GenoScreen
Innovative Genomics

INTRODUCTION TO FLOW CYTOMETRY

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Microbial Analytics Applications

Pierre Burguière, Ph.D.

Emerging Technologies in probiotic, live biotherapeutic product and microbiome analysis

USP Virtual Symposium – October 7, 2022

Affiliations disclaimer

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Flow Cytometry

Etymological definition

1. flow ⇨ moving
2. cyto ⇨ cell
3. metry ⇨ measure

Flow Cytometry - Principle

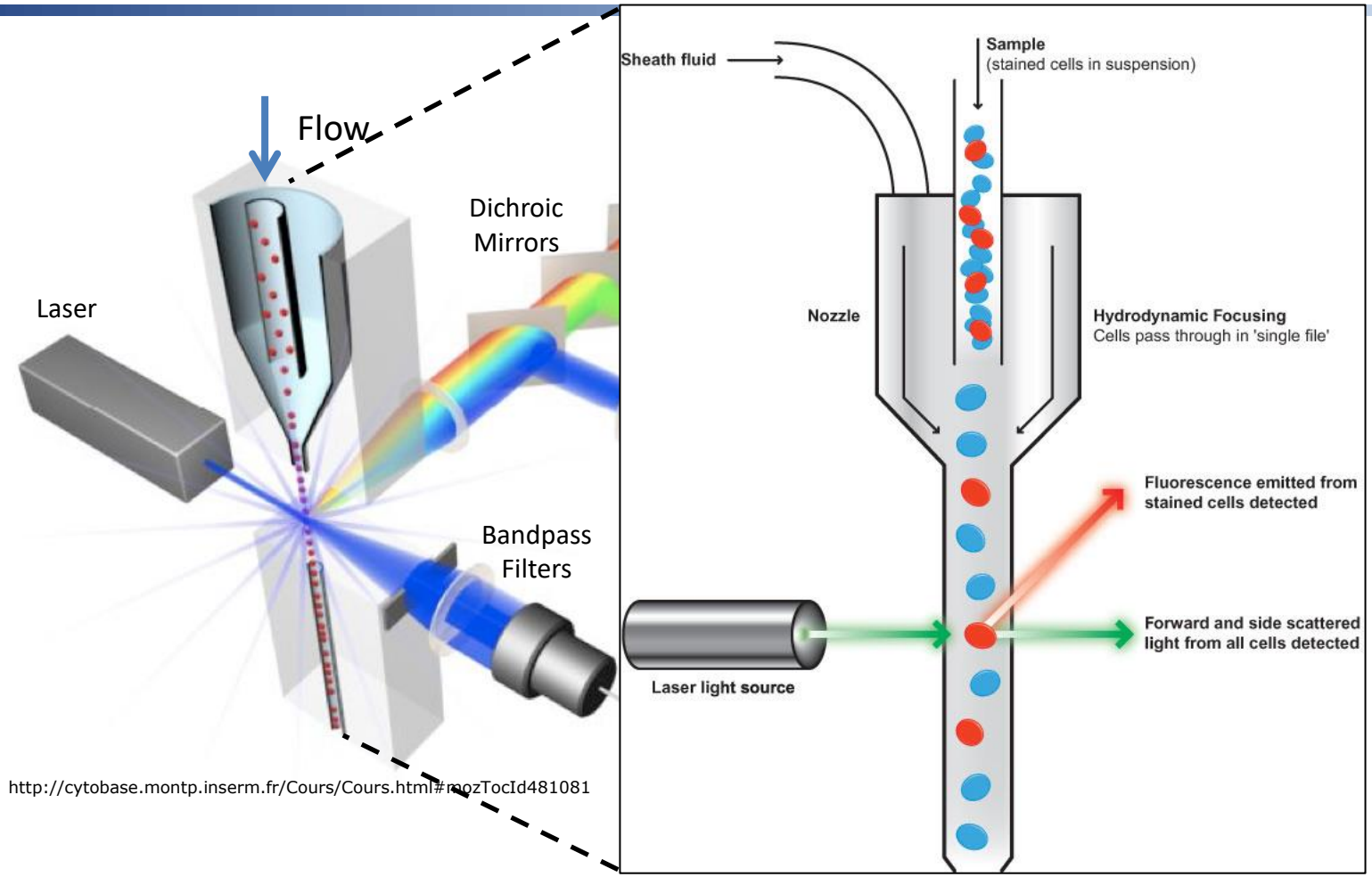
Conventional flow cytometers typically contain 3 parts

1. Fluidics ⇨ introduce & channel monodisperse particles (e.g. cells) towards light source (laser)

2. Optics ⇨ light source(s) and recovery of emitted light (fluorescence)

3. Electronics ⇨ digitalization of light signals and data interpretation

Flow Cytometry - Principle



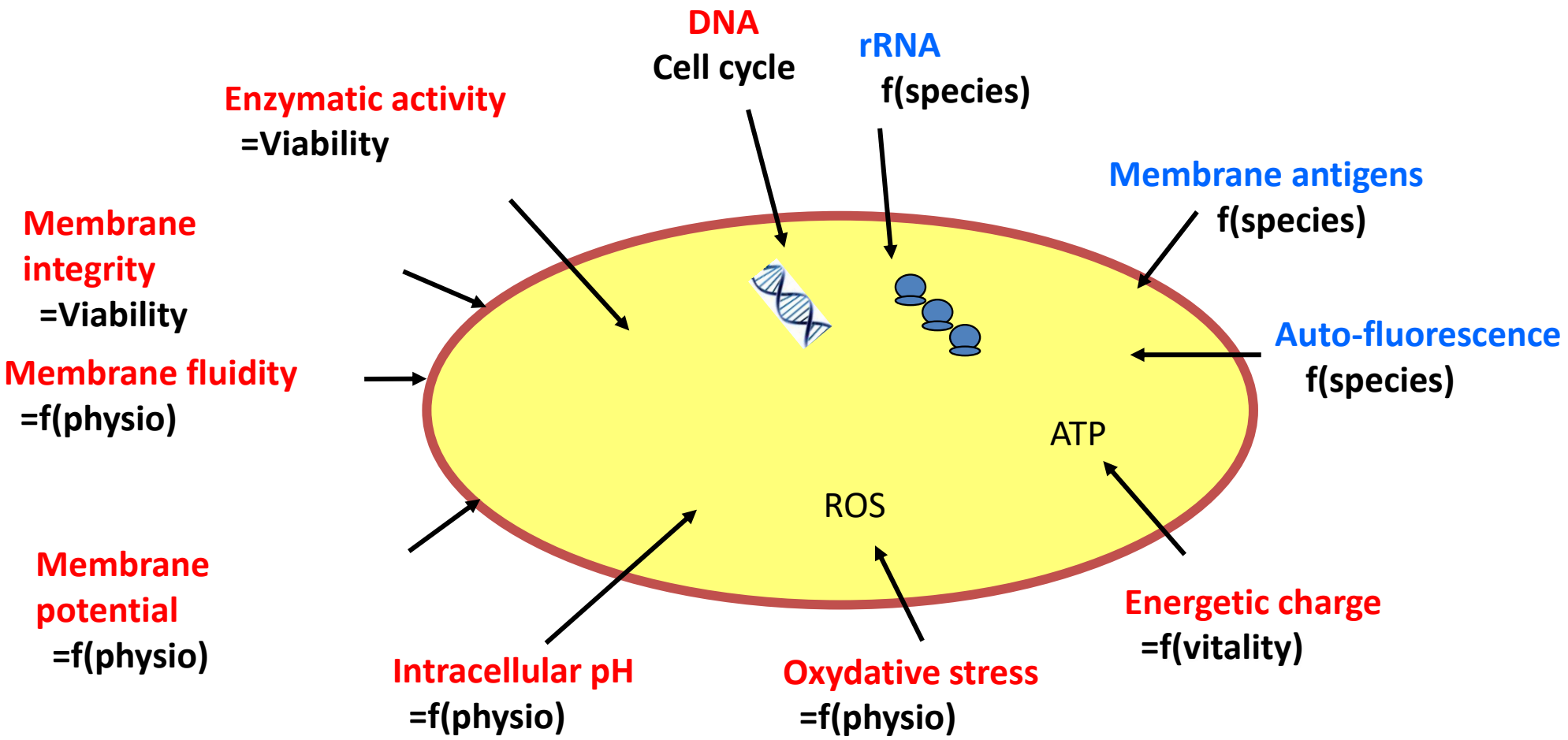
http://cytobase.montp.inserm.fr/Cours/Cours.html#mozTocId481081

- High throughput: 10,000+ cells/sec)
- Fast: few minutes
- Absolute cell quantification
- Single-cell measurements
- Multiparametric analysis: size, structure, fluorescence, etc.
- Cell physiology: viability, functionality, etc.
- Automation: 96-well

Flow cytometry advantages for microbial analytics

- detection of all microorganisms: live + VBNC & non-viable (dead)
- very short time-to-result
- analysis of multi-strain microbial products

Microbial targets labeling



Probiotics

“ Live microorganisms which when administered in adequate amounts confer a health benefit on the host ”

FAO / WHO (2001)

World Health Organization

Food and Agriculture Organization of the United Nations

Viability – physiological state

“ Bacterial viability is defined by an intact cytoplasmic membrane, protein and other cell components synthesis (nucleic acids, polysaccharides, etc.) and energy production necessary to maintain cells metabolism ; and, eventually, growth and multiplication. ”
(Breeuwer and Abee, 2000)

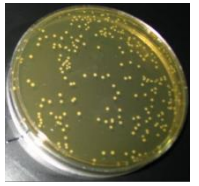
Quantity – expected concentration

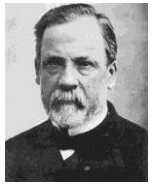
Specificity – expected strain(s)

Potency – capacity to deliver the health benefit(s)

Analytical reference: Classical Microbiology

Microorganisms growth on agar-based culture media





Viability	Culturability (i.e. ability to undergo cellular division)
Quantity	Enumeration (dilution & colony counting)
Specificity	Composition of culture media (e.g. nutrients, antibiotics)
Potency	Biomass (CFU/g)

Advantages

- ✓ Internationally recognised & widely accepted
- ✓ Low technicity
- ✓ Low-cost materials, reagents
- ✓ Sensitivity: down to 1 bacteria/g

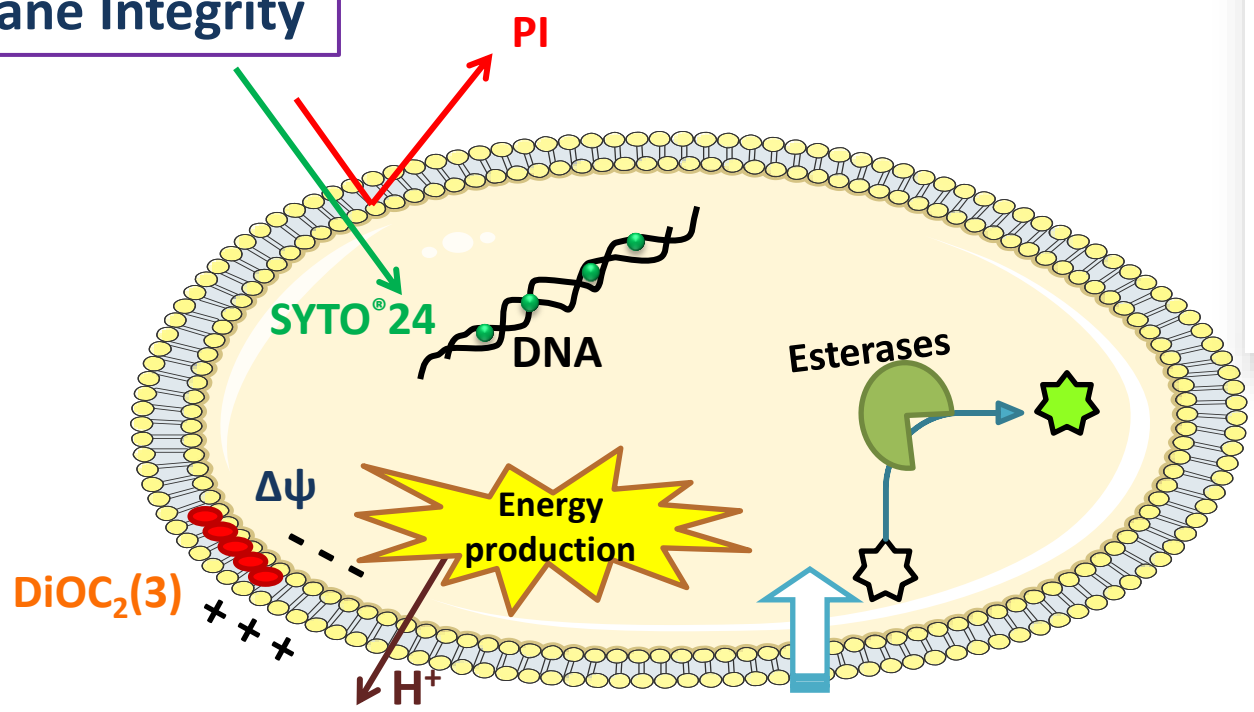
Limitations

- × Time-consuming: 2-10 days result delivery
- × Limited information: culturable cells only
- × Low specificity
- × High variability
- × Biomass: is it a relevant indicator of probiotic potency?

Quantification of viable/dead probiotics

3 different viability protocols

Membrane Integrity



Membrane Potential

Enzymatic activity

INTERNATIONAL STANDARD

ISO 19344

IDF 232

First edition
2015-12-15

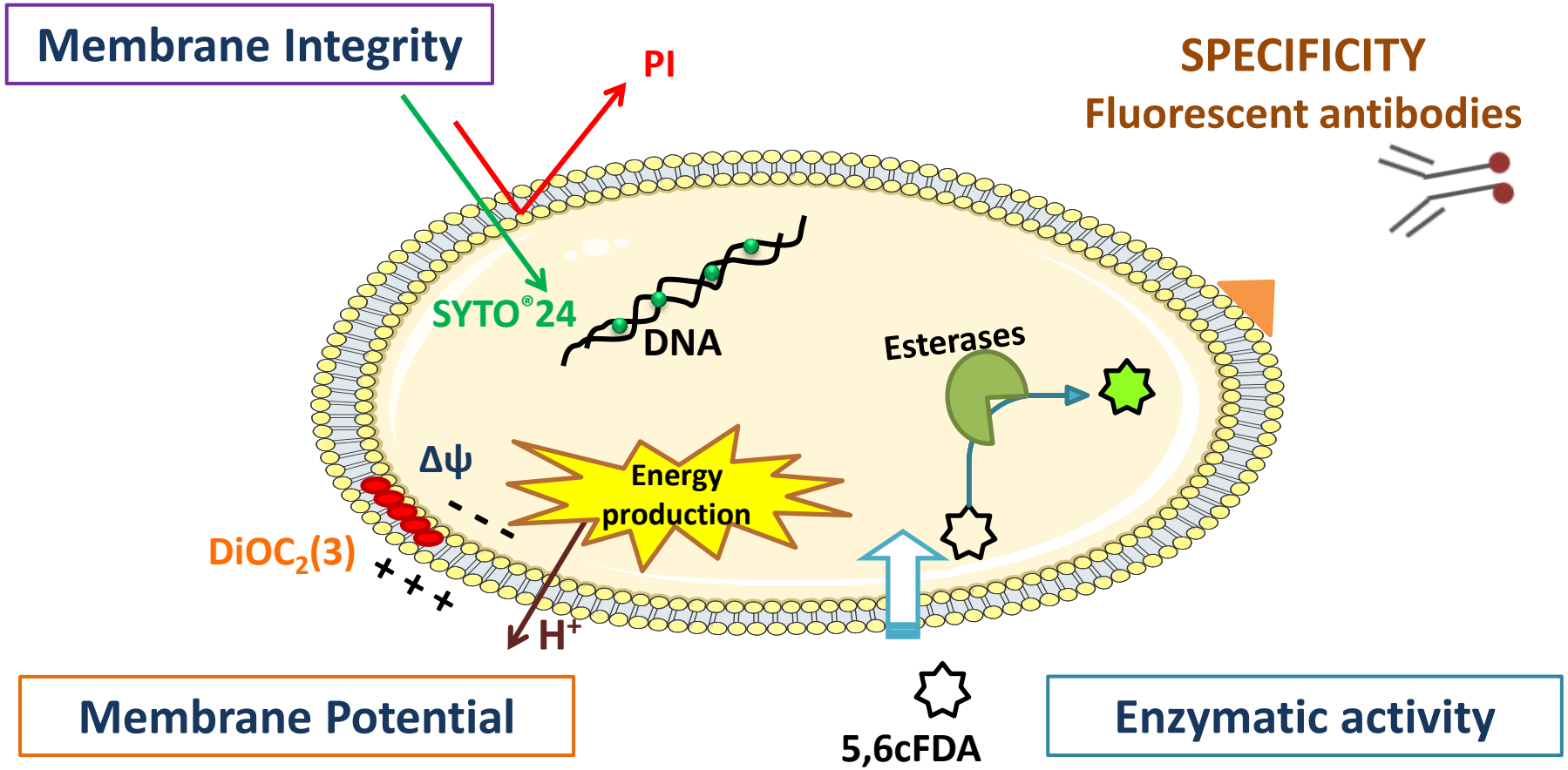
Milk and milk products — Starter cultures, probiotics and fermented products — Quantification of lactic acid bacteria by flow cytometry

Lait et produits laitiers — Cultures, probiotiques et produits fermentés — Quantification de bactéries lactiques par cytométrie en flux

Quantification of viable/dead probiotics

ISO 19344

3 different viability protocols



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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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Lallemand Health Solutions Inc., Montreal, QC, Canada

Keywords
antibodies, flow cytometry, immunofluorescence, probiotics, specific quantification, viability.

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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.

Flow cytometry analytical strategy

Principle: coupling 2 flow cytometry labeling protocols

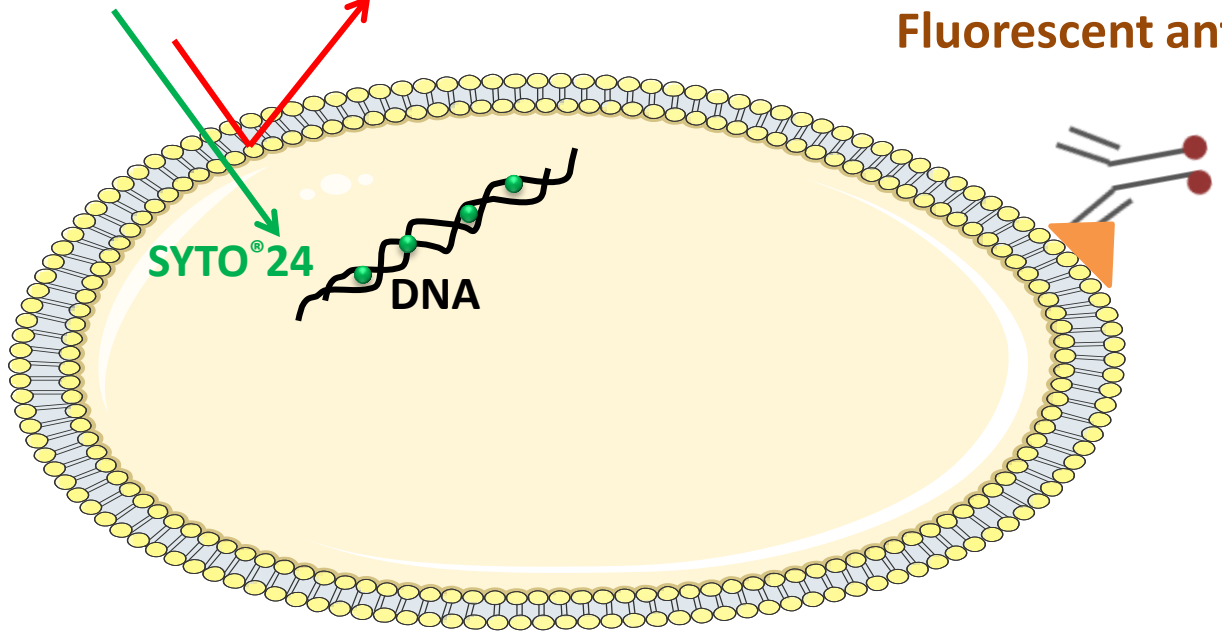
1 viability protocol
(membrane integrity)
from ISO 19344 Standard

+

1 specificity protocol
using customized
antibodies

Membrane Integrity

ISO
19344



SPECIFICITY
Fluorescent antibodies

Specific Quantification and Viability Assessment of Probiotics in Multi-strain Products : comparison FCM vs. CFU

	log10 CFU/g	Proportion
Total count	> 10,0	-
***L. rhamnosus* R0011**	> 9,98	**95%**
***L. helveticus* R0052**	> 8,70	**5%**

Shelf-life: 2 years

	log10 CFU/g	Proportion
Total count	> 10,10	-
***L. rhamnosus* R0011**	> 9,60	**33%**
***L. helveticus* R0052**	> 9,60	**33%**
***B. longum* R0175**	> 9,60	**33%**
Saccharomyces boulardii	> 9,80	

Shelf-life: 2 years

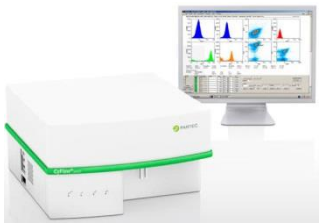
	log10 CFU/g	Proportion
Total count	> 9,88	-
***L. helveticus* R0052**	> 9,65	**60%**
***B. longum* sp. *infantis* R0033**	> 9,18	**20%**
***B. bifidum* R0071**	> 9,18	**20%**

Shelf-life: 2 years

→ 2 batches / product

→ 2 instruments & 2 operators

→ Comparison FCM vs. CFU for each product



CyFlow® Space (Partec)

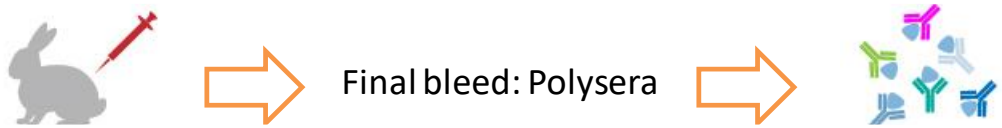
Accuri® C6 + CSampler®(BD)

Antibody generation

Development of antibodies for specific enumeration

5 Polyclonal antibodies (pAb) developed:

- 4 against inactive freeze-dried bacteria: *Lactobacillus rhamnosus* R0011
  Bifidobacterium bifidum R0071
  Bifidobacterium longum spp. infantis R0033
  Bifidobacterium longum R0175
- 1 against a purified Surface-Layer Protein (SLP) from *L. helveticus* R0052

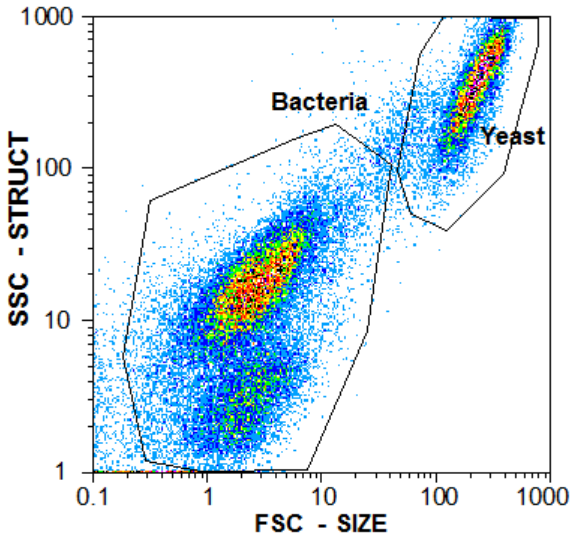


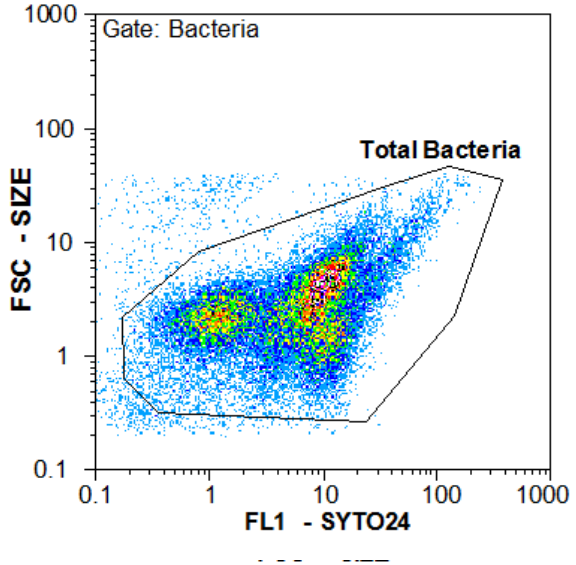
Specificity of each pAb was evaluated against 30+ strains:

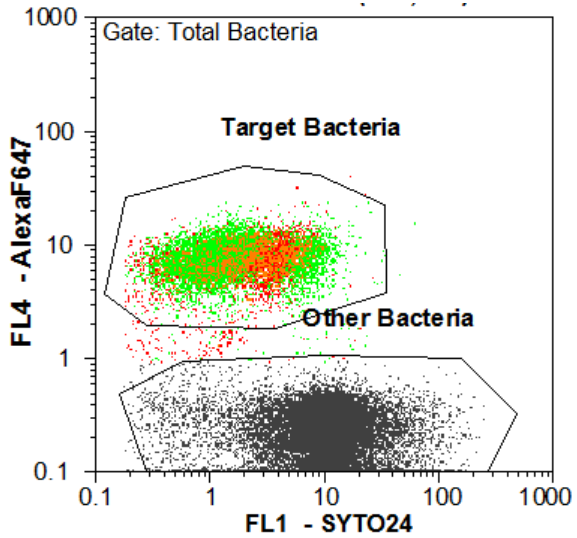
(1) Strains from different species & genera

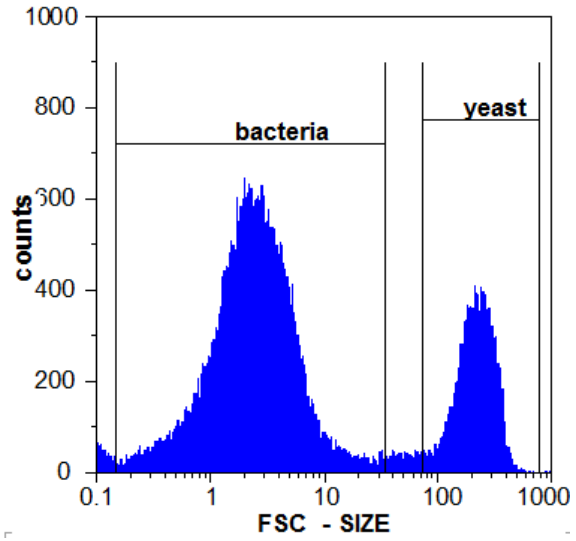
(2) Strains from the same species

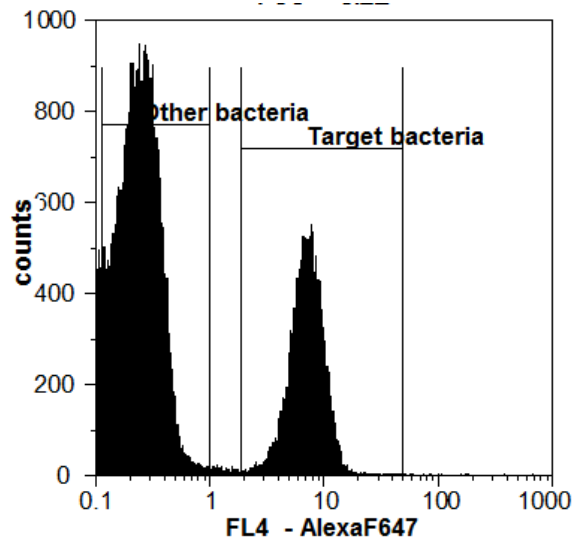
Flow cytometry raw data

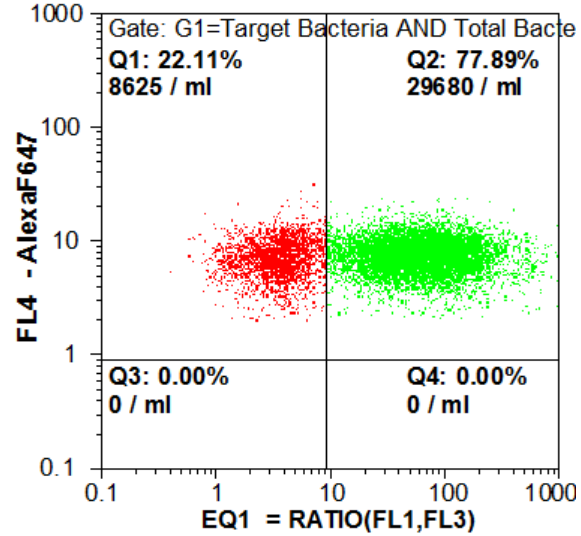












Specific Quantification and Viability Assessment of Probiotics in Multi-strain Products : comparison FCM vs. CFU

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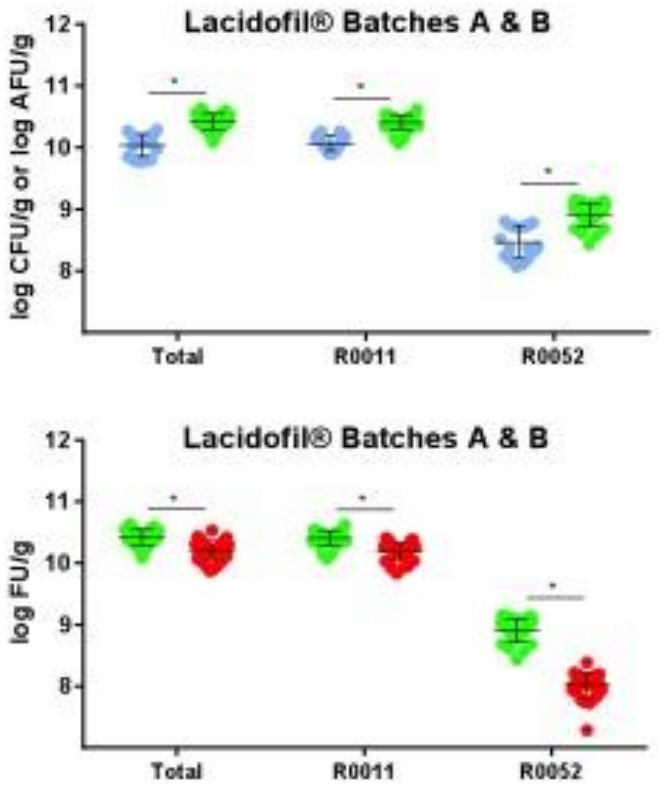
Shelf-life: 2 years

Flow Cytometry = 2h
Classical Microbiology = 48h



- CFU: Colony Forming Units
- AFU: Active Fluorescent Units
- n-AFU: Non-Active Fluorescent Units
- * *p*-value < 0.05

TAKE HOME MESSAGES

1. Flow cytometry to overcome classical microbiology numerous limitations
2. Multiple fields of application
   - R&D
   - Production Processes optimization
   - QC: positive microbiology, safety & quality
   - Analysis of complex matrices (fermented foods, clinical samples)
3. Standardization - new worldwide reference methods
   - ISO-IDF 19344 (2015)
4. Challenges
   - Paradigm Shift: from culturability to other viability parameter(s)
   - Validation: ‘reference method paradox’
   - Transfer & Implementation: need for mindset changes
   - Need to use alternative methodologies for stability and clinical trial products (not only CFU)
5. Future opportunities
   - Fastidious microorganisms (eg. strict anaerobes), inactivated bacteria (postbiotics)
   - FCM-based probiotic potency assays

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FCM-based potency assay for probiotics/LBPs

Concept

- MacPherson *et al*. (2017) showed that probiotic bioactivity is carried by a specific Surface-Layer Protein (SLP) - among other potential mechanisms of action underlying health benefits
- Chiron *et al*. (2018) developed a FCM assay coupling 1 viability protocol + 1 specificity protocol using customized antibodies (pAb)
  One pAb was developed to be specific to the probiotic SLP
- This FCM method could serve as a potency assay by assessing the level of probiotic specific bioactive SLP reflecting its capacity to deliver health benefits

PLOS ONE

RESEARCH ARTICLE

Genome-Wide Immune Modulation of TLR3-Mediated Inflammation in Intestinal Epithelial Cells Differs between Single and Multi-Strain Probiotic Combination

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OPEN ACCESS

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Received: October 27, 2016

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Published: January 18, 2017

Data Availability Statement: Information regarding the microarray platform and the expression data files can be found on the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under GEO platform no. GPL10332 and GEO series no. GSE71515.

Abstract

Genome-wide transcriptional analysis in intestinal epithelial cells (IEC) can aid in elucidating the impact of single versus multi-strain probiotic combinations on immunological and cellular mechanisms of action. In this study we used human expression microarray chips in an *in vitro* intestinal epithelial cell model to investigate the impact of three probiotic bacteria, *Lactobacillus helveticus* R0052 (Lh-R0052), *Bifidobacterium longum* subsp. *infantis* R0033 (Bl-R0033) and *Bifidobacterium bifidum* R0071 (Bb-R0071) individually and in combination, and of a surface-layer protein (SLP) purified from Lh-R0052, on HT-29 cells' transcriptional profile to poly(I:C)-induced inflammation. Hierarchical heat map clustering, Set Distiller and String analyses revealed that the effects of Lh-R0052 and Bb-R0071 diverged from those of Bl-R0033 and Lh-R0052-SLP. It was evident from the global analyses with respect to the immune, cellular and homeostasis related pathways that the co-challenge with probiotic combination (PC) vastly differed in its effect from the single strains and Lh-R0052-SLP treatments. The multi-strain PC resulted in a greater reduction of modulated genes, found through functional connections between immune and cellular pathways. Cytokine and chemokine analyses based on specific outcomes from the TNF-α and NF-κB signaling pathways revealed single, multi-strain and Lh-R0052-SLP specific attenuation of the majority of proteins measured (*TNF-α*, *IL-8*, *CXCL1*, *CXCL2* and *CXCL10*), indicating potentially different mechanisms. These findings indicate a synergistic effect of the bacterial combinations relative to the single strain and Lh-R0052-SLP treatments in resolving toll-like receptor 3 (TLR3)-induced inflammation in IEC and maintaining cellular homeostasis, reinforcing the rationale for using multi-strain formulations as a probiotic.

THANKS FOR YOUR ATTENTION !

QUESTIONS?

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