Probiotic Enumeration by Flow Cytometry: Determining both *Bacillus coagulans* and *Bacillus subtilis* Spore Counts in Mixed Populations in Foods and Dietary Supplements

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Abstract

Increasingly, the probiotic industry is using flow cytometry (FC) to provide more information about the quantity, quality, and identity of organisms in probiotic products. Previously, we (i) verified ISO 19344:2015(E)/IDF 232:2015(E) for enumerating probiotics in lyophilized powders using acoustic focusing FC[1]; (ii) expanded method utility by validating preparation modifications for several matrices[2]; and (iii) developed a method for enumerating *Bacillus coagulans*, a spore-forming probiotic[3]. Here, our objective was to develop a novel viability enumeration method using the technology to detect individual cellular subpopulations containing blends of *B. coagulans* and *Bacillus subtilis*. A gummy product containing blends of *B. coagulans* and *B. subtilis* was evaluated. The developed method is based on ISO 19344, with modifications to sample preparation (germination, filtration, heat inactivation), the dual nucleic acid staining protocol, and cytometric analysis; it does not tag cells with antibodies. A conventional *Bacillus* spp. growth medium was used for plate counting. FC and plate counting results were compared. The method developed for enumeration of *B. coagulans* and *B. subtilis* in mixtures utilizing acoustic focusing FC produced more accurate and reliable results compared to conventional plate counting. Plate count results were significantly lower with 33% recovery compared to the manufacturer-defined expected level and more variable with a Relative Standard Deviation (RSD) value of 13%. The respective species were also unable to be distinguished morphologically on the plate. FC results can discern both *Bacillus* spp. in a blend and recovered 116% compared to the expected level with an RSD value of 9%. While there are currently no established cultural plate count methodologies for the selective enumeration of *B. coagulans* and *B. subtilis* when combined in a blend, FC can accurately discern and quantify subpopulations in amalgams. FC also eliminates required incubation, reducing the time to result from 2-3 days to <8 hours.

Introduction

A flow cytometer uses laser light to detect particles in a fluid stream. Emissions scatter at different angles depending on particle size and internal complexity. The deflected light hits a series of detectors and the signals from these detectors are interpreted by computer software in real time. Using dual nucleic acid stains allows for the discernment and detection of live and dead/damaged cellular populations.

Figure 1. The Attune NxT flow cytometer.

A newer innovation, acoustic-assisted hydrodynamic flow cytometry, uses sound vibrations to align the particles and reduces the time to result.

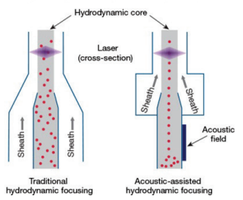

Figure 2. Rapid, improved alignment with acoustic focusing.

Objective

Evaluate a novel method for the rapid detection and enumeration of respective *B. coagulans* and *B. subtilis* spore populations when combined in a gummy matrix using an acoustic focusing flow cytometer. Sample preparation is summarized in Figure 3.

Materials and Methods

- Starch-based gummies containing known populations of *B. coagulans* MTCC 5856 and *B. subtilis* DE-111 as well as control gummies containing a single strain of *B. coagulans* or *B. subtilis* were used to develop the method.
- The gummies were inoculated with known concentrations of spores calculated using the manufacturer Certificates of Analysis and confirmed by average plate count results[4].
  - 2.9 x 10^9 CFU/serving *B. coagulans*, 7.1 x 10^9 CFU/serving *B. subtilis* for the combined sample (serving = 2 gummies).
  - 2.3 x 10^9 CFU/serving for the *B. coagulans* sample.
  - 7.1 x 10^9 CFU/serving for the *B. subtilis* sample.
- Control gummies were used to confirm the location of live strain cellular sub-populations as a function of emission properties on the data analysis graphs when run by flow cytometry.

Figure 3. Flow diagram of flow cytometry method for the enumeration of a blend of *B. coagulans* and *B. subtilis* in a gummy matrix with steps outlining a comparison to plate counting.

Results

Flow cytometry results were calculated using the following equation:

$$\text{AFU/g or mL} = (\text{Events}) \frac{(\text{Bead Conc})(\text{Bead Vol Analyzed})}{\text{Bead Events Detected}} (\text{Dilution Factor})$$

Table 1. Comparison of average quantitative enumeration results obtained by flow cytometry and by plate count evaluations for gummies containing both *B. coagulans* and *B. subtilis* with percent recovery compared to the inoculation concentration.

Sample: Mixed Population Gummy		
Method of Analysis	Count (AFU or CFU/ serving)	% Recovery (Compared to inoculation)
Flow Cytometry	1.17 x 10^{10}	116
Plate Count	3.32 x 10^9	33

Table 2. Quantification results for cellular sub-populations of *B. coagulans* and *B. subtilis* in a blend using **flow cytometry** compared to inoculation concentration.

	Flow Cytometry Enumeration Results		
Organism	Result (AFU/serving)	Inoculation (CFU/serving)	% Recovery
B. subtilis	7.99 x 10^9	7.14 x 10^9	**112**
B. coagulans	3.73 x 10^9	2.92 x 10^9	**128**

Table 3. Quantification results for cellular sub-populations of *B. coagulans* and *B. subtilis* in a blend using **plate count methods**. PNY Agar method is used for *B. coagulans* enumeration but is not selective and will grow *B. subtilis* as well. Petrifilm™ method presumed only *B. subtilis* due to being read after 24 hours incubation.

	Plate Count Enumeration Results		
Method	Result (CFU/serving)	Inoculation (CFU/serving)	% Recovery
Aerobic Plate Count (APC) Petrifilm™ – *B. subtilis*	3.71 x 10^9	7.14 x 10^9	**52**
PNY Agar – Total Spore Count	3.32 x 10^9	1.01 x 10^{10}	**33**

Table 4. Precision evaluation comparing flow cytometric results for both *B. coagulans* and *B. subtilis* to cultural plate count methods using PNY Agar and APC Petrifilm™ for gummy matrix containing both organisms.

Platform (Organism/ Media)	Average Result (AFU or CFU/ serving)	Standard Deviation	% RSD	Average % RSD
Flow Cytometry (B. subtilis)	7.99 x 10^9	8.55 x 10^8	11	**9**
Flow Cytometry (B. coagulans)	3.73 x 10^9	2.92 x 10^8	8	
Plate Count (PNY Agar)	3.32 x 10^9	3.70 x 10^8	11	**13**
Plate Count (APC Petrifilm™)	3.71 x 10^9	5.42 x 10^8	15	

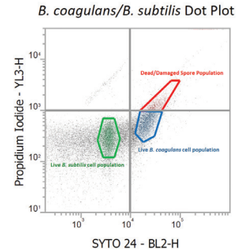

Figure 4. Dot plot showing the live and dead/damaged concentrations of cellular events for gummy sample containing both *B. coagulans* and *B. subtilis*. Dead/damaged appear in the upper right quadrant because both SYTO® 24 and propidium iodide penetrate damaged cell walls. The Live populations show up below the dead/damaged population because they exclude propidium iodide leading to a lower YL3 signal. *B. subtilis* has a lesser affinity to bind SYTO® 24 leading to the differentiation of the live populations confirmed by single strain control samples.

Conclusion

Flow cytometry can (i) be used to accurately and precisely enumerate *B. coagulans* and *B. subtilis* spores when combined in a gummy matrix; (ii) provide a distinct quantification of each strain based on dye affinity; and (iii) differentiate the live and dead/damaged cellular populations. Established cultural plate count methods cannot selectively enumerate both organisms in a blend and demonstrated poor recovery compared to the inoculation concentrations. Flow cytometry produced accurate and precise results and demonstrated a number of key advantages over cultural plate counting.

Advantages of flow cytometry methodology over classical methods are:

- **Superior precision** — Lower RSD compared to plating
- **Faster delivery** — Results in < 8 hours compared to 2 - 3 days by culture
- **Product integrity** — Discernment between live, dead, and injured cells informs product formulation, stability and guides development

References

1 Benkowski, A.A. and J.L. Schoeni. 2017. Evaluation of an acoustic focusing flow cytometer for enumeration of probiotic organisms. IPA World Congress + Probiota Americas 2017, San Francisco, CA, 7-9 June 2017. Scientific Frontiers Poster No. 5.

2 Benkowski, A.A. and J.L. Schoeni. 2017. Probiotic enumeration by flow cytometry: method scope extension of ISO 19344. AOAC Annual Meeting and Exposition 2017, Atlanta, GA, 24-28 September 2017. Poster Presentation P-T-061.

3 Benkowski, A.A., J.D. Legan and J.L Schoeni. 2018 Probiotic enumeration by flow cytometry: a method for the determination of *Bacillus coagulans* spore populations in food and dietary supplements. Institute of Food Technologists (IFT) Annual Meeting and Food Expo, Chicago, IL, 15-18 July 2018.

4 *Bacillus coagulans* GBI-30, 6086 Enumeration Method. United States Pharmacopeial Food Chemical Codex 10, First Supplement.

5 International Organization for Standardization (ISO). 2015. Milk and milk products – starter cultures, probiotics, and fermented products – quantification of lactic acid bacteria by flow cytometry. ISO 19344:2015 (E)/IDF 232:2015 (E).

Presented at IFT 2019

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