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Bacillus spore enumeration using flow cytometry: A proof of concept for probiotic application

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ABSTRACT

Use of flow cytometry (FCM) for bacteria quantification is growing in the food industry. We report here a FCM method using a double-staining LDS751/SYTO24 for the quantification of probiotic *Bacillus* viable cells and its spores, with potential application for the control of commercial product specifications.

Respect of the product composition and specifications is mandatory for marketed preparations of probiotic products. Techniques approved by regulatory bodies usually rely on standard bacterial plating methods. However, such methods suffer from limitations including inter-operator variation, low adaptability to certain forms of bacteria such as viable but non-culturable and are time consuming. This poses a challenge for product specifications testing, notably for spore-forming bacteria such as *B. subtilis* CNCM I-2745. *B. subtilis* CNCM I-2745, known, is a currently marketed spore forming bacteria that has been shown to exert safe probiotic properties (Lefevre et al., 2017; Lefevre et al., 2015). The development of alternative methods with faster turnover would be beneficial in industrial production and quality control processes. The use of flow cytometry is growing notably in the context of pathogen detection or quality control (Kennedy and Wilkinson, 2017). Interest for FCM is growing in probiotic application notably to measure bacterial viability (Chiron et al., 2018; Wilkinson, 2018). For spore-forming bacteria enumeration, it may alleviate hurdles from the conventional enumeration. Cell sorting with automated gating has been reported for *B. subtilis* to monitor germination overtime (Karava et al., 2019). However, implementation for absolute quantification in the context of industrial probiotic application is lacking. We present here the proof a concept of a FCM method to count and differentiate spores of the probiotic strain *B. subtilis* CNCM I-2745 and other *Bacillus* species.

First, *B. subtilis* CNCM I-2745 (LifeinU™ *B. subtilis* CU1, Lesaffre, France) was cultured in a sporulation medium at 30 °C at 120 rpm for up to 144 h to monitor the kinetic of spore production using standard two-step approach of total viable counting (TVC) and spore-forming cell counting using heat treatment as described in the standard ISO EN 15784. Serial dilutions in peptone salt medium (1 g peptone, 8.5 g NaCl dissolved in 1 l of water with a pH of 7.0 ± 0.2) were prepared and plated on Triptic Soy Agar (Biomerieux, Marcy-l'Etoile, France) supplemented with Yeast Extract (TSA-YE) plates and incubated 24 h at 37 °C. For spore counting, the culture was heat treated for 10 min effective at 80 °C, cooled down on ice and homogenized by sonication for 3 min 45 s with pulse of 15 s at 20% amplitude. Serial dilutions and bacterial enumeration were carried out similarly to total bacteria counting. After 24 h culture, spore concentration rose notably and reached a plateau by 48 h representing 99.0 ± 1.1% of the total population, which remained stable up to 144 h (100.4 ± 1.0%) (Fig. 1). This time point was selected for the preparation of further spore cultures.

The FCM enumeration was set up using a double staining with SYTO24 (Invitrogen, Villebon-Sur-Yvette, France), and Laser Dyes Styryl (LDS) 751 (Invitrogen). The former is a cell-permeant green-fluorescent nucleic acid stain (peak excitation at ~490 nm and peak emission at 515 nm on DNA, recommended by the standard ISO 199344:2015 for quantification of lactic acid bacteria by flow cytometry for starters cultures, probiotics and fermented products. The latter is a cell-permeant nucleic acid stain (peak excitation at ~543 nm and peak emission at ~712 nm. on double-stranded DNA; ~20-fold fluorescence enhancement upon DNA binding). Mixes of different spores: vegetative cells ratios (100%:0%, 90%:10%, 50%:50%, 10%:90%, and 0%:100%) were prepared using spore culture as described above and vegetative cell culture obtained from overnight culture of *B. subtilis* CNCM I-2745 in TSB-YE with no shaking at 37 °C. Preparations of bacteria/spores were

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stained in 0.1% peptone salt medium with 100 nM SYTO24 during 10 min at 37 °C in the dark and then with 20 ng/ml LDS751 for 5 min at 37 °C in the dark. After vortexing, the suspension was enumerated by FCM with an Attune® NxT Acoustic Focusing Cytometer (Thermofisher, Villebon-Sur-Yvette, France) equipped with blue laser (λex = 488 nm) and band pass filters as following (BL1 BP 530/30; BL-2 BP 574/26; BL-3 BP 695/40; BL-4 BP 780/60). With this cytometer, all events are automatically counted, and particle counts or concentrations can be viewed. The analysis was performed with 150 µl of stained bacteria preparation with a flow rate of 100 µl/min. The gating strategy is presented in.

Fig. 2 and relied on three subpopulations: LDS751+ / SYTO24 dim as spores, LDS751 + / SYTO24 + as vegetative cells, and LDS751 - / SYTO24 + as VBNC or dead cells. For a given sub-population, the concentration was computed as following: $[\boldsymbol{n}] = ([\boldsymbol{n}]_{\boldsymbol{singulets}} + (\mathbf{2} \times \%\boldsymbol{n}_{\boldsymbol{singulets}} \times [\boldsymbol{doublets}])) \times \boldsymbol{dilution\ factor}$ and expressed as Active Fluorescent Unit (AFU)/ml, with $[\boldsymbol{n}]_{\boldsymbol{singulet}}$ being the concentration of singulets in the subpopulation $\boldsymbol{n}$ (in event/µl), $\%\boldsymbol{n}_{\boldsymbol{singulets}}$ the proportion of the subpopulation singlulets $\boldsymbol{n}$ gated (in %), and $[\boldsymbol{doublets}]$ the concentration of doublets (in event/µl). The proportion of subpopulations was assumed to be the same between the singulets population and the doublets populations. The relative distribution of vegetative cells and spores measured by FCM showed a strong correlation with the theoretical one with Pearson correlation coefficient = 0.9947 (Pearson correlation coefficient test $p < 0.001$) for vegetative cells and 0.9939 (Pearson correlation coefficient test $p < 0.001$) for spores (Fig. 2).

To evaluate the robustness of the gating strategy for spores and vegetative cells, kinetic experiments were performed. An aliquot of pure spore inoculum was cultured in TSB-YE medium at 37 °C with shaking to induce germination. A two-log decrease in the concentration of spores was observed within 35 min of culture while vegetative cells concentration increased drastically within 10 min and plateaued around 7 Log_{10} AFU/ml. As a control experiment, another spore inoculum was cultured in peptone salt medium at 20 °C without shaking. As expected, the concentration of spores remained stable while proliferation of vegetative cells was also largely inhibited (Supplementary Fig. 1).

The FCM method was then applied to commercial *B. subtilis* CNCM I-2745 lyophilizate that was rehydrated based on a method adapted from ISO EN 15784. Two grams of lyophilizate were dissolved in 198 ml PBS-1% Tween®80. The solution was homogenized with T25 Ultra-Turrax® (IKA, Staufen, Germany) for 2 min at 22,000 RPM. Then, 10 ml of the solution was sonicated for 3 min 45 s with 15 s pulses on / off at 20% amplitude. Recovery rates were assessed as Log_{10} AFU/ml over Log_{10} CFU/ml both for spores and total viable count. For FCM analysis, total viable count was obtained by summing spores and vegetative cells concentrations. Replication assays on the same lyophilizate showed no significant difference in spore concentration (mean difference − 0.021 ± 0.083 Log_{10} cfu/ml, Wilcoxon Signed-Rank test for paired samples p-value 0.7893) and in TVC (mean difference − 0.214 ± 0.044 Log_{10} cfu/ml; Wilcoxon Signed-Rank test for paired samples p-value 0.1814) between FCM and bacterial plating (Fig. 1 D). Recovery rates were 0.998 ± 0.008 and 0.981 ± 0.004 for spores and total count respectively (n = 3). When testing five different batches, no significant difference was observed between the total concentrations from the two methods (mean difference 0.005 ± 0.088 Log_{10} cfu/ml; Wilcoxon Signed-Rank Test for a

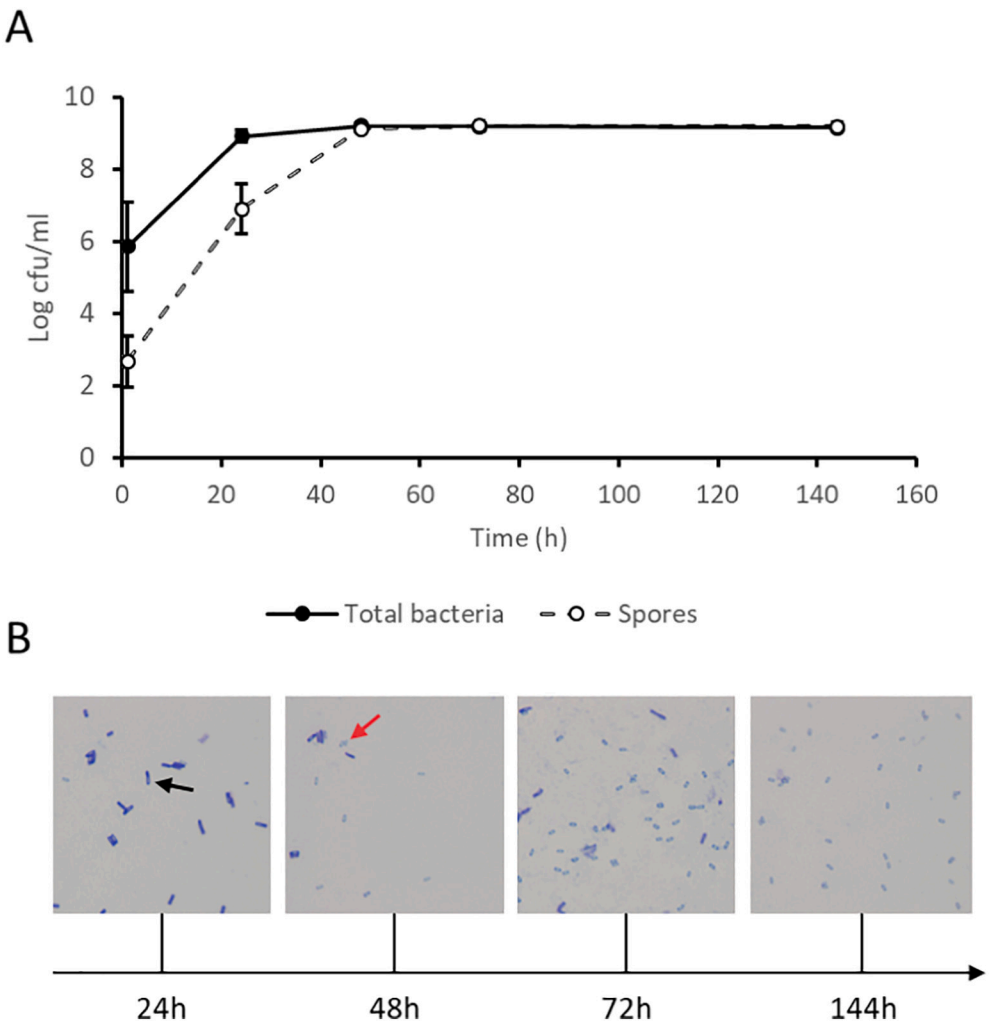

Fig. 1. Evolution of spore concentration in spore-forming medium.
The concentration of spores peaked at 48 h and was maintained up to 144 h culture in a sporulation medium at 30 °C at 120 rpm. Microscopic analysis confirmed the progressive enrichment in spores.
A-Concentration in Log cfu/ml of total cells (black circle) and spores (blank circle) in spore-forming medium using conventional total viable count (mean, sem; n = 3). B-Methylene blue staining of bacterial smear of samples collected from the culture in spore-forming media at different time points (representative result). Pictures were captured using a digital camera (Olympus *E*-M5 markII) placed on the ocular port of the microscope using a x100 objective. Black and red arrow indicates vegetative cells containing an endospore and spores respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

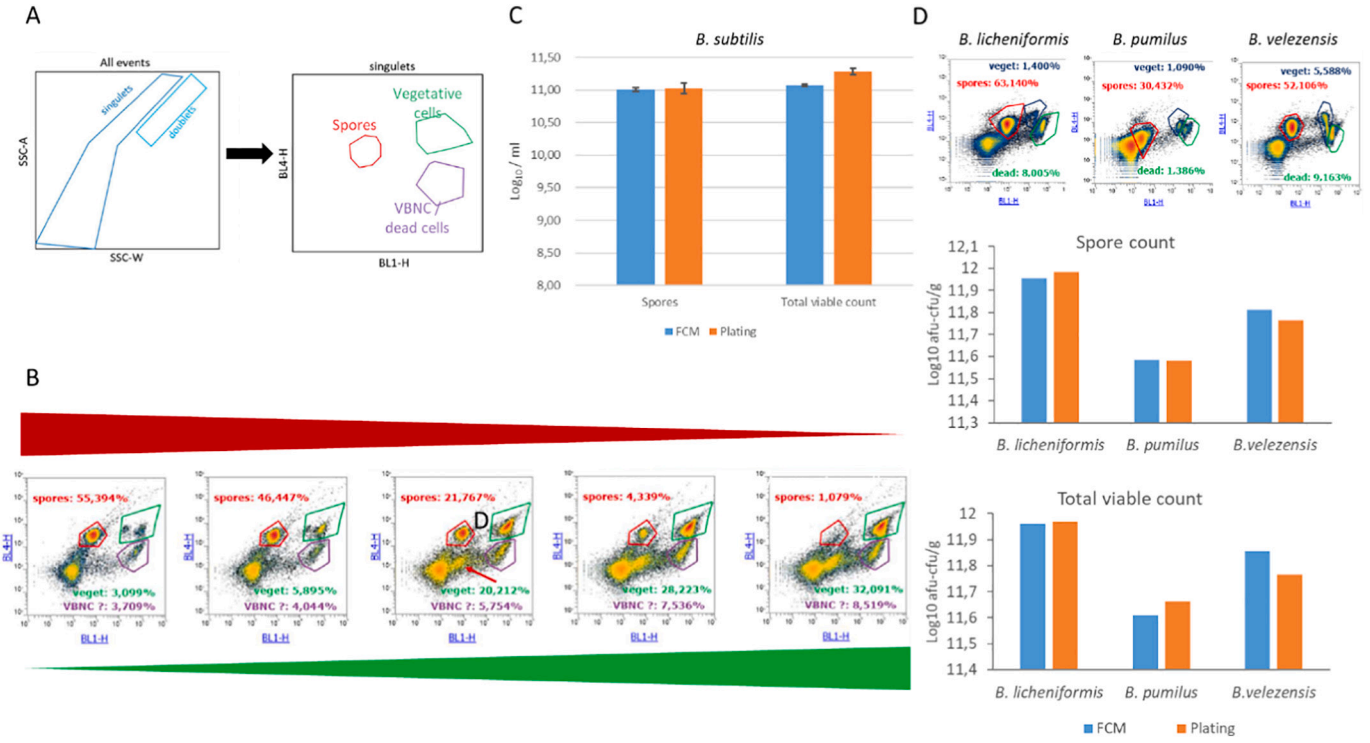

Fig. 2. Double staining LDS751 + Syto24 flow cytometry analysis.
The flow cytometry using double staining LDS751 + Syto24 was able to differentiate three subpopulations: spores, vegetative cells and VBNC or dead cells. Relative proportions of spores and vegetative cells mirrored strongly those resulting from different spores: vegetative cells ratios. FCM enumeration of *Bacillus* strains showed no difference with the one obtained with plating.
A –Gating of three separated populations from the singulet sub-population: LDS751+ / SYTO24 dim as spores, LDS751 + / SYTO24 + as vegetative cells, and LDS751 - / SYTO24 + as VBNC or dead cells. B From left to right, proportion of spores:vegetative cells culture of *B. subtilis* CNCM I-2745 were 100%:0%, 90%:10%, 50%:50%, 10%:90%, and 0%:100% prepared from individual preparations of fresh vegetative cell cultures and spore cultures. C- Application of LDS751 / SYTO24 double staining and FCM analysis (blue bar graph) on five different batches of lyophilizate of *B. subtilis* CNCM I-2745 and comparison of the spore count and total viable count with the plating method (orange bar graph). D- Application of LDS751 / SYTO24 double staining and FCM analysis (blue bar graph) on lyophilizates of *B. licheniformis* NRRL B-67649, *B. pumilus* NRRL B-67648 and *B. velezensis* NRRL B-67647R and comparison of the spore count and total viable count with the plating method (orange bar graph).
Note: VBNC: viable not culturable cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Single Sample p-value 0.7874) and the recovery rate was 1.000 ± 0.008 (n = 5). These results supported good and stable recovery rate of the FCM method. Preliminary tests on other *Bacillus* lyophilizates such as *B. licheniformis* NRRL B-67649, *B. pumilus* NRRL B-67648 and *B. velezensis* NRRL B-67647R produced similar results as shown by the recovery rates of 1.001 ± 0.003 for spores and 1.001 ± −0.006 for TVC. These data suggested that the method could be applied to other probiotic species of *Bacillus* (Fig. 2).

Complementary tests may be warranted to further understand the mechanisms of labeling with these dyes. Previous work reported unsuccessful use of LDS-751 to stain bacteria (Shapiro, 2005) but *B. subtilis* CNCM I-2745 could be distinguished from the background, consistently with a recent study that used LDS-751 in association with CFDA to monitor membrane permeabilization during spore germination process in *Bacillus* (Trunet et al., 2019). The authors showed however that impermeable dormant spores were not labeled with LDS-751. Such impermeable spores required unfavorable culture conditions (prolonged culture in water at 4 °C). Here, the presence of such spores was highly unlikely as culture settings were aligned with industrial conditional favorable to probiotic production. Originally thought as nucleic acid dye, LDS-751 has been shown to bind mitochondria in eukaryotic cells and not the nucleus (Prowse et al., 2012; Snyder and Small, 2001). More specifically, data suggest that LDS-751 binds to polarized membrane as shown by the decrease of LDS-751 related fluorescence when mitochondrial depolarizing agents phenyl arsine oxide and carbonyl cyanide *m*-chlorophenylhydrazone are used (Snyder and Small, 2001). However, this mechanism may not be involved for spore binding as known membrane potential related dyes such as $DiOC_6(3)$ (3,3¢-dihexyloxacarbocyanine Iodide) has been shown to label *Bacillus* spore independently of its membrane polarization (Magge et al., 2009).The mechanism behind the ability SYTO24 to differentiate vegetative cells from spores remains elusive.

To appraise further the subpopulations identified by LDS751/SYTO24 from a *B. subtilis* CNCM I-2745 lyophilizate, an external laboratory (TWB Technical Research Institute, Toulouse, France) adapted the FCM method on a Moflo Astrios EQ cell sorter (Beckman Coulter) in order to perform live microscopic evaluation in a wet mount (Fig. 3). The vegetative cells fraction showed a mix of small motile rod-shape bacteria while the spores fraction was composed of refractile forms with thick wall on microscopy as expected for spores (Pandey et al., 2013). The third population hypothesized as VBNC/dead cells were a mix of small non-motile non-refracting rods. In some of them, non-refracting round forms were observed in the apical side. These observations supported the correct separation of the three populations by LDS751/SYTO24 staining. Further analyses are required to confirm these observations, notably through culture of the isolated fractions. This would help to determine whether the hypothesized VBNC/dead cells fraction is indeed not culturable.

In conclusion, we report here a simple method with a fast turnover for enumeration of spores and total viable cell count to monitor industrial production of spore-based preparations for market application. Compared to classical method, this assay would enable close evaluation

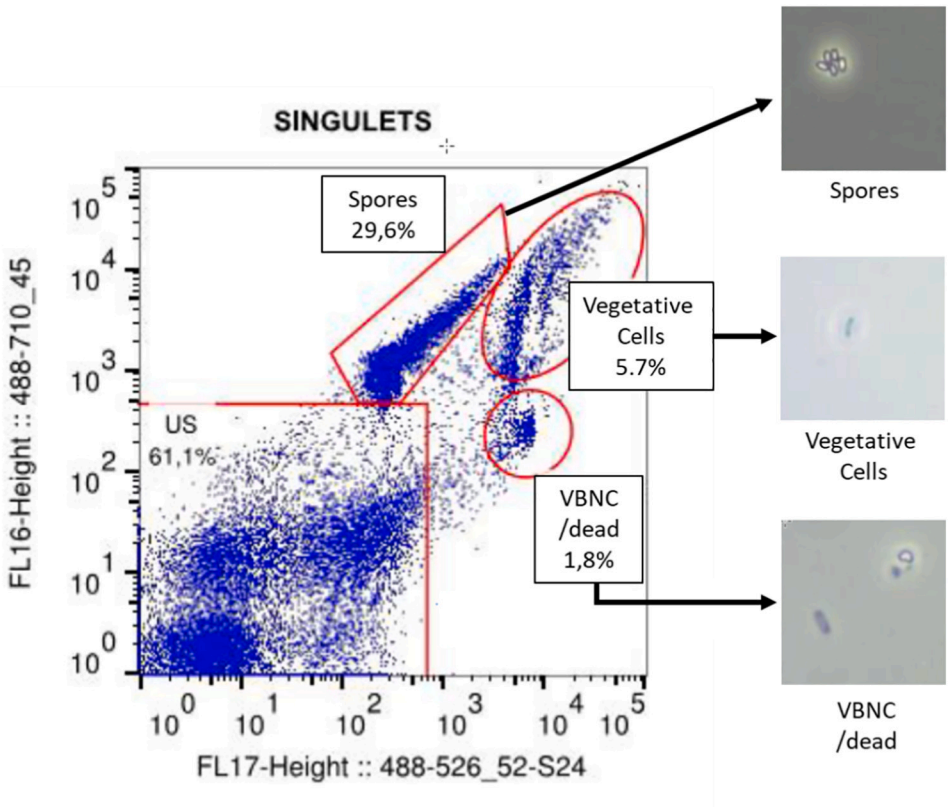

Fig. 3. Live microscopic observation of the sorted populations resulting from double staining LDS751 + Syto24.
The method was successfully transferred to another laboratory using a cell sorter. The three subpopulations were separated and observed under light microscope in a wet mount to confirm the isolation of spores, vegetative cells and VBNC/dead.
B. subtilis CNCM I-2745 lyophilizate were prepared, double stained with LDS751 + Syto24 and analyzed in Moflo Astrios EQ cell sorter (Beckman Coulter) with a protocol adapted from the one developed for the Attune® NxT Acoustic Focusing Cytometer (Thermofisher). The three subpopulations could be distinguished and sorted for live microscopic visualization in a wet mount.
Note: VBNC, Viable But Not Culturable; US, Unstained.

of batch productions in a reduced timeframe nearly real time, thus allowing better control of production and quality control process. Further work is required using other spore-forming probiotic strains or species with market application to evaluate the generalizability of the method.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106336.

Declaration of Competing Interest

Maxime Genovese, Edith Poulain, Florie Doppler, Renaud Toussaint, Mickaël Boyer are employees of Lesaffre International R&D (Marcq-en Baroeul, France).

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