To count or not to count ?

Pro and cons of Probiotic Enumeration Methods

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6th Microbiome R&D and Business Collaboration Forum: USA

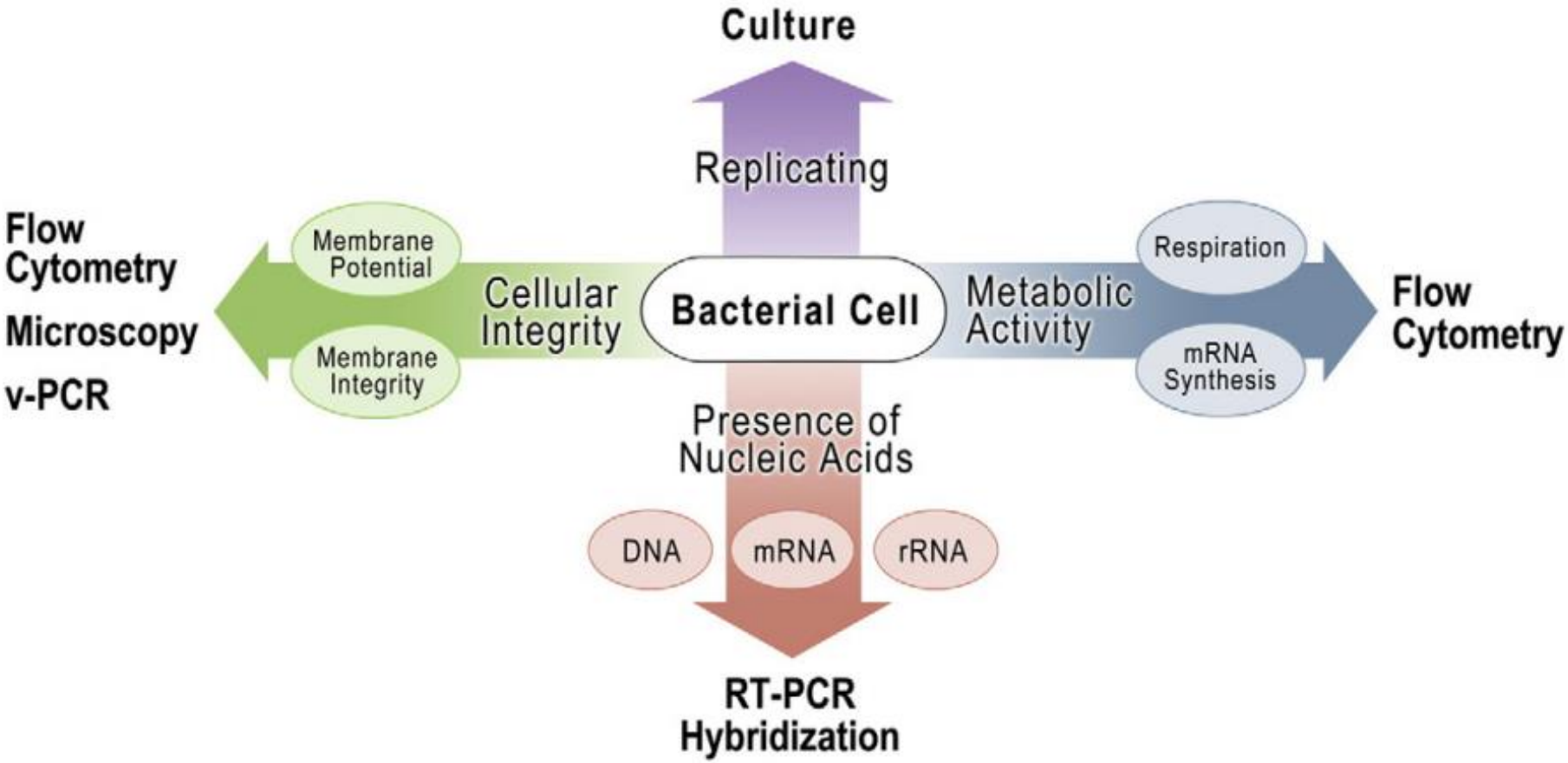
Probiotic Definition

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host (FAO 2001)[1]

[1]Food and Agriculture Organization of the United Nations (FAO). 2001. Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria, http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf

Different methodologies for probing bacterial cells

C. Davis / Journal of Microbiological Methods 103 (2014) 9–17



Evolution of analytical methods

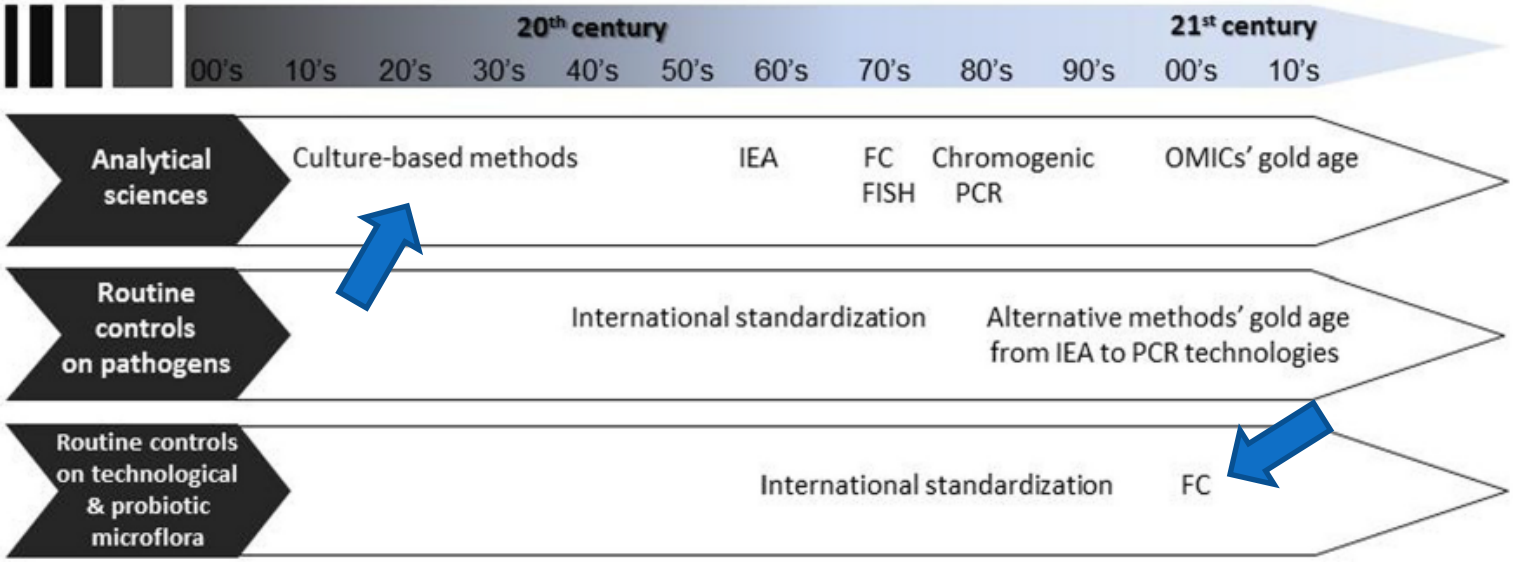
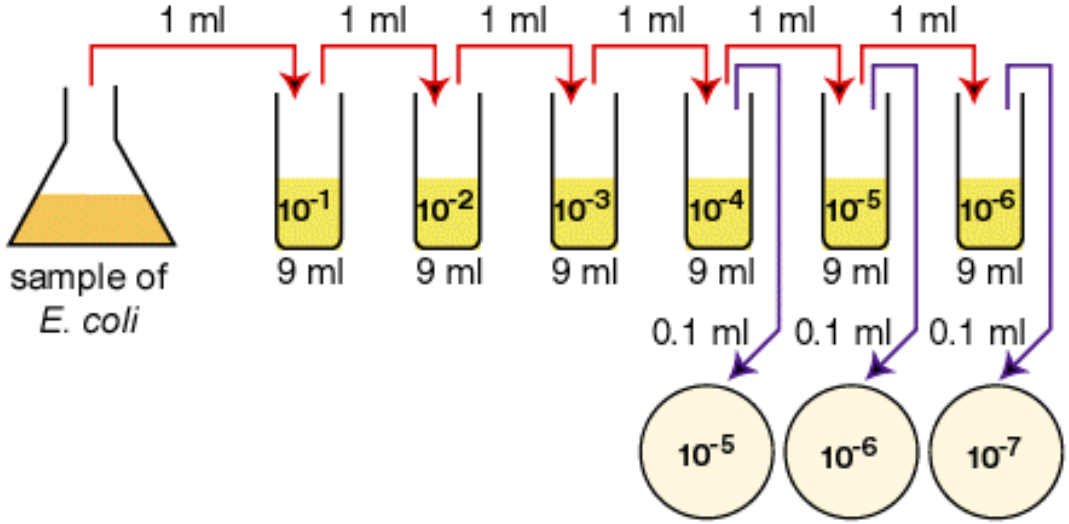
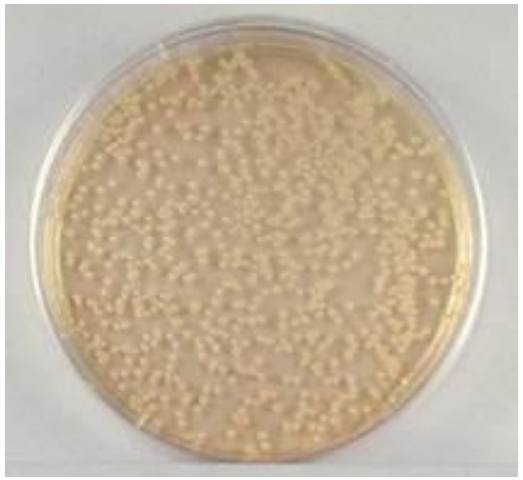


FIGURE 1 | Evolution of analytical methods, and the gaps between routine controls depending on the targeted microbiota. IEA, immuno-enzymatic assay; FC, flow cytometry; FISH, fluorescent *in situ* hybridization; PCR, polymerase chain reaction.

doi: 10.3389/fmicb.2014.00016

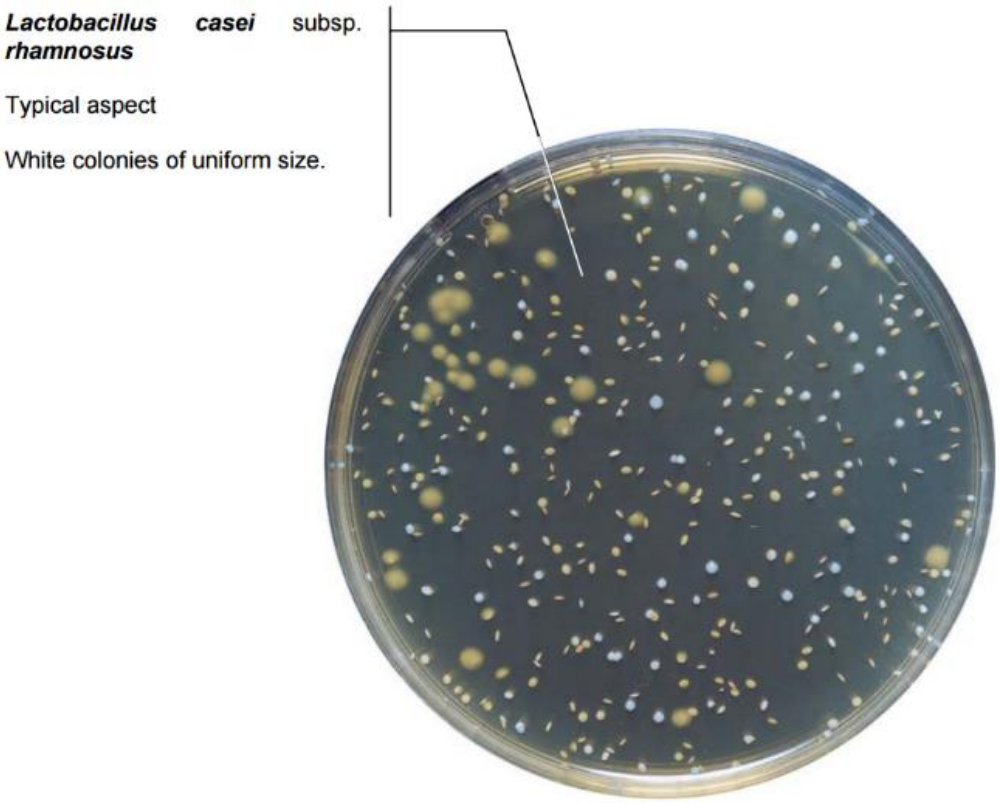
Plate count methods





The concept of plate count methods

The plate count method is based on the premise that a single bacterium can growth and divide to give an entire colony



Culture-base methods detect only bacteria that are able to replicate under specific condition (different from their native ecosystem!)

Interpretation of plate count methods

Observed result	Usual interpretation	Alternative interpretations
A colony is formed	A viable cell gave rise to the colony	At least one viable cell gave rise to the colony—but it may have been two or more cells coinciding at the same place on the plate or a clump of cells that contained at least one viable individual
No colony is formed	There were no viable cells in the sample	(i) The growth medium and/or incubation conditions were incorrect (ii) The cells were damaged/stressed and therefore unable to grow on solid medium (iii) The population density was low and therefore cell-cell communication could not take place, resulting in no observable growth (iv) Insufficient time was allowed for visible colony development in slowly growing cells

doi:10.1128/AEM.00744-11

"...certain plates in any series made from a given sample are more satisfactory for use in computing a total than other.
The matter of selecting plates to be used in computing a count becomes a matter of considerable judgment."

Breed and Dotterrer, **1916**

Ministero della Salute

DIPARTIMENTO SANITA' PUBBLICA VETERINARIA, SICUREZZA ALIMENTARE E ORGANI COLLEGIALI
PER LA TUTELA DELLA SALUTE
DIREZIONE GENERALE IGIENE E SICUREZZA DEGLI ALIMENTI E DELLA NUTRIZIONE
UFFICIO IV EX DGSAN

COMMISSIONE UNICA PER LA DIETETICA E LA NUTRIZIONE

Revision May 2013

Guidelines on probiotics and prebiotics

*"**The amount of cells present must be listed on the label, and moreover, this amount has to be guarantee until the end of the shelf-life, at the specified storage conditions, with uncertainty of 0,5 log**. It is emphasized that the analytical method of quantification of living bacterial cells may differ from specie to specie."*

Compositional Quality and Potential Gastrointestinal Behavior of Probiotic Products Commercialized in Italy

TABLE 2 | Enumeration of the organisms contained in a unit dose of each probiotic formulation.

Formulation	Dose	Labeled cell no.	Total CFU	CFU from spores only
Enterogermina	1 vial	2×10^9	$1.15 \pm 0.50 \times 10^9$	$1.65 \pm 0.71 \times 10^9$
Enterolactis Plus	1 capsule	2.4×10^{10}	$2.71 \pm 0.30 \times 10^{12}$	
Lactoflorene Plus	1 bottle	2×10^9	$6.02 \pm 5.73 \times 10^7$	$1.35 \pm 1.50 \times 10^7$
Reuflor	5 drops	1×10^9	$8.72 \pm 1.53 \times 10^{11}$	
Codex	1 capsule	5×10^9	$2.68 \pm 2.4 \times 10^9$	
Prolife	1 bottle	1.25×10^{11}	$2.16 \pm 0.36 \times 10^{11}$	$3.51 \pm 1.49 \times 10^{10}$
Dicoflor	5 drops	5×10^9	$9.65 \pm 1.95 \times 10^9$	
Enterelle	1 capsule	3×10^9	$5.74 \pm 0.99 \times 10^{10}$	
Yovis	1 sachet	2.97×10^{11}	$3.51 \pm 3.13 \times 10^{12}$	
VSL3	1 sachet	4.5×10^{11}	$4.53 \pm 0.47 \times 10^{13}$	

45300 billion\dose = approx. 10.000 billion\g

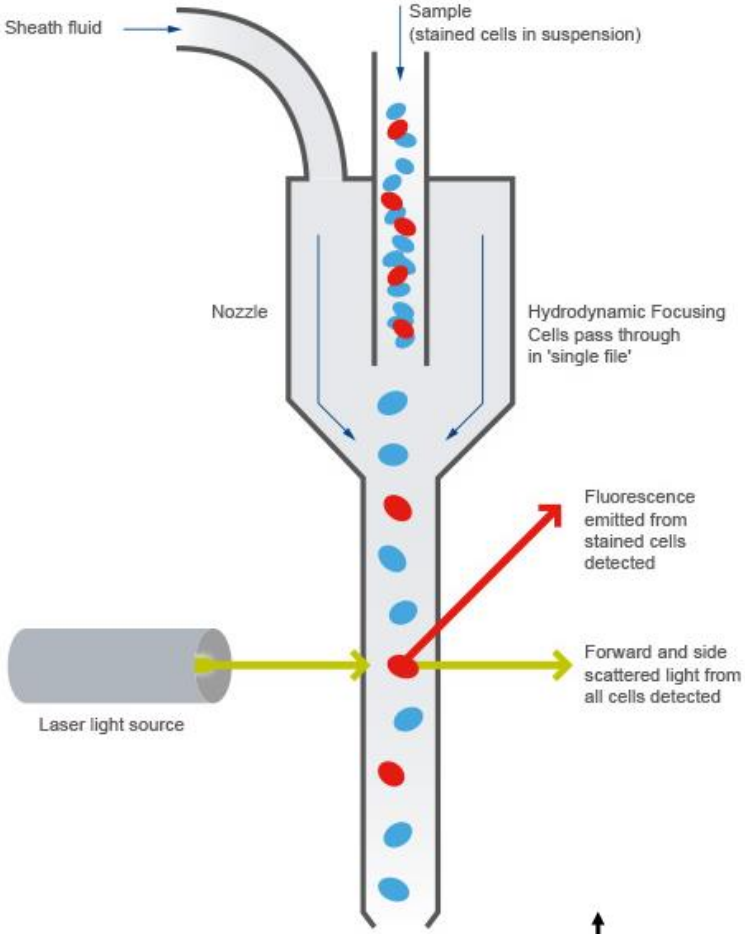
Limits of plate count methods

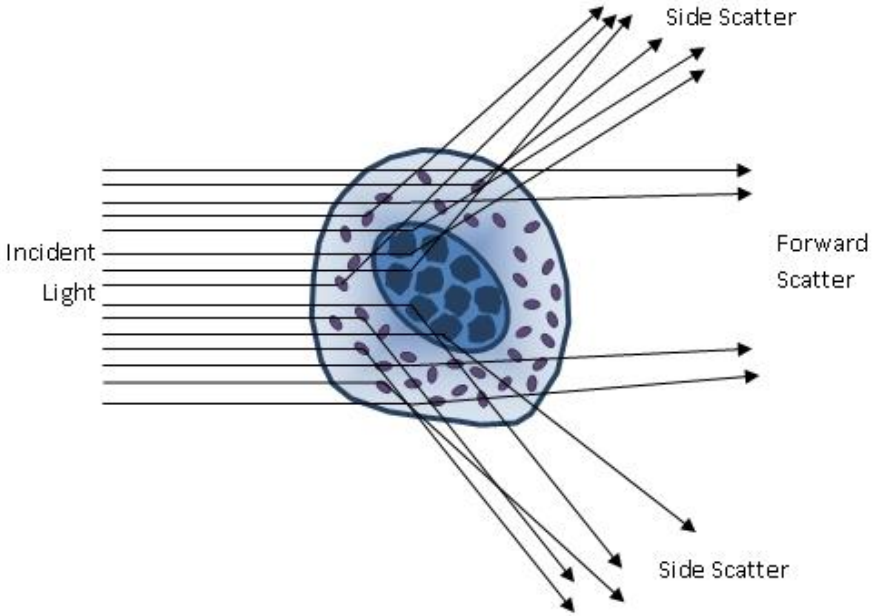
- Time-to-results (48-72h)
- ISO relative only to few species!
- Different Internal method for the same species!!
- Relatively narrow countable range (25-250 CFU/petri)
- Impossibility to recover viable but not-culturable (VBNC) cells, which are seen as dead
- Even when totally in compliance with ISO or Validated Internal Methods difficulties arisa
- Lack of standardized microbiological references!!!

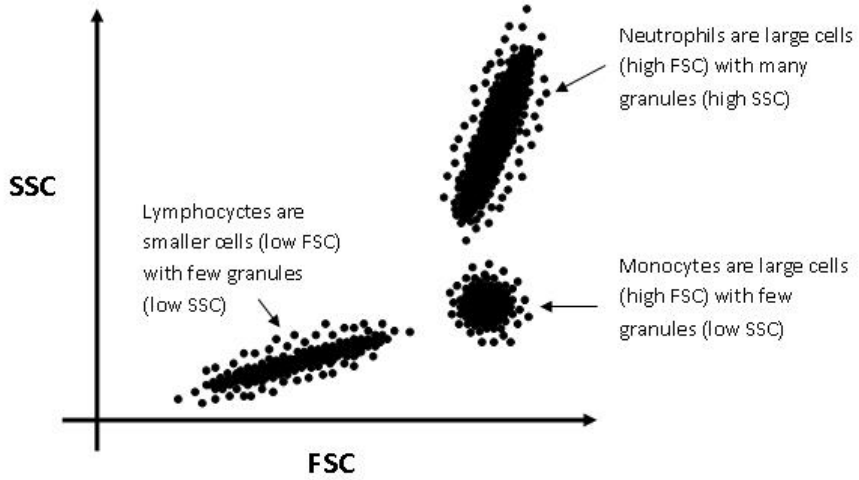
http://dx.doi.org/10.1016/j.mimet.2014.04.012

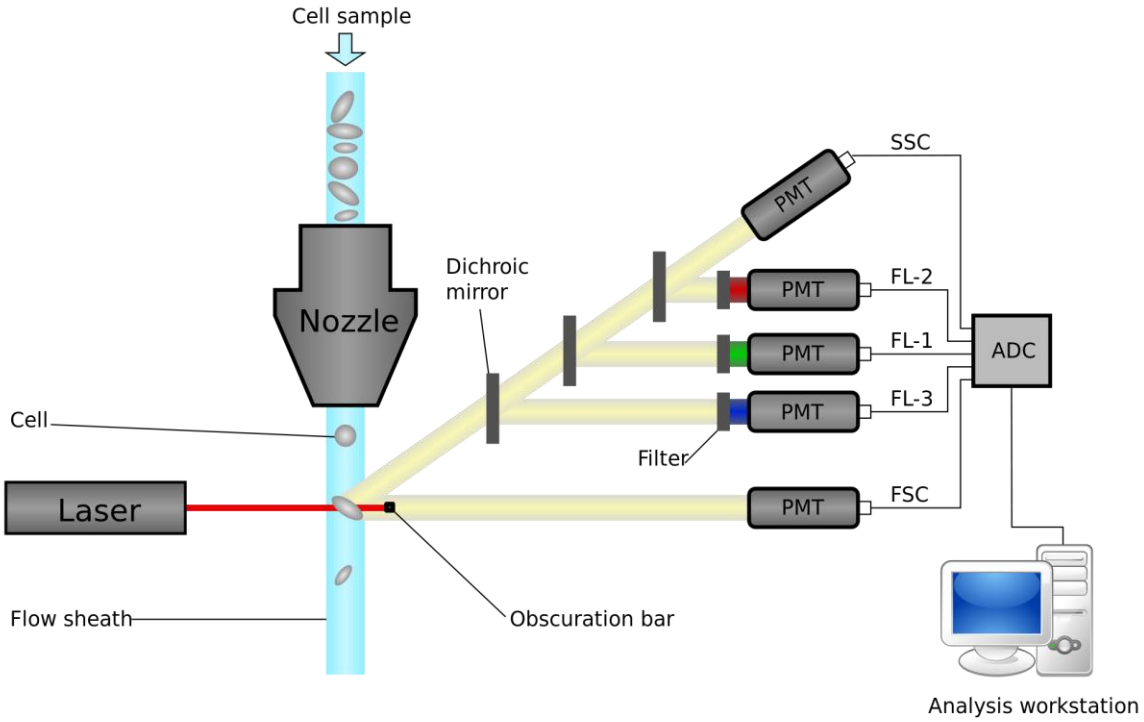
Flow cytometry

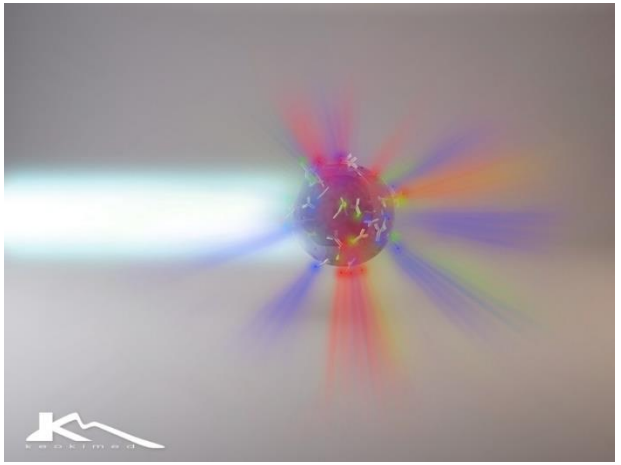
A ***fast***, ***accurate*** and ***universal*** tool for bacterial enumeration


Sheath fluid
Sample
(stained cells in suspension)
Nozzle
Hydrodynamic Focusing
Cells pass through
in 'single file'
Fluorescence
emitted from
stained cells
detected
Forward and side
scattered light from
all cells detected
Laser light source


Side Scatter
Incident
Light
Forward
Scatter
Side Scatter


SSC
FSC
Neutrophils are large cells
(high FSC) with many
granules (high SSC)
Lymphocytes are
smaller cells (low FSC)
with few granules
(low SSC)
Monocytes are large cells
(high FSC) with few
granules (low SSC)





Click on the picture for a youtube demo of FC

Forward and side scattered light and fluorescence from stained cells are split into defined wavelengths and channeled by a set of filters and mirrors within the flow cytometer. The fluorescent light is filtered so that each sensor will detect fluorescence only at a specified wavelength. These sensors are called photo - multiplying tubes (PMTs).

December 2015

ISO 19344 IDF 232

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

- **15** labs (5 Countries)
- **9** different FCM models
- **10** samples (2 different batches)
  - **8** pure starter cultures
  - **1** blend starter culture
  - **1** yoghurt sample
- **3** staining protocols
- **2** repetition per lab per strain per protocol
- **1800** tests

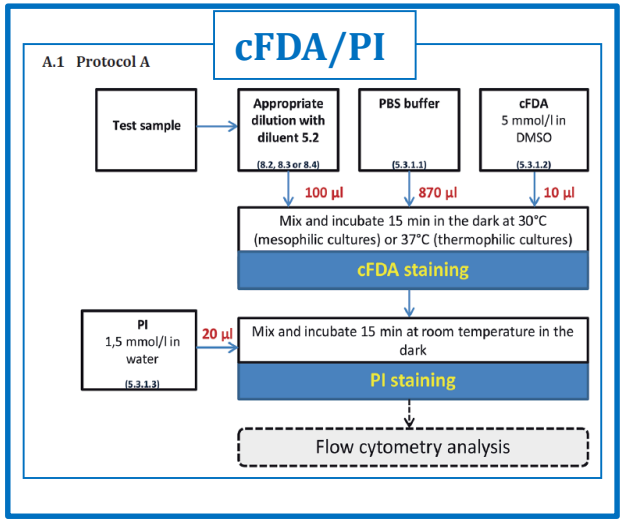
- **SAME method for ALL the lactic bacteria!**

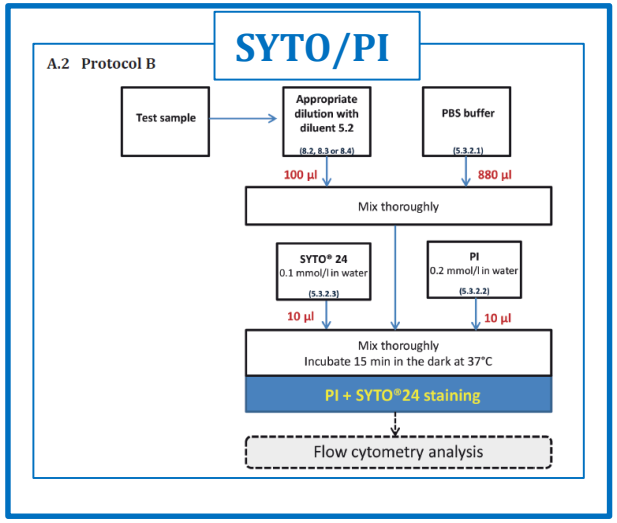
ISO 19344 IDF 232

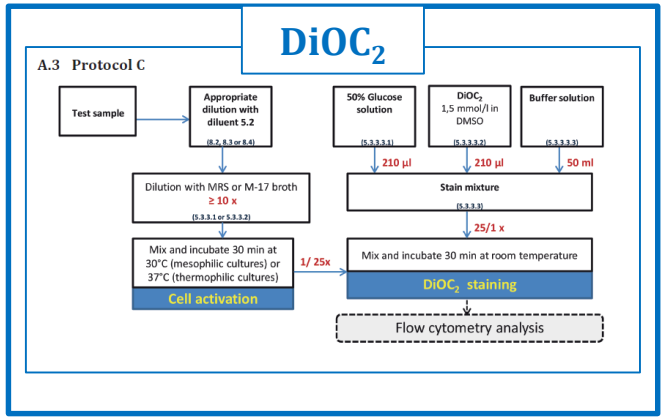
3 different protocols
for enumeration of

- **AFU (Active Fluorescent Unit) and**
- **TFU (total cells)**

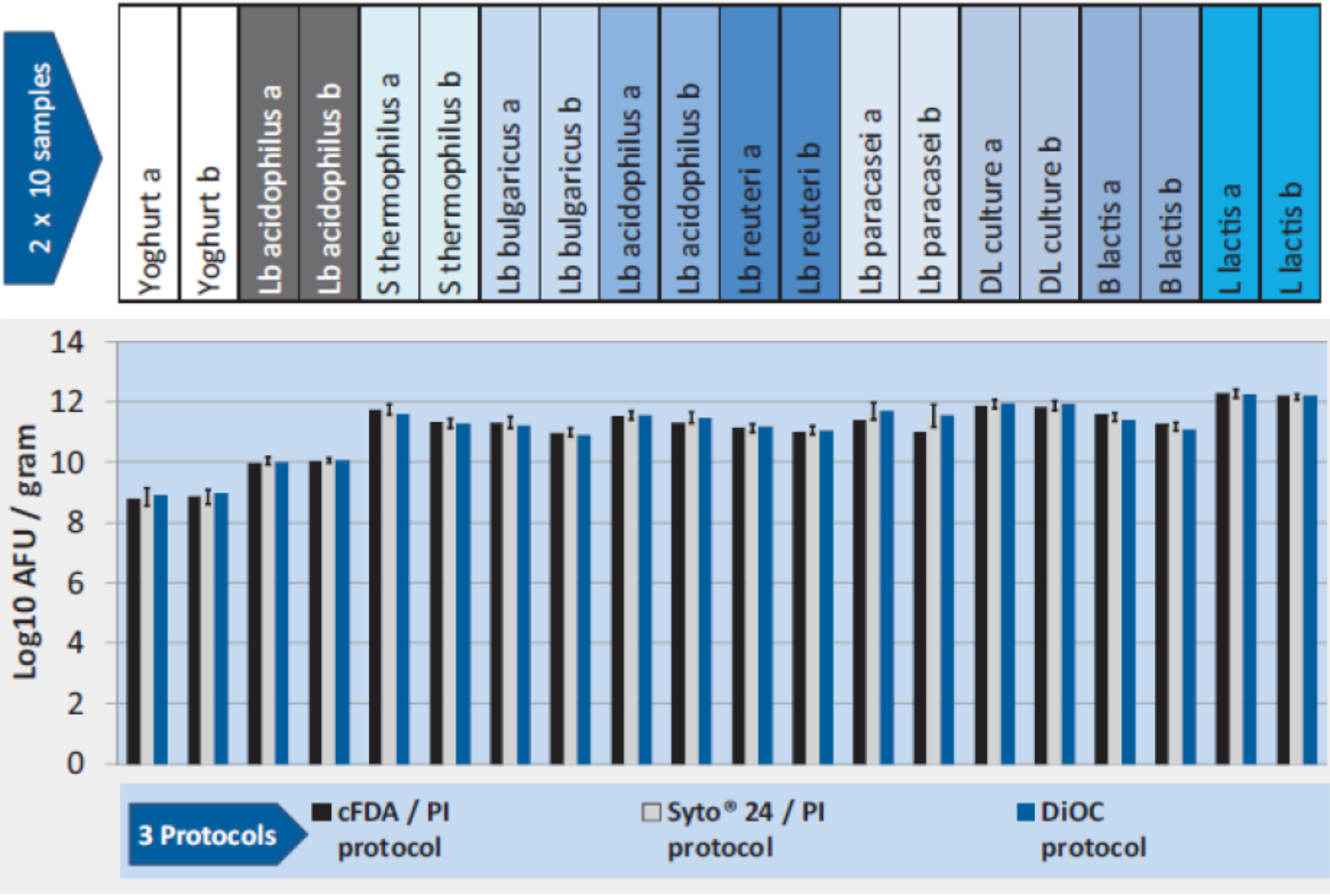
Enzymatic activity **Membrane Integrity** **Membrane potential**







Equivalency of the three staining protocol



ISO 19344 IDF 232
Repeatability (r) and Reproducibility (R)
Collectively defined (all protocols, 15 labs, 9 different FC equipment)

Parameters[a]	Product type	r*	R*
AFU/g	Frozen and freeze-dried lactic acid bacteria or probiotic strains	0,06	0,45
TFU/g	Frozen and freeze-dried lactic acid bacteria or probiotic strains	0,07	0,38

*All precision data are expressed as decadic logarithms of AFU or TFU per gram sample

MOFIN ALCE GROUP

Probiotical
Repeatability (r) and Reproducibility (R)
Collectively defined (all protocols, 2 labs, same FC equipment)

Parameters[a]	Product type	r*	R*
AFU/g	freeze-dried probiotic strains	0,05	0,07
TFU/g	freeze-dried probiotic strains	0,07	0,06

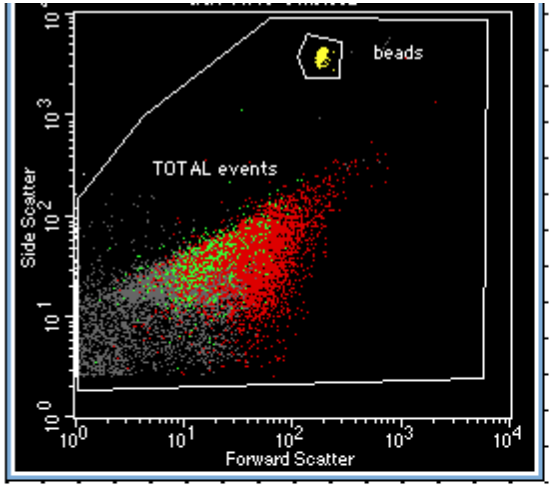
*All precision data are expressed as decadic logarithms of AFU or TFU per gram sample

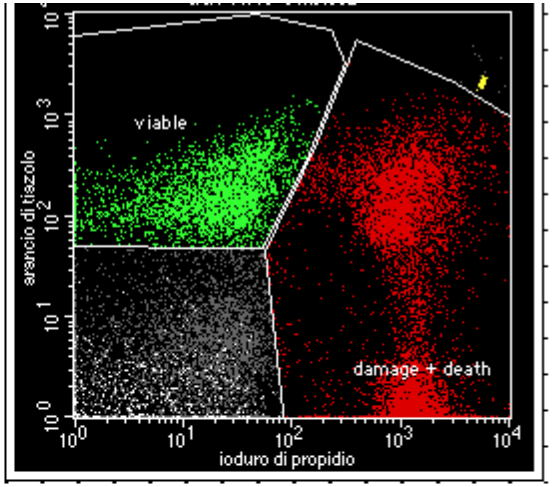
With proper training Reproducibility (R) is close to Repeatability (r)

Probiotic Enumeration Example (membrane integrity protocol)

Forward Scatter (FS) = dimension

Side Scatter (SS) = granularity and morphology





Thiazole orange (TO) or equivalent penetrates all bacteria and stain the **nucleic acid** with green fluorescence (y axis)

Propidium iodide (PI) penetrates only bacteria with damaged membranes with red fluorescence (x axis)

33% density to increase discrimination between clouds and draw better squares

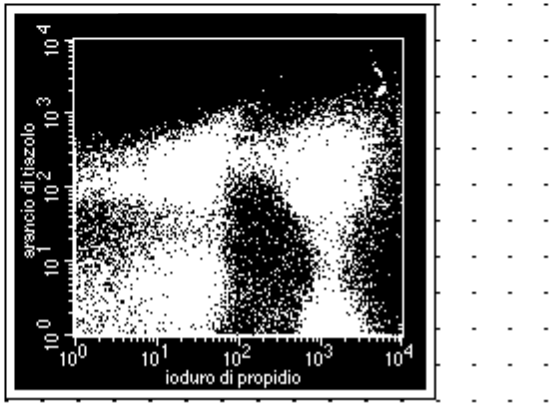
Cultivable + VBNC

viable 152.16 billion

damage + death 352.30 billion

Region Statistics

Region	Events	% Gated	% Total
beads	684	1.37	1.37
viable	11060	22.12	22.12
damage + death	25608	51.22	51.22
TOTAL events	47761	95.52	95.52



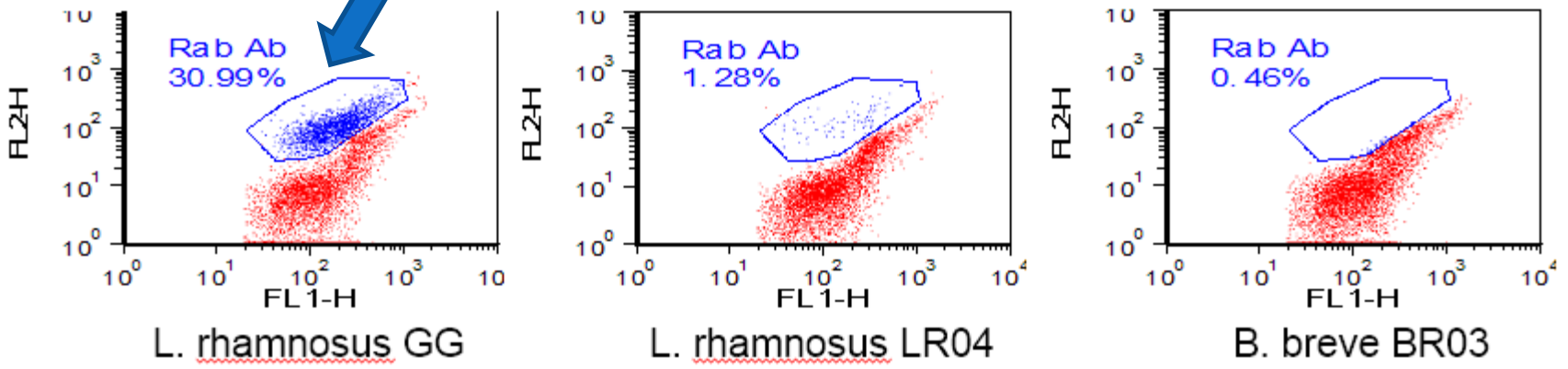
100% density (B&W to better discriminate clouds)

Flow Cytometry as a Superior Method for the Enumeration of Specific Microbial Product Formulations

Dana Buckman[1], Arthur A. Keegan[2], Marco Pane[3]
[1]BioForm Solutions, San Diego, CA; [2]Pharmachem Laboratories, Anaheim, CA; [3]Probioticals, Novara, Italy

Strain specific antibodies

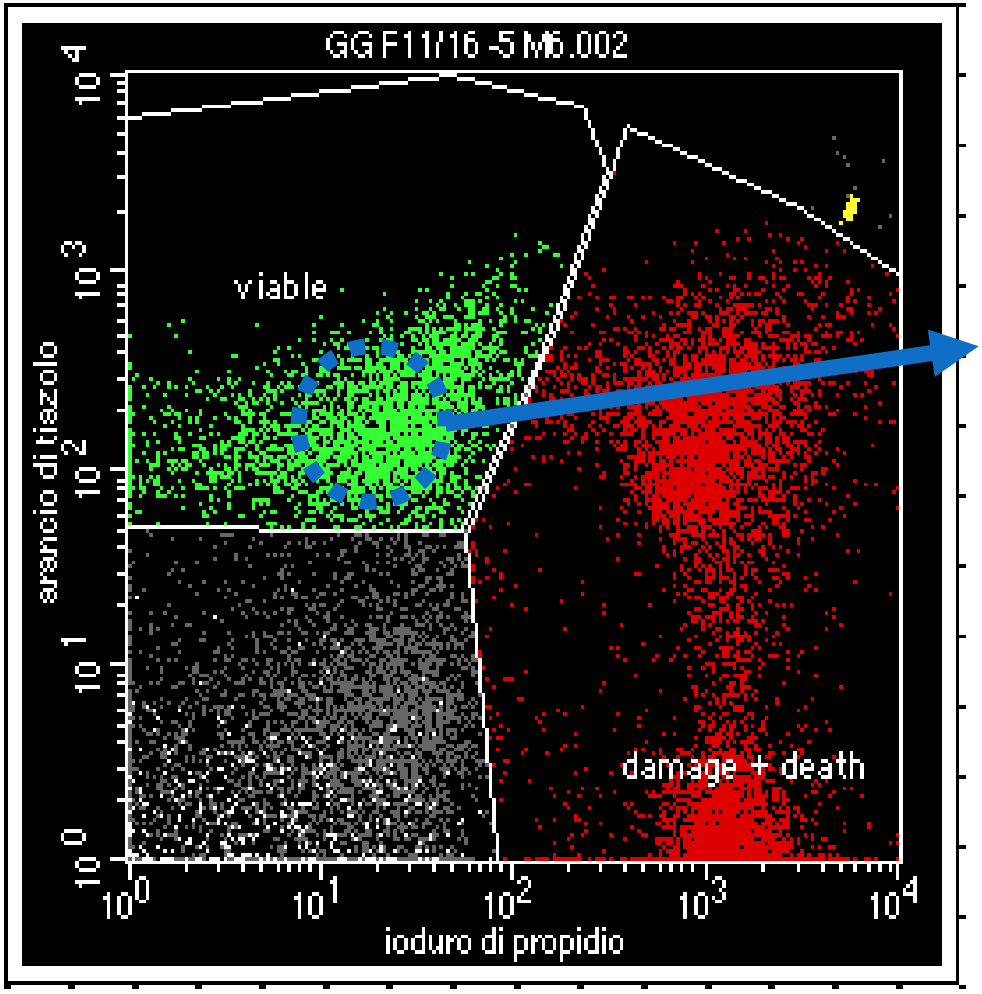
STRAIN SPECIFIC ANTIBODY



L. rhamnosus GG | L. rhamnosus LR04 | B. breve BR03

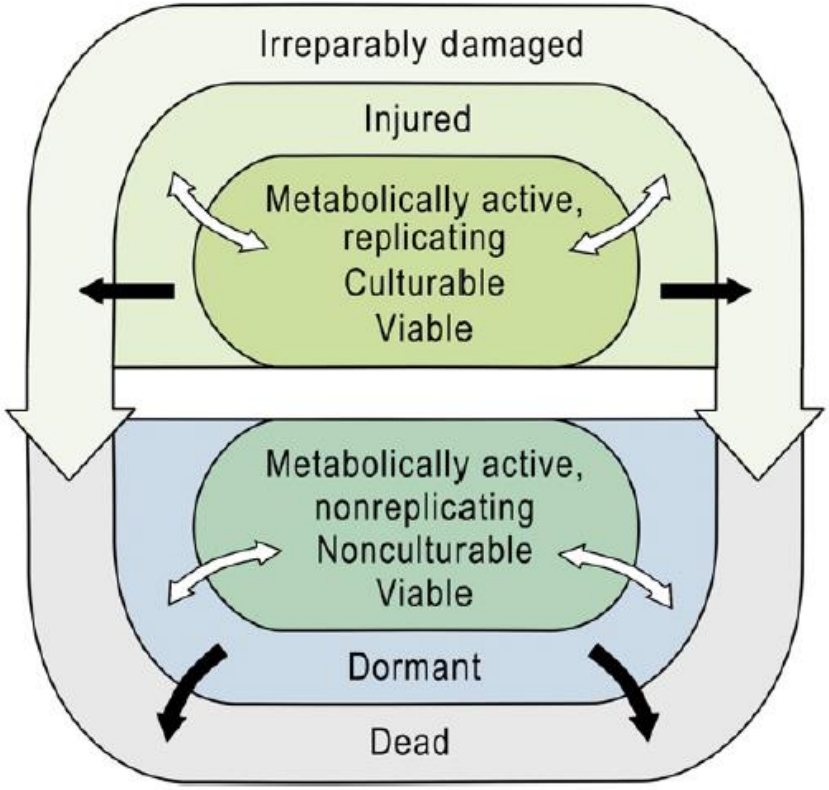
Purified rabbit serum was screened for binding to L rhamnosus GG, L rhamnosus LR04, and B. Breve BR03. A PE labeled secondary antibody was used to detect binding of the antibody to the bacteria. The polyclonal antibody generated against L Rhamnosus GG was specific for that strain and not a bacteria in the same species or a different species.

Viable but not Culturable Cells



Culturable cells (plate count) + Viable but Not Cultivable (VBNC) < Active Fluorescent Unit (AFU) < Total Fluorescent Unit (TFU)

Viable But Non-Cultivable (VBNC) cells



"Microbes exists in a variety of growth phases and metabolic states depending on environmental conditions and stressor, and only a subset of these involve active replication. The convention that viable microbes must be capable of forming colonies excludes not only dead or irreparably damaged organisms but also live microbes that have adapted to environmental stress by becoming dormant (VBNC state)"
from Davis, 2014

Fig. 1. A concept map for probiotic strains that describes metabolically active, replicating/culturable/viable states and the transitions that are possible. The arrow on the perimeter and the black one-way arrows indicate that once a cell is non-viable/dead it does not return to a viable state.

http://dx.doi.org/10.1016/j.mimet.2014.04.012

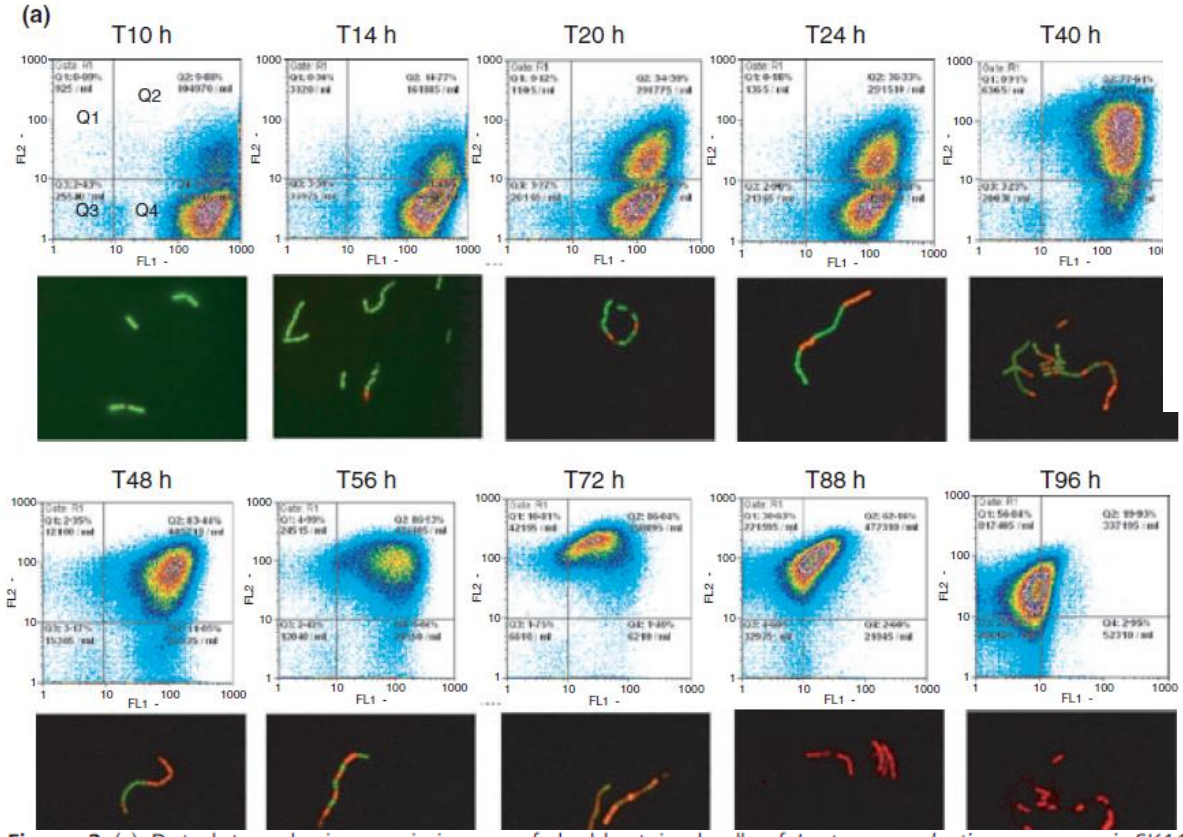
VBNC: Myth or reality ?

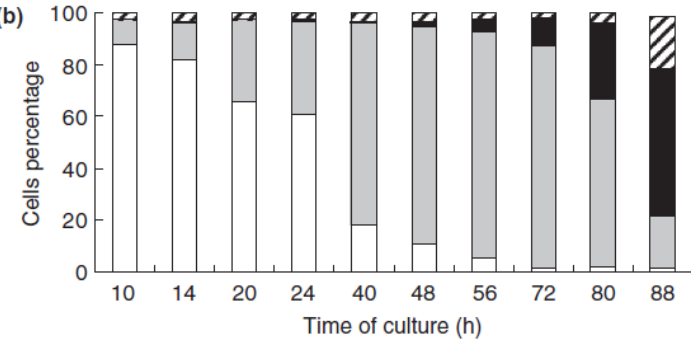
VBNC-forming food-borne bacteria
Campylobacter jejuni
Campylobacter coli
Enterococcus faecalis
Escherichia coli
Helicobacter pylori
Salmonella enterica serovar, Enteritidis
S. Typhimurium
Shigella dysenteriae type 1
Shigella flexneri
Vibrio cholerae
Vibrio parahaemolyticus
Yersinia enterocolitica

- *Shigella dysenteriae* in VBNC (environmental samples) produce biologically active Shiga toxin
- *Vibrio alginolyticus* e V. *parahaemolyticus* lose virulence in VBNC but after resuscitation in the mouse it is restored after two passage in the rat ileal loop
- *Aeromonas hydrophila* in VBNC lose its ability to lyse human erythrocytes but after temperature-induced resuscitation (5°C to 23°C) regain its virulence properties
- *Listeria monocytogenes* in VBNC due to distilled water exposure regain its virulence using embryonated eggs
- *RpF is a resuscitation promoting factor which is a bacterial cytokines found in both Gram+ and Gran- bacteria*

doi: 10.3389/fpubh.2014.00103

VBNC during production (fermentation)



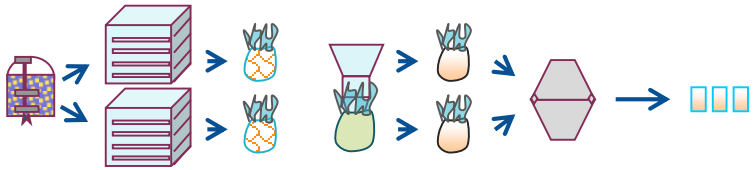


- White bar = cultivable
- Grey bar = VBNC
- Black bar = damaged/dead
- Stipe bar = cells fragments

Figure 2 (a) Dot plots and microscopic images of double-stained cells of *Lactococcus lactis* ssp. *cremoris* SK11 with carboxyfluorescein diacetate and propidium iodide dyes during growth. (b) Relative percentage of each population discriminated in dot plots. (□) Green cells; ▨ double-stained cells; (■) red cells and ▧ no stained cells.

doi:10.1111/j.1365-2672.2011.05114.x

VBNC during production (stability)



***Bifidobacterium longum* 46** ***Lactobacillus acidophilus* LA-5** ***Bifidobacterium lactis* Bb-12**

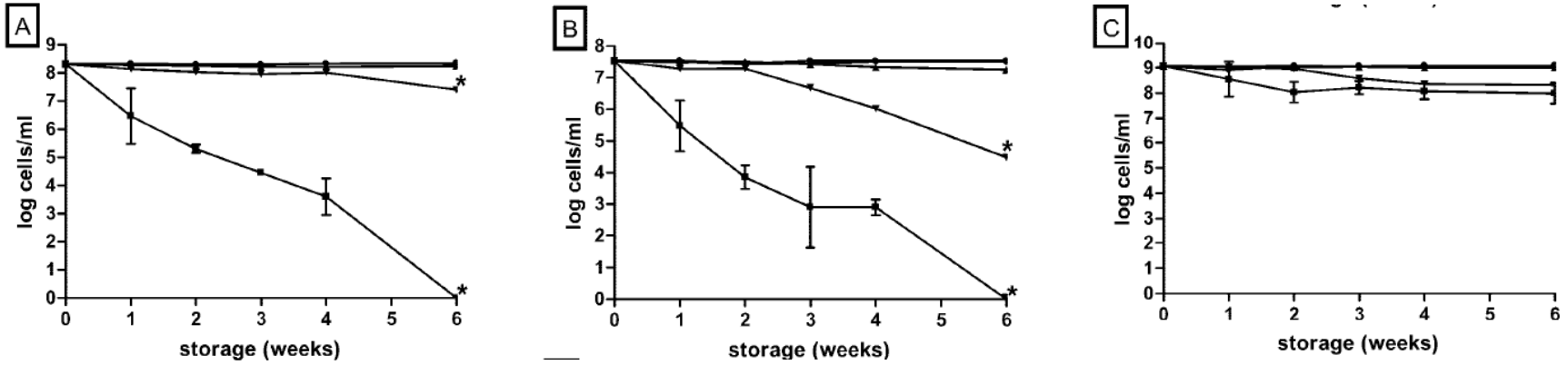
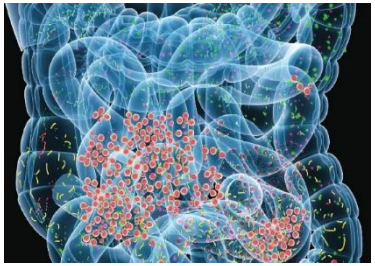
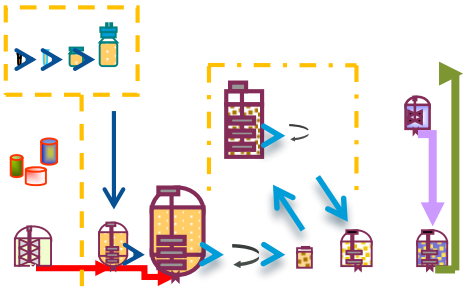


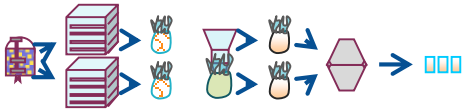
FIG. 1. Changes in the intrinsic properties of *Bifidobacterium longum* 46 (A), *Lactobacillus acidophilus* La-5 (B), and *B. lactis* Bb-12 (C) during storage in fermented milk. The cell counts are presented as averages for duplicate samples. *, statistically significant change ($P \leq 0.05$). cF, cFDA.

doi:10.1128/AEM.02897-05

Plate count
Membrane integrity
Esterase activity cF
Esterase activity cF-SE
pH gradient cF-SE







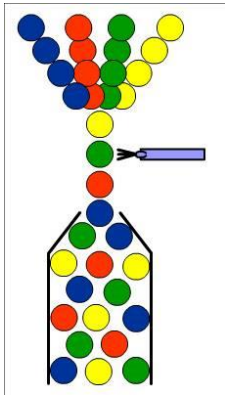
What a stressful life!

After an industrial process that is stressful for the probiotic cell we seal them in a finished product formulation that represent a new closed system that is far away from the natural condition where the microorganism belong, and we count them with a method that put the same microorganisms in an new closed system that is as distant as possible to their original native one.

But reality is different …

… even more for ANAEROBIC MICRORGANISMS!

Flow cytometry:



- Fast (30 minutes for a triplicate)
- Accurate
- Universal (same method for all the species)
- With standards (beads or absolute standard)
- Can be further developed (e. strain specific antibodies)
- It give a complete «picture» of the bacterial population (TFU and AFU) based on enzymatic activity, membrane integrity and membrane polarization
- Not related to the «cultivability of the strain» thus potentially suitable for strictly anaerobic micro-organisms
- **HOWEVER No easy correlation with Plate Count (CFU) during stability**

Contents lists available at ScienceDirect

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journal homepage: www.elsevier.com/locate/tifs

Review

Flow cytometry as a potential method of measuring bacterial viability in probiotic products: A review

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To count or not to count ?

Pro and cons of Probiotic Enumeration Methods

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6th Microbiome R&D and Business Collaboration Forum: USA