM agar (Reinforced Clostridial Medium; Oxoid) for total count of bacteria in Protecflor® and ProbioKid®. Specific media were used for enumeration of each strain in commercial products: *L. rhamnosus* R0011 was selected with MRS agar containing 1 mg l^{-1} of vancomycin hydrochloride (Sigma-Aldrich, Oakville, ON, Canada) as it is intrinsically resistant to this antibiotic; *L. helveticus* R0052 was selectively cultured on MRS agar containing clindamycin 0·25 mg l^{-1} (Sigma-Aldrich); strains of *Bifidobacterium* were selected following ISO 29981 standard with TOS propionate agar (Merck, Darmstadt, Germany) supplemented with lithium mupirocin (1 g l^{-1}) (Merck) (Standardization, 2010); yeast *S. boulardii* was selected with YEPD (Yeast Extract Peptone Dextrose) (Difco, BD, Franklin Lakes, NJ) and chloramphenicol 25 mg l^{-1} (Sigma-Aldrich). All bacteria were grown for 48 h at 37°C under anaerobic conditions; the yeast was grown for 48 h at 30°C in aerobic conditions. Each product was analysed by two operators with at least three independent experiments.

Data analysis

Precision (repeatability and reproducibility) of flow cytometry and classical microbiology was determined by calculation of the mean standard deviations (SD) obtained for each analysis with one or two operators. Calculation was performed for each instrument and considering two instruments. An unpaired Student's *t*-test was used to determine statistical significance with PRISM software ver. 6 (GraphPad software, San Diego, CA, USA). Difference was considered statistically significant when $P < 0{\cdot}05$.

Results

Polyclonal antibodies specificity evaluation

Five rabbit polyclonal antibodies were produced. Four antibodies were raised against whole heat-inactivated bacteria (*L. rhamnosus* R0011, *B. longum* ssp. *longum* R0175, *B. longum* ssp. *infantis* R0033 and *B. bifidum* R0071) and one against a surface-layer protein extract from *L. helveticus* R0052. Specificity of purified IgG (anti-*L. rhamnosus* R0011, anti-*B. longum* ssp. *longum* R0175 and anti-*L. helveticus* SLP-R0052) was first evaluated by an ELISA-based test on different probiotic genera and species, involving a total of 34 strains (Table 2). As ELISA is more sensitive than flow cytometry, the ELISA-negative strains were considered not cross-reacting. A second specificity evaluation was performed by flow cytometry on ELISA-positive strains only. Cross-reactivity was defined as bacterium with relative percentage of antibody binding above 5% cut-off by flow cytometry. This cut-off has been arbitrarily determined given the analytical error we would accept internally. The studied microbial products, originally concentrated at >5e9 micro-organisms per g, are diluted prior to being analysed by flow cytometry (final concentration around 1e4 to 1e5 micro-organisms per ml). Thus, a 5% cut-off on flow cytometry analytical data corresponds to less than 1% of variation, when reported to the initial microbial concentration, making that threshold value acceptable for our applications. The ELISA-based analysis revealed that anti-*L. rhamnosus* R0011 antibodies bound 16 of 34 strains tested, anti-*B. longum* ssp. *longum* R0175 Ab bound 8 of 34 strains tested and 2 of 34 strains tested bound with anti-*L. helveticus* SLP-R0052 leaving a limited number of cross-reactive strains following the primary screen. Flow cytometry analysis of ELISA-positive

Table 3 Specificity evaluation of pAb on micro-organisms found in probiotic supplements

Antibody	Antigen	Cross-reactivity*
Anti-*B. bifidum* R0071	Whole bacteria	*L. fermentum* (1 strain)
Anti-*B. longum* ssp. *infantis* R0033	Whole bacteria	0
Anti-*B. longum* ssp. *longum* R0175	Whole bacteria	*B. bifidum* (2 strains); *S. boulardii* (1 strain)
Anti-*L. helveticus* R0052	R0052-SLP	0
Anti-*L. rhamnosus* R0011	Whole bacteria	0

*Cross-reactivity was considered with >5% reactivity with antibodies (flow cytometry).

strains showed no cross-reactivity towards all strains tested for polyclonal antibodies produced against *L. rhamnosus* R0011 and *L. helveticus* SLP-R0052 (Table 3). The anti-*B. longum* ssp. *longum* R0175 antibody cross-reacted with two strains out of 34 tested (*B. bifidum* R0071 and *S. boulardii* CNCM I-1079). Specificity of anti-*B. bifidum* R0071 and anti-*B. longum* ssp. *infantis* R0033 antibodies was evaluated against all strains by flow cytometry only. Results showed no cross-reactivity for the *B. infantis* antibodies, whereas anti-*B. bifidum* cross-reacted with *L. fermentum* HA-179.

Epifluorescence microscopy

Epifluorescence microscopy analysis indicated that anti-*L. rhamnosus* R0011 antibody potentially indicates cell surface antigens (Alexa Fluor 647—red) (Fig. S1). Bacteria stained with antibodies exhibited a bright fluorescence signal (Alexa Fluor 647) which did not interfere with the green fluorescence channel (SYTO®24). No background noise was observed. Same results were observed for anti-*L. helveticus* SLP-R0052 and anti-*B. longum* ssp. *longum* R0175 antibodies (data not shown).

Enumeration of probiotics in multistrain products

Flow cytometry and classical microbiology methods were performed in parallel on two batches of the multistrain probiotic products Protecflor®, Lacidofil® and ProbioKid® (Table 1). Analyses were conducted by two operators on two flow cytometry instruments with three or four replicates. Populations of bacteria stained with antibodies were well separated from the other bacteria in both instruments, which corresponds with microscopy data. Viable and nonviable bacteria populations were also well separated (Fig. 2). An intermediate population stained with both PI and SYTO®24 was considered as nonviable and included in the nonviable gate, as recommended in the ISO 19344 standard.

Enumeration of probiotics in the different products by flow cytometry (plotted values are the mean of data obtained by two instruments) and classical microbiology methods is reported in Fig. 1a. Total count of each batch was compliant with the manufacturer's specifications (Table 1). There was no significant difference between bacterial counts obtained by classical microbiology and flow cytometry for Protecflor Batch A ($P > 0{\cdot}05$), except for *B. longum* R0175 ($P = 0{\cdot}050$). Enumeration by flow cytometry produced significantly higher counts than microbiology for two batches of Lacidofil & ProbioKid ($P < 0{\cdot}05$) and one batch of Protecflor (Batch B) (total: $P < 0{\cdot}05$; R0011: $P < 0{\cdot}05$; R0052: $P < 0{\cdot}05$; *S. boulardii*: $P < 0{\cdot}05$). However, enumeration of *B. longum* R0175 by flow cytometry was lower than classical microbiology in Batch A ($P = 0{\cdot}050$) and B ($P < 0{\cdot}05$). Classical microbiology methods were not able to differentiate *B. infantis* R0033 and *B. bifidum* R0071 in ProbioKid. Correlation of viable bacteria (AFU) data between the two flow cytometry instruments was $R^2 = 0{\cdot}9185$ and 0·9247 with each operator (Fig. S2). When combining all data, correlation was $R^2 = 0{\cdot}9424$, indicating that both instruments gave linear results as well as both operators (Fig. S2). Classical microbiology and flow cytometry methods gave a correlation of $R^2 = 0{\cdot}8222$ (Fig. S3).

The precision of flow cytometry (i.e. repeatability and reproducibility) was also determined (Table 4). Repeatability, the variation in replicates performed by one operator, was $0{\cdot}07 \pm 0{\cdot}07$ log AFU per g with Cyflow Space (Sysmex-Partec) and $0{\cdot}06 \pm 0{\cdot}05$ log AFU per g with Accuri with auto-loader station (BD). Reproducibility between two operators was $0{\cdot}10 \pm 0{\cdot}05$ log AFU per g and $0{\cdot}08 \pm 0{\cdot}05$ log AFU per g for flow cytometry using the Cyflow and the Accuri, respectively. Reproducibility between two flow cytometry instruments was $0{\cdot}12 \pm 0{\cdot}07$ log AFU per g (one operator) and $0{\cdot}13 \pm 0{\cdot}05$ (two operators). Similar results were obtained with classical microbiology: repeatability was $0{\cdot}07 \pm 0{\cdot}04$ log CFU per g and reproducibility (including two operators) was $0{\cdot}13 \pm 0{\cdot}06$ log CFU per g.

Flow cytometry viable and nonviable cell counts are represented in Fig. 1b. The total amount of viable bacteria from each product batch varied between 53 and 68% (Table S1). Viability of *L. rhamnosus* R0011 (55–70% of viable cells) was similar among Protecflor and Lacidofil products. The viability of *L. helveticus* R0052 was 87–89% in Lacidofil and 68–76% in Protecflor and ProbioKid. Viability of *B. longum* R0175 and *S. boulardii* was 66–74% in Protecflor. Finally, the viability of *B. bifidum* R0071 was *c.* 35% in ProbioKid, whereas *B. longum* ssp. *infantis* R0033 varied from 21% to 40% in Batch A and B, respectively.

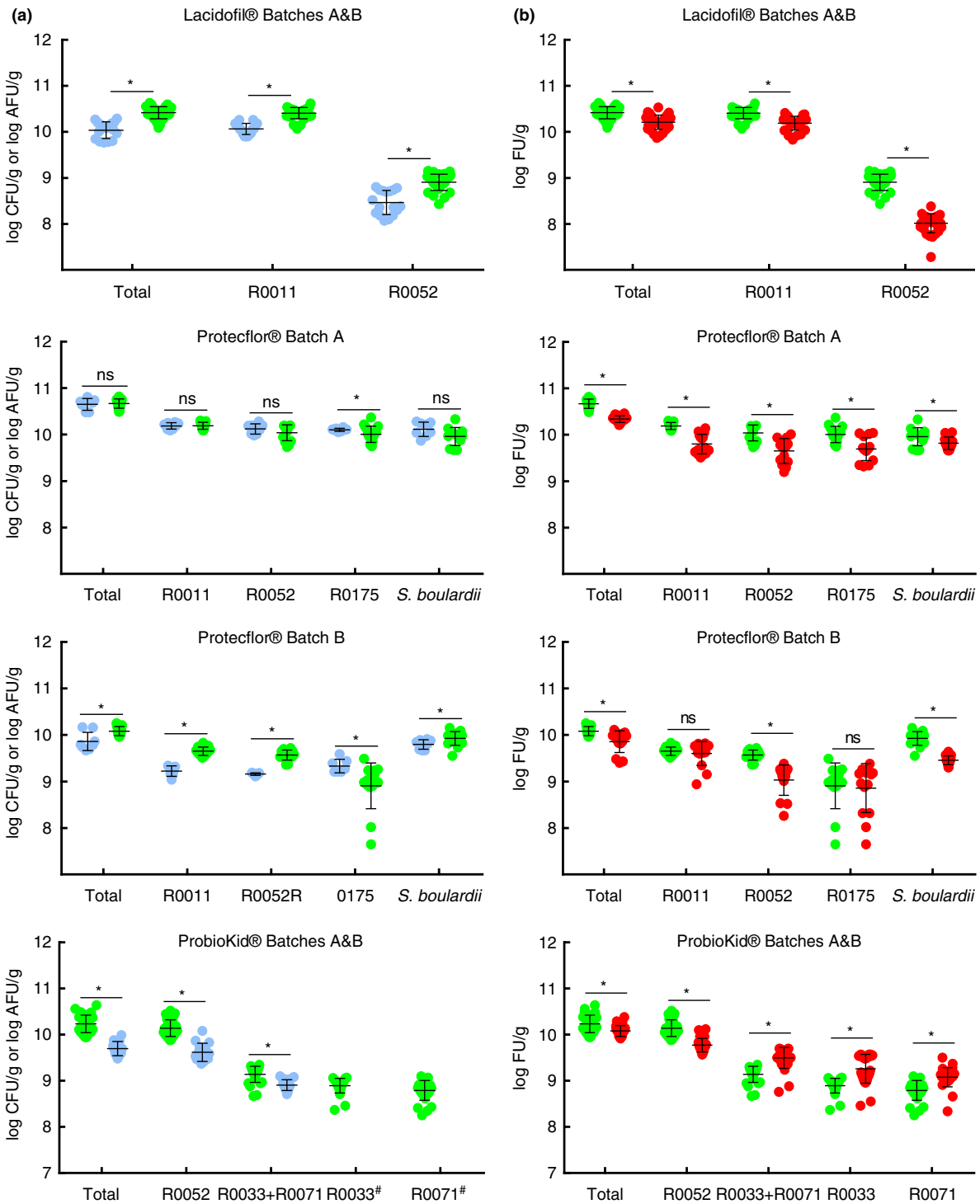

Figure 1 (a) Specific quantification of probiotic bacteria in Lacidofil, Protecflor and ProbioKid multistrain products, comparison between classical microbiology (● CFU) and flow cytometry (● AFU) results. (b) Viable (● AFU) and nonviable (● n-AFU) micro-organisms quantification by flow cytometry. Two batches were analysed by two operators with at least three replicates. Flow cytometry data were obtained from two instruments using manufacturer's software: CyFlow Space+FloMax software (Sysmex-Partec) and Accuri C6 and CSampler+BD CSampler (BD). Blue and green dots correspond to CFU enumerations and AFU (Active Fluorescent Units) flow cytometry data, respectively. Data are reported in log g^{-1}. Statistical significant differences were determined using Student's *t*-test on Prism (GraphPad) and are represented by symbols: *($P < 0{\cdot}05$); ns, nonsignificant ($P > 0{\cdot}05$). #CFU data N/A.

Table 4 Precision of flow cytometry and classical microbiology

	Flow cytometry			
	Partec	Accuri	2 Instruments	Classical microbiology
	Mean SD, SD (log cells per g)			Mean SD, SD (log CFU per g)
Analyses with 1 operator	0·07* ± 0·07	0·06* ± 0·05	0·12† ± 0·07	0·07* ± 0·04
Analyses with 2 operators	0·10‡ ± 0·05	0·08‡ ± 0·05	0·13§ ± 0·05	0·13‡ ± 0·06

*Repeatability (same operator and same flow cytometry instrument).
†Reproducibility considering multiple instruments.
‡Reproducibility considering multiple operators.
§Reproducibility considering multiple operators and multiple flow cytometry instruments.

Discussion

Antibody production

Custom antibodies targeting probiotic strains were produced as no commercial antibodies were available. Polyclonal antibodies have been previously used for probiotic enumeration by flow cytometry (Geng *et al.* 2014). Our custom polyclonal antibodies exhibited high specificity considering that they were raised against whole freeze-dried bacteria (Table 3). An additional screening against different strains of the same species showed that antibody specificity is beyond subspecies level (data not shown). Cross-reactivity of anti-*B. longum* antibody with yeast is likely due to the presence of residual yeast extract used during production process of freeze-dried bacteria powder. However, this would not interfere with specific enumeration, as flow cytometry can easily differentiate yeast and bacteria based on particle size. Anti-*B. longum* R0175 and anti-*B. bifidum* R0071 cross-reacted with *B. bifidum* and *L. fermentum*, respectively; this must be considered when analysing product formulas containing both species (which was not the case in the present study). One approach to overcoming this would be to remove cross-reacting antibodies from serum by a series of adsorption with the cross-reactive bacteria. However, this could potentially lower the diversity of epitopes targeted by the antibodies and potentially lead to antibody titre decrease and significant loss of fluorescent signal.

The use of polyclonal antibodies is a convenient strategy for research purposes and proof of concept studies (Geng *et al.* 2014), because it is faster to produce (*c.* 2–3 months), compared with monoclonal antibodies (4–6 months) (Lipman *et al.* 2005). However, it becomes a barrier for long-term use as quantity of polyclonal antibodies is limited and production varies from batch to batch, that is, animal to animal. Moreover, a recent article highlighted the necessity to develop validated antibodies for clinical applications (Bradbury and Pluckthun 2015), a matter that is also very important for QC applications that require the use of standardized protocols, and thus validated reagents. Polyclonal antibodies would be less suitable for long-term QC applications unless batch to batch reproducibility can be demonstrated. Monoclonal antibodies (mAbs) are more stable and reproducible and would be an attractive alternative for QC applications. A few studies reported the mAbs applied to LAB and probiotics, such as Stuknyte and colleagues who developed a mAb targeting *L. helveticus* SLP (from a different strain) for its detection in cheese by western blot (Stuknyte *et al.* 2014). Another study also reported the development of anti-bifidobacteria mAbs used for immuno-culture methods (Amrouche *et al.* 2006). However, mAbs production is more expensive and time consuming than pAbs. Besides, mAbs specificity is against a single epitope which can result in lower sensitivity or no binding at all if the target epitope is not accessible or in the correct conformation in native conditions (Baker 2015). Another solution could be the use of aptamers; however, identification of specific nucleotide sequences is still expensive and potentially less reliable because the technology is relatively new (Gold *et al.* 2012). Therefore, custom antibodies (mAbs and pAbs) remain the best choice for the specific quantification of probiotics by flow cytometry.

Identification of specific surface proteins, such as *L. helveticus* R0052-SLP, would facilitate antibody production. This is exemplified in the work of Prévost and colleagues, who developed a bead-based assay for the detection of staphylococcal enterotoxin in food matrices using polyclonal antibodies following a specific patented preparation (Barasino *et al.* 2012). Moreover, previous studies on *L. helveticus* R0052-SLP showed *in vitro* immunomodulatory properties (MacPherson *et al.* 2017) as well as *E. coli* O157:H7 anti-adhesion activity (Johnson-Henry *et al.* 2007). Therefore, identification of specific targets could be also useful for host–microbe interaction studies as surface-exposed protein might have a role in promoting health benefits. Flow cytometry applied to the quantification of identified bacterial

markers of health, such as the *L. helveticus* R0052-SLP, could potentially allow for the development of probiotic bio-efficacy tests that could be complementary to viability analysis and biomass quantification. However, we believe that specific detection of bacteria in a complex environment such as the human microbiota would be difficult to achieve using antibodies since there could be cross-reactivity appearing with other closely related strains.

Evaluation of flow cytometry for specific quantification of probiotics

In this study, we compared flow cytometry with a classical microbiology reference method to allow specific enumeration of viable probiotics in Protecflor, Lacidofil and ProbioKid. Two flow cytometry instruments with equivalent optical benches were used for inter-instrument comparison. Accuri C6 was equipped with auto-loading station for 96-well plates, whereas CyFlow Space analyses single tubes individually. Our study showed that both instruments and methods gave equivalent precision results. Moreover, our data were comparable to the limit of repeatability reported by ISO 19344|IDF 232 ($0{\cdot}06 \pm 0{\cdot}023$). However, the limit of reproducibility given by ISO 19344|IDF 232 ($0{\cdot}45 \pm 0{\cdot}16$) cannot be compared with our data because our analyses were performed in the same laboratory, whereas ISO involved multiple sites. Viable probiotic counts by flow cytometry were significantly higher than classical microbiology CFU counts for most of the strains studied, with the exception of *B. longum* R0175 for which classical microbiology data were higher. We hypothesize that higher counts obtained with flow cytometry could be due to a dormant or VBNC bacteria population as all the tested products were stored for at least a year (Lahtinen *et al.* 2005). Alternatively, with *B. longum* R0175, flow cytometry may have given a count lower than classical microbiology because *B. longum* R0175 antibodies were less specific and fluorescent signal was relatively lower compared with the other antibodies (Fig. 2). This could be due to bacteria and antibodies agglutination, as the concentration of secondary antibodies might not be in excess. Although tested without success in our study (data not shown), a primary labelled antibody could avoid this phenomenon. In addition, the SYTO®24 and PI double-stained population could contain culturable viable bacteria as reported with *B. cereus* in the literature (Want *et al.* 2011).

Overall, data showed that all products were compliant with the specifications provided by the manufacturer (Fig. 1 & Table 1), although the Protecflor batches A and B gave different trends and we chose not to combine data obtained from these two batches. Counts obtained for Protecflor batch B were globally lower and showing higher variation than those of batch A. These results could be explained by differences in the preparation of the two batches. Batch A was stored under strict controlled conditions and retained in specific packaging with controlled atmosphere settings enabling greater stability over time; conversely, batch B was prepared without as stringent packaging standards as batch A, and under these less stringent conditions, we observed reduced viability. Production process, packaging and storage conditions are very important to ensure product stability over time.

One major advantage of flow cytometry over classical microbiology is the reduced time of sample analysis due to an absence of cultivation. As described, it is possible to quantify viable and nonviable micro-organisms within 2 h, whereas classical microbiology took a minimum of 48 h and considered culturable bacteria only. Moreover, strain specificity has been achieved with flow cytometry combining antibodies, whereas we did not obtain the differential count of bifidobacteria species as in Probiokid with classical microbiology methods. TOS-mupirocin as described in ISO 29981|IDF 220 method was used for specific enumeration of strains belonging to *Bifidobacterium* (ISO 29981 2010). However, ISO 29981 was not able to differentiate the two bifidobacteria species in Probiokid (*B. longum* ssp. *infantis* and *B. bifidum*). Additionally, the RAF 5·1 media, developed for specific enumeration of bifidobacteria, was also tested on ProbioKid (Roy 2001; Farnworth *et al.* 2007). Yet, our tests did not show any improvement with this media as colonies were difficult to differentiate on the plate (data not shown).

In this study, a single flow cytometry method was applied to quantify and assess viability of several probiotic micro-organisms, including yeasts, whereas different culture conditions were used in classical microbiology. Flow cytometry has a greater versatility and could be applied to many different probiotic bacteria species and subspecies. Having a single protocol is another advantage in high-throughput laboratories. Interestingly, several papers also reported the use of these dyes to spore-forming bacteria (Comas-Riu and Vives-Rego 2002; Cronin and Wilkinson 2007; Tracy *et al.* 2008), lending support for the flow cytometry application on spore-forming probiotics such as *Bacillus subtilis*. Flow cytometry can also detect the nonviable bacteria population, which is not achievable with the culture-based methods. Recent studies have demonstrated that inactive probiotics elicit positive health benefits, such as *in vitro* immunomodulation associated with anti-inflammatory properties (Zhang *et al.* 2005; Demont *et al.* 2015). A group working on oral health in Germany also found that inactive probiotics had a higher impact than live strain on cariogenic bacteria, in artificial mouth models (Schwendicke *et al.* 2014).

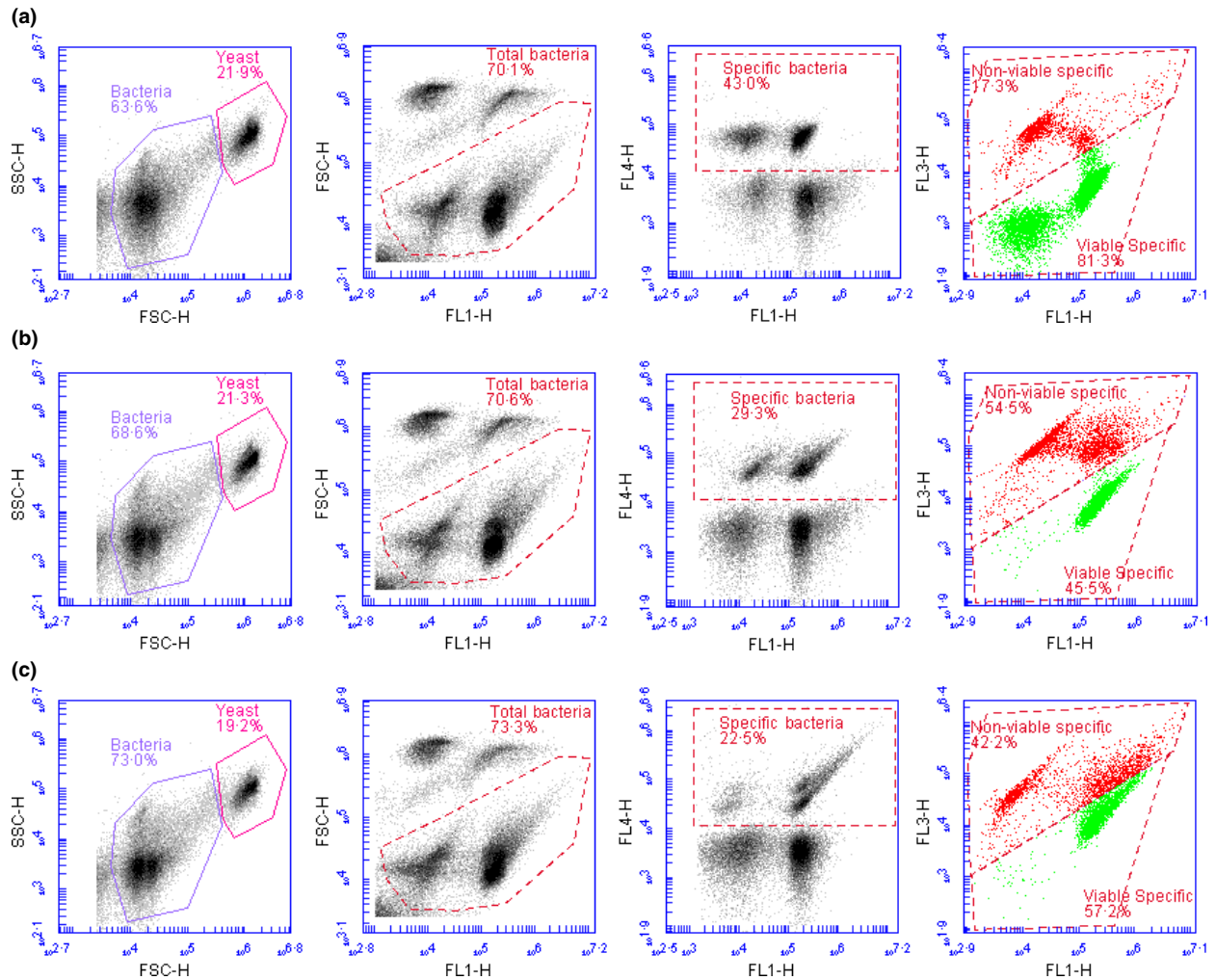

Figure 2 Flow cytometry dot plots showing antibody labelling on Protecflor. (a) Anti-*Lactobacillus rhamnosus* R0011, (b) antibodies anti-*L. helveticus* R0052, and (c) antibodies anti-*Bacillus longum* ssp. *longum* R0175.

Another advantage of flow cytometry is the possibility to detect the VBNC or dormant bacteria population (Lahtinen *et al.* 2006). Information about viability percentage is a significant element for the optimization of production process and product stability; flow cytometry could help to identify and rectify stability difficulties encountered with specific strains. It could also be useful in R&D such as gastrointestinal tract digestion simulation studies used to characterize probiotic strains.

Molecular methods such as PMA-qPCR, using strain-specific primers, is another example of a quantification technique where PMA (Propidium MonoAzide) enables detection of viable bacteria based on membrane integrity (Nocker *et al.* 2006). However, these techniques can be tedious as they require several steps (DNA extraction, amplification) increasing time of analysis to *c.* 12–15 h at best and require higher technical expertise than flow cytometry. Therefore, the latter is better adapted than molecular techniques for high-throughput experiments such as industrial quality control analysis. Moreover, recent development of flow cytometry for detection of pathogens and contaminants in various matrices (Buzatu *et al.* 2014) also demonstrates the high versatility of this method and high interest to the global quality assurance of food supplements and products. From a broader perspective, flow cytometry is an attractive method compared with classical microbiology approaches as this technology can easily be adapted for the analysis of numerous other sample types, including dairy products, food matrices, upper gastrointestinal tract and clinical samples.

Taken together, flow cytometry has many advantages compared with classical microbiology techniques and will be useful for probiotic and nonviable bacteria

quantification during industrial production and quality control as well as a valuable research tool.

The present study aimed to assess the suitability of flow cytometry as a novel method for probiotic-specific enumeration and viability assessment. We demonstrated that specific antibodies can be produced against probiotic strains and used in combination with viability staining for specific enumeration and viability assessment of probiotic micro-organisms. Flow cytometry enables access to more information (VBNC, dead cells) in a time-efficient manner, compared to the classical microbiology methods currently used. Also, the advantage of widespread versatility makes flow cytometry methods an attractive approach for application in research and QC laboratories. Moving forward, the results presented here will contribute to the recognition and standardization of flow cytometry methods not only in research but also for industrial and commercial applications—a paradigm shift that remains the next challenge for acceptance of flow cytometry as a new standard in probiotic industry.

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Conflict of Interest

Authors declare being employees of LHS, a company manufacturing and selling probiotic microbes business-to-business but not to the consumer.

References

Amor, K.B., Breeuwer, P., Verbaarschot, P., Rombouts, F.M., Akkermans, A.D., De Vos, W.M. and Abee, T. (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**, 5209–5216.

Amrouche, T., Boutin, Y., Moroni, O., Kheadr, E. and Fliss, I. (2006) Production and characterization of anti-bifidobacteria monoclonal antibodies and their application in the development of an immuno-culture detection method. *J Microbiol Methods* **65**, 159–170.

Apajalahti, J.H., Kettunen, H., Kettunen, A., Holben, W.E., Nurminen, P.H., Rautonen, N. and Mutanen, M. (2002) Culture-independent microbial community analysis reveals that inulin in the diet primarily affects previously unknown bacteria in the mouse cecum. *Appl Environ Microbiol* **68**, 4986–4995.

Ashraf, R. and 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**, 194–208.

Baker, M. (2015) Reproducibility crisis: blame it on the antibodies. *Nature* **521**, 274–276.

Barasino, C., Keller, D., Masoud, K. and Prévost, G. (2012) *Depleted anti-staphylococcal enterotoxins polyclonal antibodies, preparation and uses thereof [Online]*. Available at: https://www.google.ca/patents/WO2012117342A1?cl=en.

Bradbury, A. and Pluckthun, A. (2015) Reproducibility: standardize antibodies used in research. *Nature* **518**, 27–29.

Buzatu, D.A., Moskal, T.J., Williams, A.J., Cooper, W.M., Mattes, W.B. and Wilkes, J.G. (2014) An integrated flow cytometry-based system for real-time, high sensitivity bacterial detection and identification. *PLoS ONE* **9**, e94254.

Charteris, W.P., Kelly, P.M., Morelli, L. and Collins, J.K. (1997) Selective detection, enumeration and identification of potentially probiotic Lactobacillus and Bifidobacterium species in mixed bacterial populations. *Int J Food Microbiol* **35**, 1–27.

Chen, S., Cao, Y., Ferguson, L.R., Shu, Q. and Garg, S. (2012) Flow cytometric assessment of the protectants for enhanced in vitro survival of probiotic lactic acid bacteria through simulated human gastro-intestinal stresses. *Appl Microbiol Biotechnol* **95**, 345–356.

Comas-Riu, J. and Rius, N. (2009) Flow cytometry applications in the food industry. *J Ind Microbiol Biotechnol* **36**, 999–1011.

Comas-Riu, J. and Vives-Rego, J. (2002) Cytometric monitoring of growth, sporogenesis and spore cell sorting in Paenibacillus polymyxa (formerly Bacillus polymyxa). *J Appl Microbiol* **92**, 475–481.

Cronin, U.P. and Wilkinson, M.G. (2007) The use of flow cytometry to study the germination of *Bacillus cereus* endospores. *Cytometry A* **71**, 143–153.

Davis, C. (2014) Enumeration of probiotic strains: review of culture-dependent and alternative techniques to quantify viable bacteria. *J Microbiol Methods* **103**, 9–17.

Demont, A., Hacini-Rachinel, F., Doucet-Ladeveze, R., Ngom-Bru, C., Mercenier, A., Prioult, G. and Blanchard, C. (2015) Live and heat-treated probiotics differently modulate IL10 mRNA stabilization and microRNA expression. *J Allergy Clin Immunol* **137**, 1264–1267.

Doherty, S.B., Wang, L., Ross, R.P., Stanton, C., Fitzgerald, G.F. and Brodkorb, A. (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**, 301–310.

FAO/WHO. (2001) *Health and nutritional properties of probiotics inclunding powder milk with live lactic acid bacteria*.

Farnworth, E.R., Mainville, I., Desjardins, M.P., Gardner, N., Fliss, I. and Champagne, C. (2007) Growth of probiotic bacteria and bifidobacteria in a soy yogurt formulation. *Int J Food Microbiol* **116**, 174–181.

Galat, A., Dufresne, J., Combrisson, J., Thepaut, J., Boumghar-Bourtchai, L., Boyer, M. and Fourmestraux, C. (2016) Novel method based on chromogenic media for discrimination and selective enumeration of lactic acid bacteria in fermented milk products. *Food Microbiol* **55**, 86–94.

Geng, J., Chiron, C. and Combrisson, J. (2014) Rapid and specific enumeration of viable Bifidobacteria in dairy products based on flow cytometry technology: a proof of concept study. *Int Dairy J* **37**, 1–4.

Gold, L., Janjic, N., Jarvis, T., Schneider, D., Walker, J.J., Wilcox, S.K. and Zichi, D. (2012) Aptamers and the RNA world, past and present. *Cold Spring Harb Perspect Biol* **4**().

Grootaert, C., Boon, N., Zeka, F., Vanhoecke, B., Bracke, M., Verstraete, W. and Van de Wiele, T. (2011) Adherence and viability of intestinal bacteria to differentiated Caco-2 cells quantified by flow cytometry. *J Microbiol Methods* **86**, 33–41.

Gunasekera, T.S., Attfield, P.V. and Veal, D.A. (2000) A flow cytometry method for rapid detection and enumeration of total bacteria in milk. *Appl Environ Microbiol* **66**, 1228–1232.

ISO 19344|IDF 232. (2015) *Milk and milk products – Starter cultures, probiotics and fermented products – Quantification of lactic acid bacteria by flow cytometry*.

ISO 20128|IDF 192. (2006) *Milk products – Enumeration of presumptive Lactobacillus acidophilus on a selective medium – Colony-count technique at 37 Degrees C*.

ISO 29981|IDF 220. (2010). *Milk products – Enumeration of presumptive bifidobacteria – Colony count technique at 37 Degrees*.

Johnson-Henry, K.C., Hagen, K.E., Gordonpour, M., Tompkins, T.A. and Sherman, P.M. (2007) Surface-layer protein extracts from Lactobacillus helveticus inhibit enterohaemorrhagic Escherichia coli O157:H7 adhesion to epithelial cells. *Cell Microbiol* **9**, 356–367.

Joux, F. and Lebaron, P. (2000) Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbes Infect* **2**, 1523–1535.

Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.R. and Barer, M.R. (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie Van Leeuwenhoek* **73**, 169–187.

Koch, C., Harms, H. and Muller, S. (2014) Dynamics in the microbial cytome-single cell analytics in natural systems. *Curr Opin Biotechnol* **27**, 134–141.

Kramer, M., Obermajer, N., Bogovic Matijasic, B., Rogelj, I. and Kmetec, V. (2009) Quantification of live and dead probiotic bacteria in lyophilised product by real-time PCR and by flow cytometry. *Appl Microbiol Biotechnol* **84**, 1137–1147.

Lahtinen, S.J., Gueimonde, M., Ouwehand, A.C., Reinikainen, J.P. and Salminen, S.J. (2005) Probiotic bacteria may become dormant during storage. *Appl Environ Microbiol* **71**, 1662–1663.

Lahtinen, S.J., Ouwehand, A.C., Reinikainen, J.P., Korpela, J.M., Sandholm, J. and Salminen, S.J. (2006) Intrinsic properties of so-called dormant probiotic bacteria, determined by flow cytometric viability assays. *Appl Environ Microbiol* **72**, 5132–5134.

Lahtinen, S.J., Ahokoski, H., Reinikainen, J.P., Gueimonde, M., Nurmi, J., Ouwehand, A.C. and Salminen, S.J. (2008) Degradation of 16S rRNA and attributes of viability of viable but nonculturable probiotic bacteria. *Lett Appl Microbiol* **46**, 693–698.

Lipman, N.S., Jackson, L.R., Trudel, L.J. and Weis-Garcia, F. (2005) Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. *ILAR J* **46**, 258–268.

MacPherson, C.W., Shastri, P., Mathieu, O., Tompkins, T.A. and Burguiere, P. (2017) Genome-wide immune modulation of TLR3-mediated inflammation in intestinal epithelial cells differs between single and multi-strain probiotic combination. *PLoS ONE* **12**, e0169847.

Muller, S. and Nebe-von-Caron, G. (2010) Functional single-cell analyses: flow cytometry and cell sorting of microbial populations and communities. *FEMS Microbiol Rev* **34**, 554–587.

Nebe-von-Caron, G., Stephens, P.J., Hewitt, C.J., Powell, J.R. and Badley, R.A. (2000) Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. *J Microbiol Methods* **42**, 97–114.

Nocker, A., Cheung, C.Y. and Camper, A.K. (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**, 310–320.

Novo, D.J., Perlmutter, N.G., Hunt, R.H. and Shapiro, H.M. (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**, 827–834.

Oliver, J.D. (2005). The viable but nonculturable state in bacteria. *J Microbiol*, **43**, 93–100.

Rault, A., Beal, C., Ghorbal, S., Ogier, J.C. and Bouix, M. (2007) Multiparametric flow cytometry allows rapid assessment and comparison of lactic acid bacteria viability after freezing and during frozen storage. *Cryobiology* **55**, 35–43.

Raymond, Y. and 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. and Roederer, M. (2015) History of science. Flow cytometry strikes gold. *Science* **350**, 739–740.

Roy, D. (2001) Media for the isolation and enumeration of bifidobacteria in dairy products. *Int J Food Microbiol* **69**, 167–182.

Schwendicke, F., Horb, K., Kneist, S., Dorfer, C. and Paris, S. (2014) Effects of heat-inactivated Bifidobacterium BB12 on cariogenicity of Streptococcus mutans in vitro. *Arch Oral Biol* **59**, 1384–1390.

Sohier, D., Pavan, S., Riou, A., Combrisson, J. and Postollec, F. (2014) Evolution of microbiological analytical methods for dairy industry needs. *Front Microbiol* **5**, 16.

Stuknyte, M., Brockmann, E.C., Huovinen, T., Guglielmetti, S., Mora, D., Taverniti, V., Arioli, S., De Noni, I. *et al.* (2014) *Lactobacillus helveticus* MIMLh5-specific antibodies for detection of s-layer protein in grana padano protected-designation-of-origin cheese. *Appl Environ Microbiol* **80**, 694–703.

Taverniti, V., Stuknyte, M., Minuzzo, M., Arioli, S., De Noni, I., Scabiosi, C., Cordova, Z.M., Junttila, I. *et al.* (2013) S-layer protein mediates the stimulatory effect of *Lactobacillus helveticus* MIMLh5 on innate immunity. *Appl Environ Microbiol* **79**, 1221–1231.

Tracy, B.P., Gaida, S.M. and Papoutsakis, E.T. (2008) Development and application of flow-cytometric techniques for analyzing and sorting endospore-forming clostridia. *Appl Environ Microbiol* **74**, 7497–7506.

Want, A., Hancocks, H., Thomas, C.R., Stocks, S.M., Nebe-von-Caron, G. and Hewitt, C.J. (2011) Multi-parameter flow cytometry and cell sorting reveal extensive physiological heterogeneity in *Bacillus cereus* batch cultures. *Biotechnol Lett* **33**, 1395–1405.

Zhang, L., Li, N., Caicedo, R. and Neu, J. (2005) Alive and dead Lactobacillus rhamnosus GG decrease tumor necrosis factor-alpha-induced interleukin-8 production in Caco-2 cells. *J Nutr* **135**, 1752–1756.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Phase contrast and epifluorescence microscopy pictures of Protecflor. Pictures obtained with Leica DMIRE2 inverted microscope (Leica Microsystems Canada) equipped with a Hamamatsu cooled charge-coupled-device camera, at ×630 magnification. (a) Brightfield channel; (b) Green fluorescence channel. SYTO®24 staining of all micro-organisms; (c) Far-red fluorescence channel. Anti-*Lactobacillus rhamnosus* R0011 antibodies + Alexa Fluor 647 conjugated secondary antibodies. (d) Overlay (A+B+C). Antibody and SYTO®24 fluorescence overlay is indicated in yellow.

Figure S2 Correlation curve of total & specific quantification of probiotic strains from Lacidofil, Protecflor and ProbioKid obtained by two flow cytometry instruments (CyFlow Space, Partec & Accuri C6, BD). (a) Mean data from each operator. (b) Mean data from two operators. Each dot represents the mean concentration with SD (log AFU per g) of one probiotic target (specific or total count) obtained by one or two operators, with at least three replicates (except for one product lot and one operator where only two replicates were available)

Figure S3 Correlation curve of total and specific quantification of probiotic strains from Lacidofil, Protecflor and ProbioKid obtained by classical microbiology and flow cytometry. Each dot represents the mean concentration with SD of one probiotic target (specific or total count) obtained by two operators, with at least three replicates. Flow cytometry data are the mean from the two instruments. Classical microbiology and flow cytometry data are expressed in log CFU per g and log AFU per g, respectively.

Table S1 Viability percentage of probiotic in multi-strain products. Viability determined by flow cytometry using SYTO®24 and PI viability staining. AFU, active fluorescence units; n-AFU, nonactive fluorescent units. SD, standard deviation. Each data correspond to the mean of at least three replicates by two operators and two flow cytometry instruments.

