SIMULTANEOUS ENUMERATION OF SPORE-FORMING PROBIOTICS AND POSTBIOTICS IN MULTI-STRAIN BLENDS USING FLOW CYTOMETRY: ADAPTATION OF ISO 19344 PROTOCOL B

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ABSTRACT

Objective: To develop and validate a single-panel flow cytometry method for simultaneous enumeration of spore-forming probiotics and postbiotics in multi-strain blends, adapted from ISO 19344 Protocol B.

Methods: Six strains were evaluated individually using ISO 19344 Protocol B-adapted flow cytometry with added sonication to disrupt aggregates: three spore-forming probiotics (*Heyndrickxia coagulans* (f.k.a. *Bacillus coagulans*), *Shouchella clausii* (f.k.a. *Bacillus clausii*), *Bacillus subtilis* complex) and three heat-inactivated postbiotics (*Lactococcus lactis*, *Akkermansia muciniphila*, *Lactobacillus crispatus*). Enumeration was then performed on three blended formulations: a multi-strain spore blend, a multi-strain postbiotic blend, and a combined spore–postbiotic blend. Gating strategies based on scatter and fluorescence characteristics were developed to resolve spore and postbiotic populations within each blend.

Results: For individual spore strains, flow cytometry showed strong agreement with standard plate counts for all probiotic spore-formers, with log differences of +0.16 (*H. coagulans*), −0.18 (*S. clausii*), and +0.10 (*B. subtilis*). Analysis of blended formulations demonstrated accurate and distinct population recovery relative to theoretical inputs. Measured values closely matched expected counts for the spore-forming probiotic blend (+0.19 log difference), the postbiotic blend (−0.13 log difference), and the combined spore–postbiotic blend (−0.03 log difference for the postbiotic component and +0.16 log difference for the spore component).

Conclusions: This method enables rapid, simultaneous enumeration and differentiation of spore-forming probiotics and postbiotics in complex blends, providing a scalable tool for quality assurance, safety verification, and label claim substantiation.

INTRODUCTION

Postbiotic and spore-forming probiotics are among the most popular supplement products on the market. However, there is a notable gap in current testing methodologies, as no standardized approach exists for accurately enumerating both components simultaneously in blended products.

METHODS

- Modified ISO 19344 Protocol B flow cytometry with an added sonication step was applied to three spore-forming probiotic and postbiotic strains, using BL1-H and YL2-H channels (SYTO 24 and Propidium Iodide).
- Plate counts were performed for each spore material to assess accuracy, and spore blends were enumerated to compare total fluorescing units (TFU) against individual strain counts (CFU) for blend retention
- A final mixed blend was analyzed using population ratios and prior data to calculate theoretical TFU, enabling recovery assessment of initial spore and postbiotic populations within the blend.

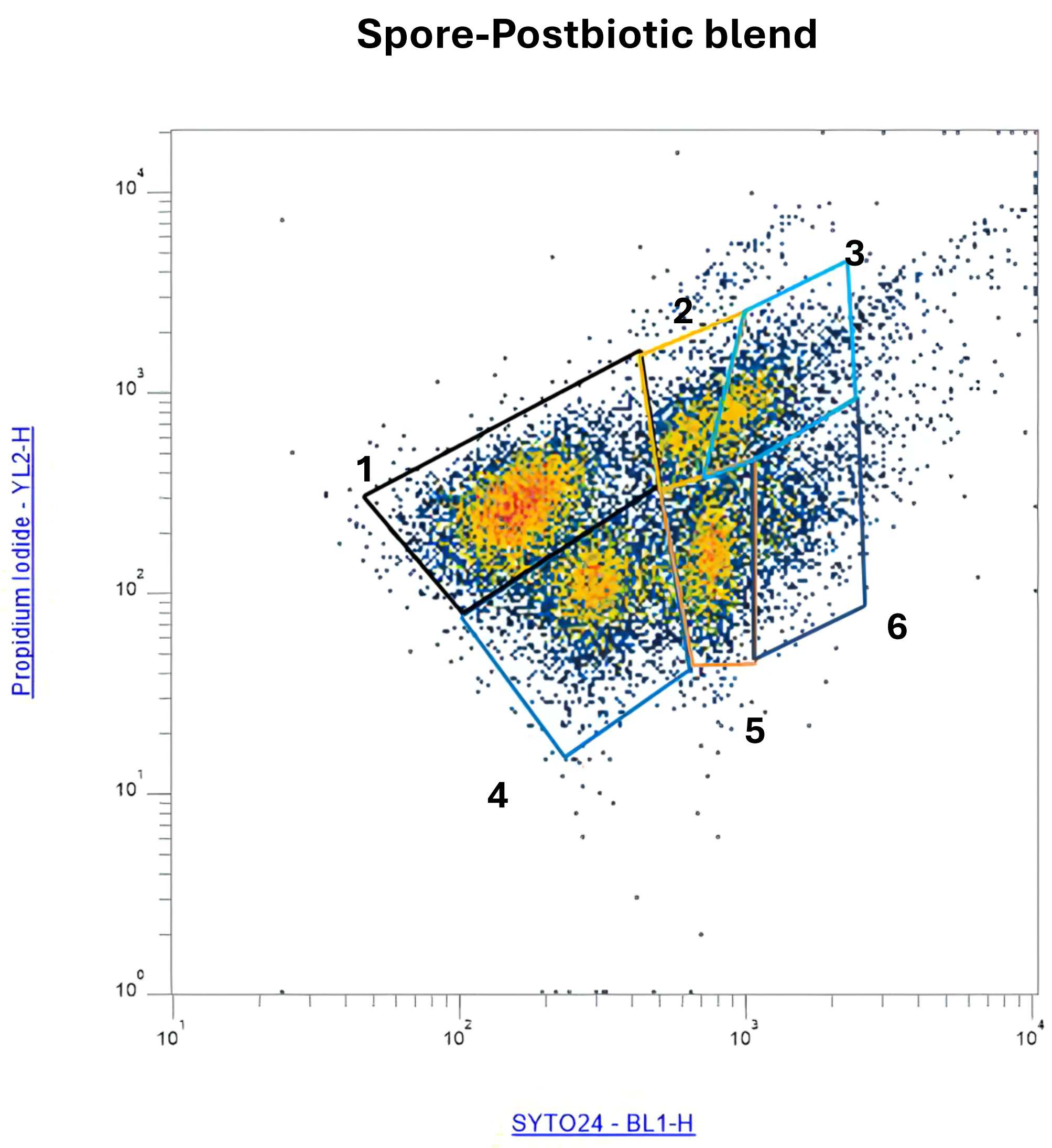

Figure 1. A blend of postbiotic (1) *Lactobacillus crispatus,* (2) *Akkermansia muciniphila,* and (3) *Lactococcus lactis* and probiotic spores (4) *Bacillus subtilis* complex, (5) *Shouchella clausii,* and (6) *Heyndrickxia coagulans*.

RESULTS

Strong correlation with reference methods

Spore plate count vs flow cytometry:

- *H. coagulans*: +0.16 log
- *S. clausii*: −0.18 log
- *B. subtilis* complex: +0.10 log

Accurate enumeration in blends

- Spore blend: +0.19 log expected count from individual strains
- Postbiotic blend: −0.13 log expected count from individual strains

Good recovery of both ingredients in a spore–postbiotic blend

CATEGORY	THEORETICAL TFU	MEASURED TFU	RETENTION (LOG)	RESOLVED POPULATIONS
Spores	6.02×10^{10}	9.03×10^{10}	+0.16	4, 5, 6
Postbiotics	1.53×10^{11}	1.37×10^{11}	−0.03	1, 2, 3

≤ ±0.2 log difference across all conditions

CONCLUSIONS

Modified ISO 19344 Protocol B flow cytometry method enables accurate, simultaneous enumeration of spore-forming probiotics and postbiotics in complex multi-strain blends, with strong agreement to traditional plate counts for spores and theoretical input for postbiotics.

Method shows consistent recovery across both strain level and postbiotic / spore populations, with sonication improving population resolution and supporting robust gating.

Flow cytometry provides a scalable, efficient approach for analyzing increasingly complex biotic formulations.

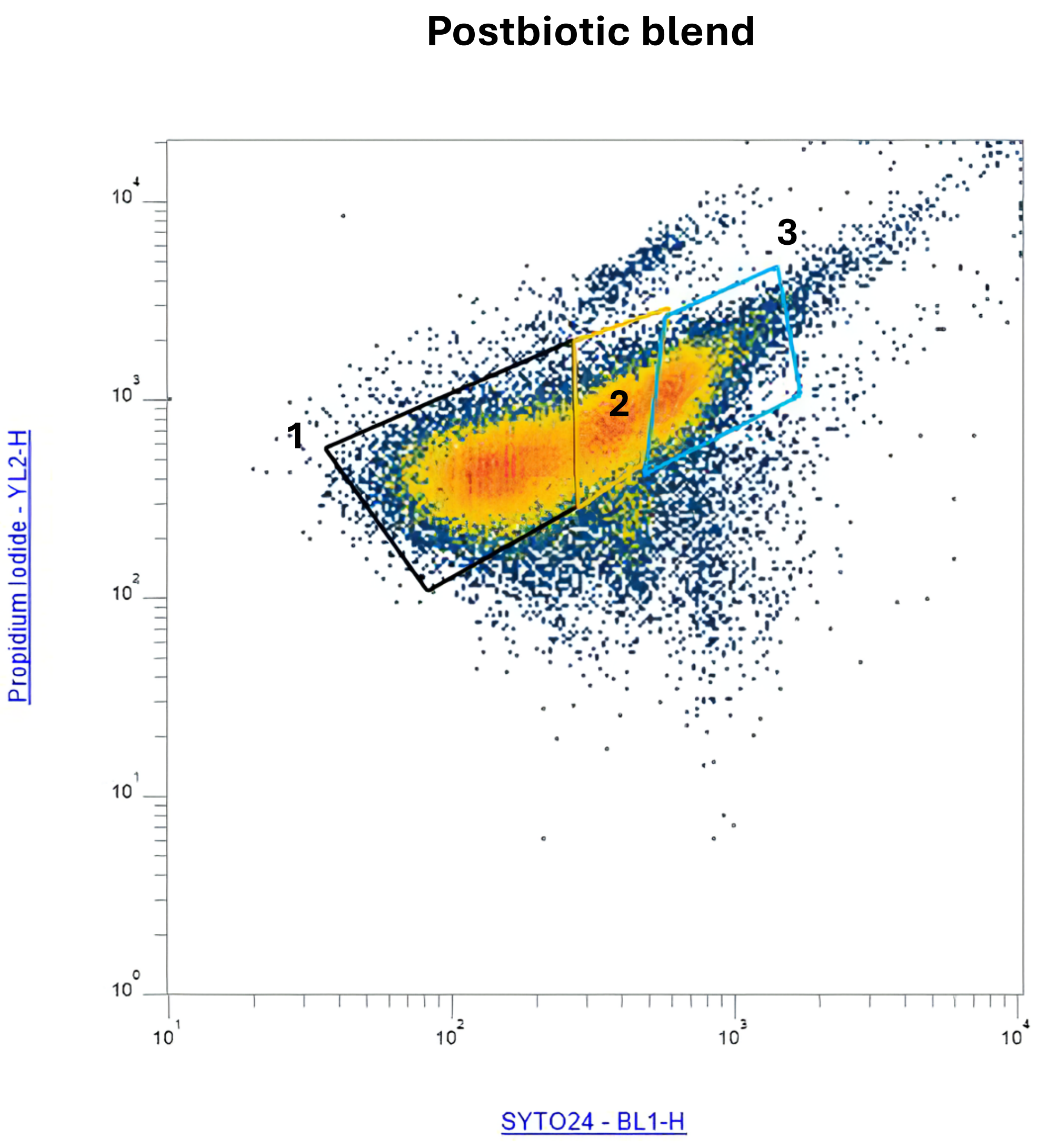

Figure 2. Postbiotic blend of (1) *L. crispatus,* (2) *A. muciniphila,* and (3) *L. lactis*.

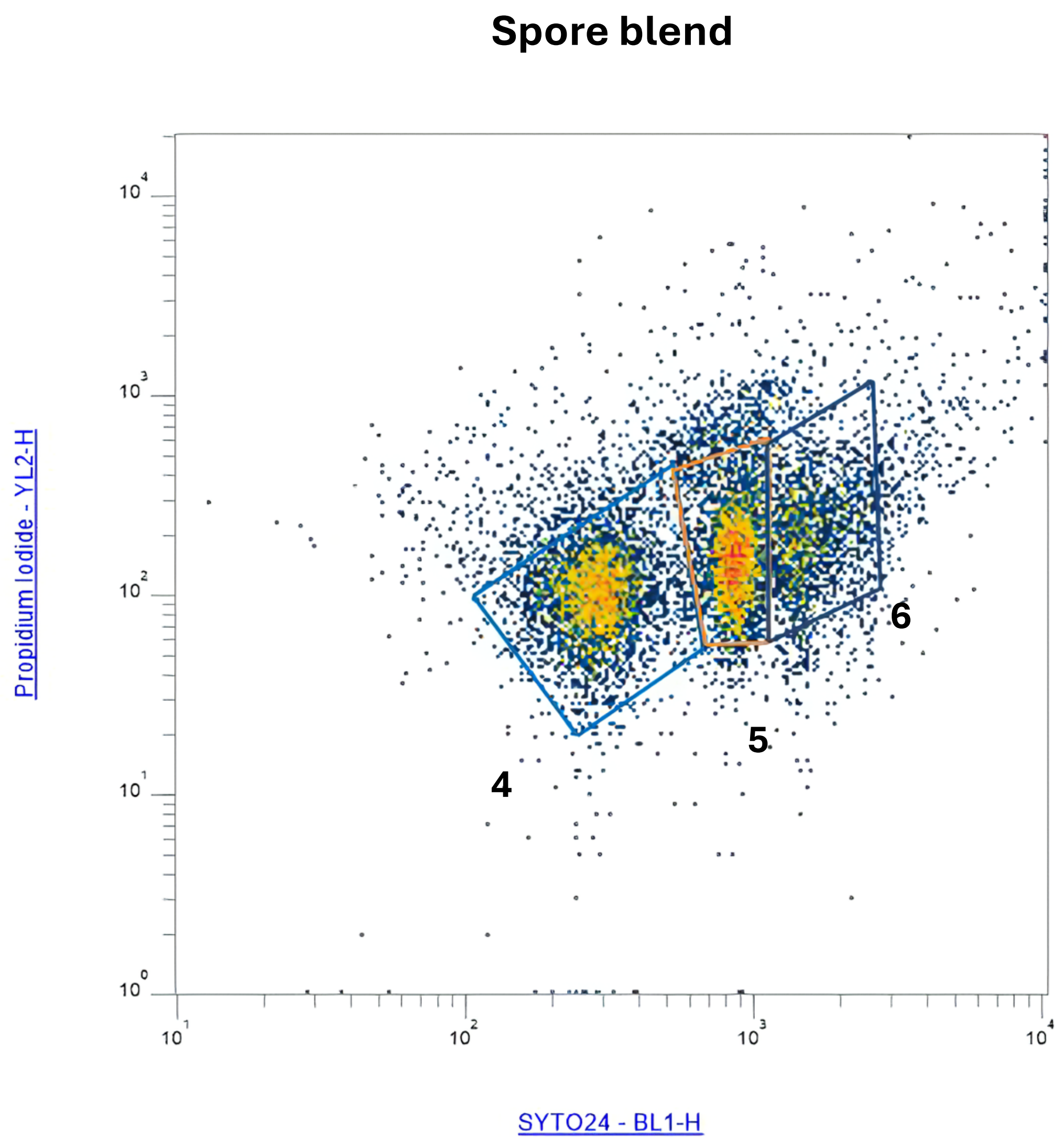

Figure 3. Spore blend of (4) *B. subtilis* complex, (5) *S. clausii*, and (6) *H. coagulans*.

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