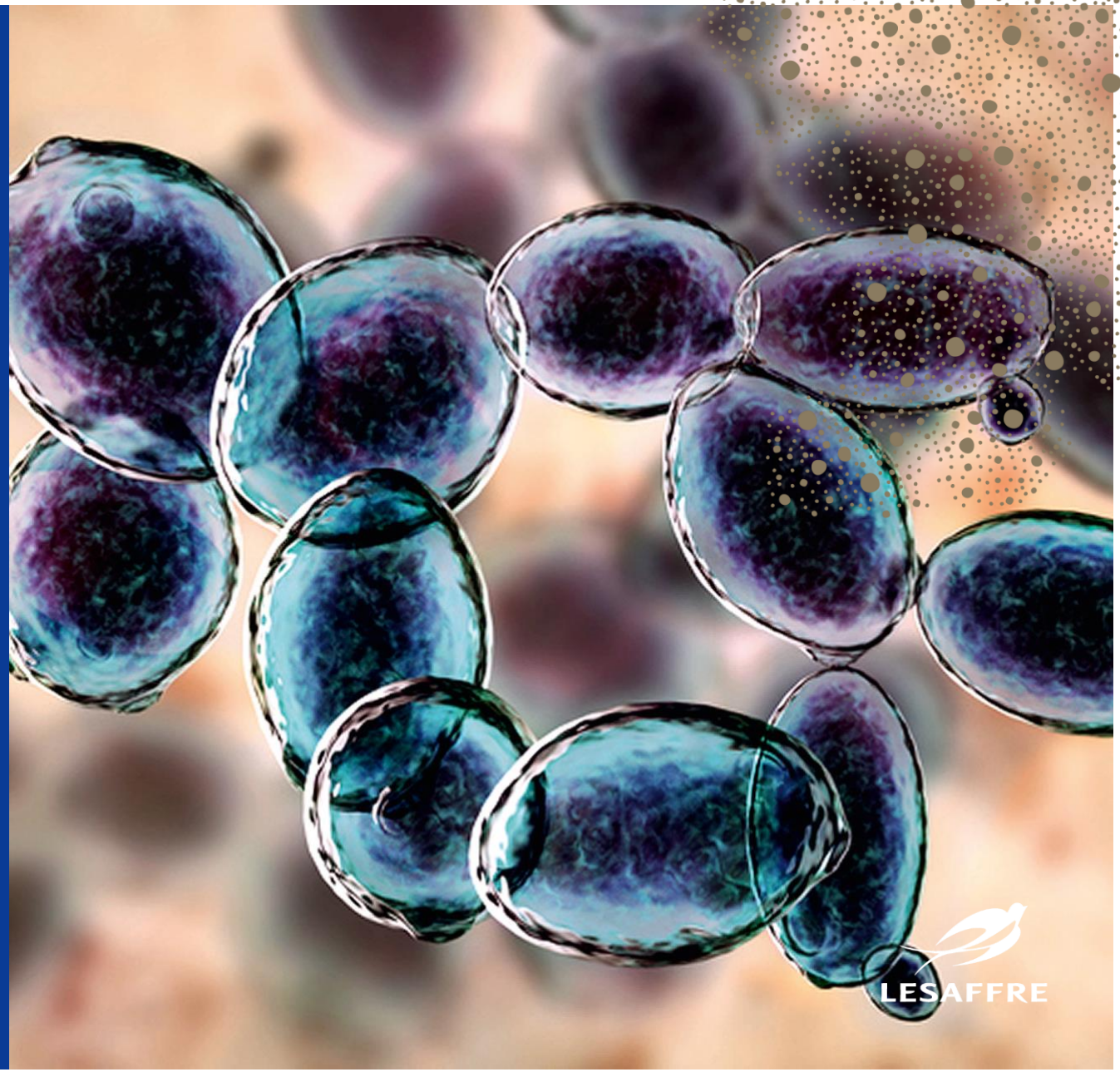
Advanced Molecular Tools for the Analysis of Beneficial Microbes in Foods

MICKAËL BOYER

06/10/2022



FOOD TESTING THROUGH ANALYTICAL TOOLS, WHY?

Food testing is the key point to reach this

Need of analytical tools and methods with the highest robustness

FOOD TESTING, BAD AND GOOD MICROBES

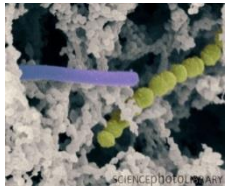
Pathogens, Spoilage Microorganisms

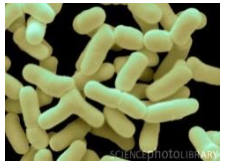
Bacterial contamination, yeast & mould…..

Live Ingredients :

Lactic Acid Bacteria

Probiotics….



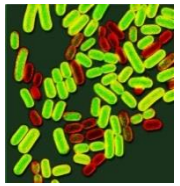


From cell or product we need to detect and/or quantify microorganisms (good & bad one).
Analytical technics must be :

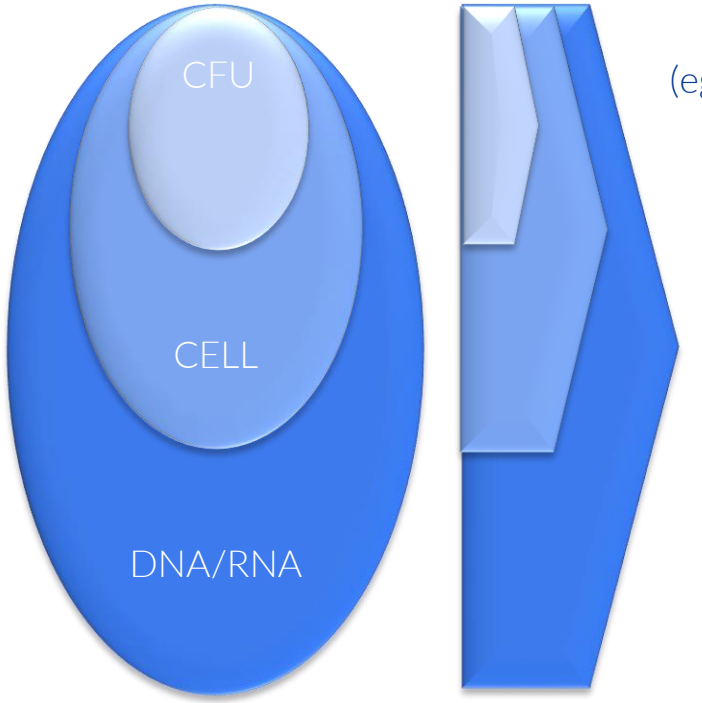
In any case:
Sensitivity : 100%
Specificity :100%
Robust
Validated

Next generation of tools should be :
Flexible
“All in One”
Ease-of-use
In real time, on-line
Cheaper as possible

DETECTION & COUNTS : 3 ANALYTICAL LEVELS



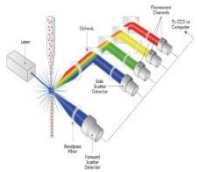
Food sample



CULTIVABLE
(eg selective medium)

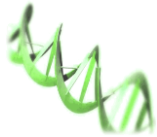
Petri Dishes 24h to 5 days
(eg selective medium)

VIABLE
(eg Flow cytometry)



Flow cytometry
1 or 2h w/o preincubation

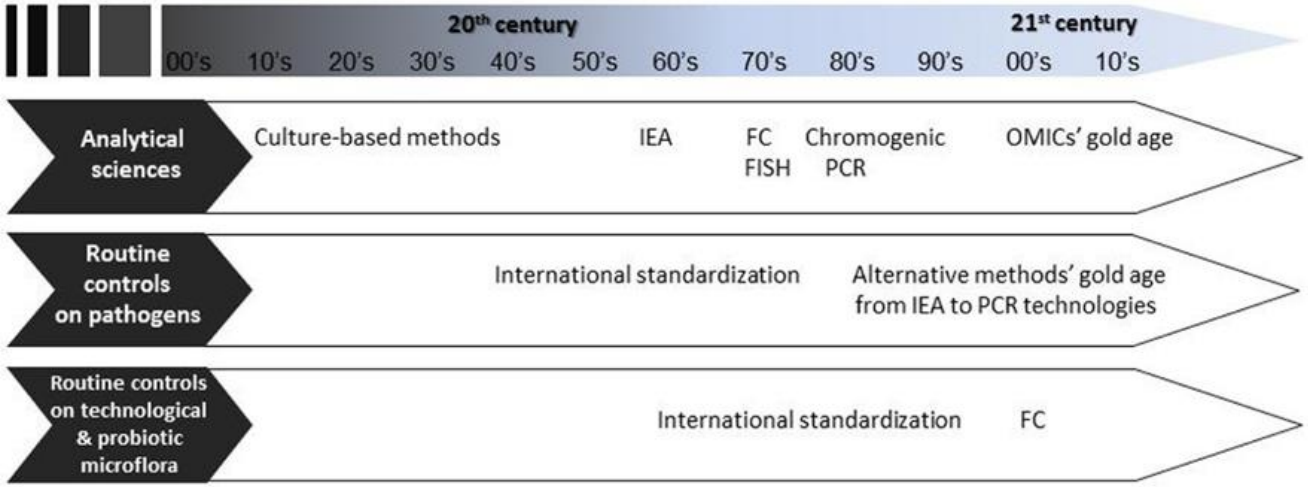
TOTAL
(eg qPCR)



DNA :
- Pre enrichment : 16h to 21h
- DNA extraction : 1h to 2h
- qPCR : 2h

DEFINITION OF VIABILITY IS CONTROVERSAL, THE RELEVANT BIOMARKER OF VIABILITY WITH THE MOST RELEVANT TECHNOLOGY SHOULD BE SELECTED

EVOLUTION OF MICROBIOLOGICAL ANALYTICAL METHODS



A broaden choice

Sohier et al., 2014, Frontiers in Microbiology 5:16

How to decide which method is the best?

ANALYTICAL MANAGEMENT SYSTEM

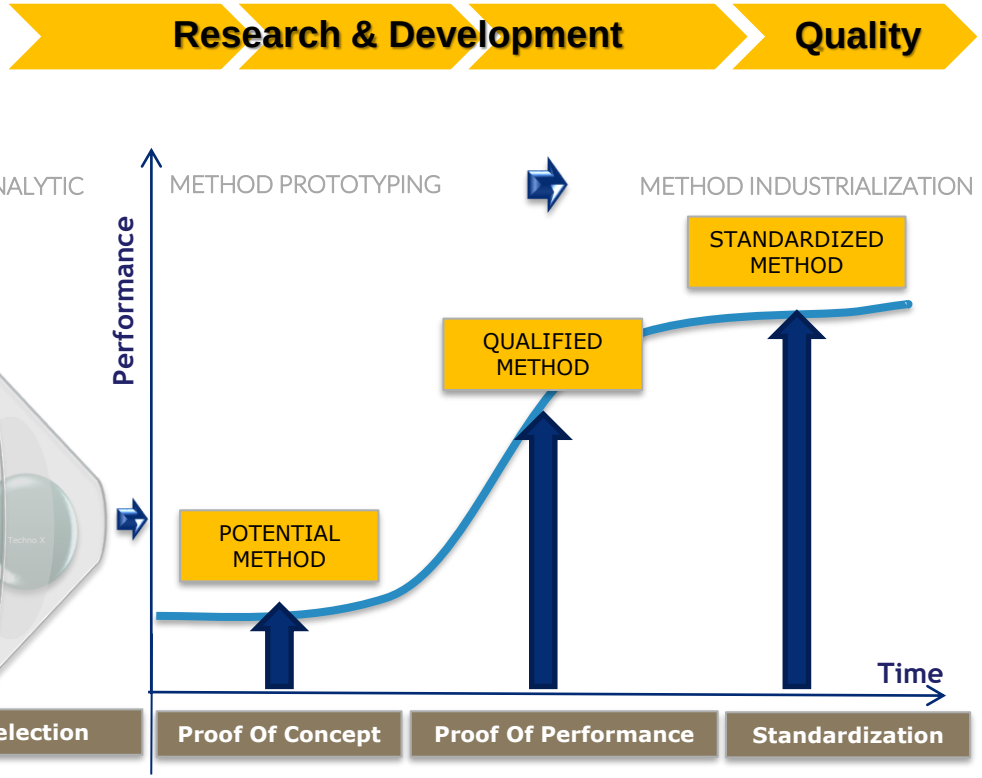
Food Microbio Platform

Flow Cyto-metry Platform

PCR & qPCR Platform

Digital PCR Platform

Sequencing Platform



Geng et Boyer, 2014, New Food 17:59-61

MAIN STAKES OF BENEFICIAL MICROBES/PROBIOTICS COUNT IN FOOD

➢ **More and more food products associated with several fermenting microorganisms/probiotics**

➢ Need to characterize a mix of species composing a beneficial microflora in the final product (e.g. kefir, cheese, kombucha, dietary supplement ,…).

➢ **Definitions of some fermented foods (e.g. yoghurt, kefir,…) and probiotic categories, (CODEX and WHO/FAO-ISAPP, respectively) state that microorganisms have to be alive and in a sufficient number in final products.**

➢ Need to quantify beneficial microbes alive and in high number in the product

➢ **Expected positive effects of probiotics on health could be related to the applied dose and to the ability to remain viable in the gut**

➢ Need to evaluate survival of probiotics in complex gut content like faeces

WHAT IS NEEDED?

- HIGH DISCRIMINATORY POWER TO QUANTIFY VIABLE CELLS OF FERMENTING MICROBES/PROBIOTICS IN COMPLEX FOOD PRODUCTS,
- INFORMATION RELATED TO THEIR ABILITY TO SURVIVE ALONG THE SHELF LIFE AND ALSO THROUGH THE INTESTINAL TRANSIT.

PROS/CONS OF CULTURE/MOLECULAR BASED METHODS IN BENEFICIAL MICROBES ENUMERATION?

- **Most of Reference methods (ISO, …) are based on culture approach**

- **Molecular methods (PCR, Flow cytometry) offer new opportunities**
  - to reduce time to result,
  - quantify all viable population (including VBNC),
  - higher discriminatory power, particularly with the boom of omics
  - automation.

Table 5
Parameters for consideration in selection of approach to enumeration probiotic species.

	Method	Material cost	Time to execute	Time to availability of results	Specificity	Automation	Challenges (examples)
Culture based	Culture	Inexpensive	++	+++	++	No	Identifies replicating cells only if placed on appropriate synthetic media; Fermentation patterns may be similar between strains; Tedious to prepare some media; Some media incorporate antibiotics
Molecular based	EMA/PMA-PCR (v-PCR)	+	++	++	+++	Yes	Toxic materials; Sensitive to small variations in sample preparation
	RT-PCR	+	+++	++	++	No	
	Fluorescent microscopy	+	+	<2 h	++	No	Optimization of permeabilization of cell wall methods for penetration fluorescent probe
	MALDI-TOF mass spectrometry	+	+	++	+	No	Variability in reproducibility reported
	Flow cytometry/FACS	+++	++	++	+++	Yes	LOD 1×10^4 cells/mL, however, most probiotic preparations contain $\geq 1 \times 10^6$ cells per preparation

Davis, 2014, J Micro Meth 103:9-17

- **Drawbacks of qPCR counts: not be directly associated with cell viability**

VIABLE BENEFICIAL BACTERIA QUANTIFICATION WITH PCR BASED METHODS?

✓**Technology based on DNA-intercalating dye: Ethidium Monoazide – EMA; Propidium Monozazide – PMA**

EMA/PMA-(q)PCR = Viability-PCR (V-PCR)

Viability criteria = cell wall integrity

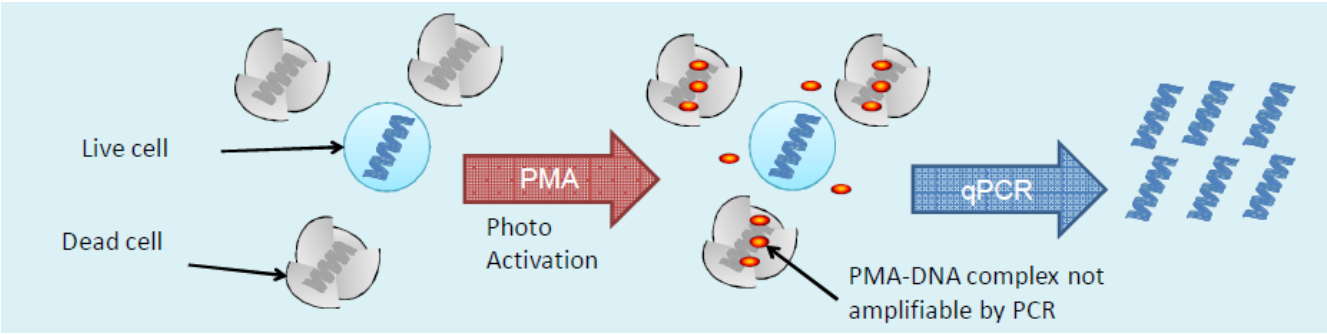


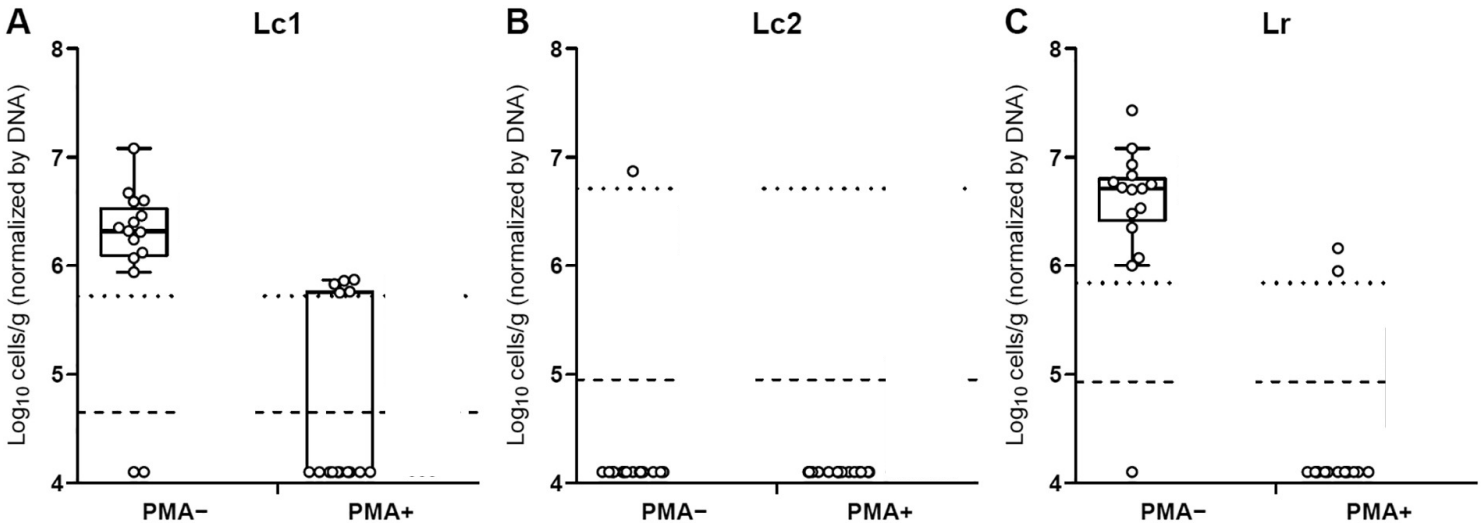
Figure 1: Schematic representation of qPCR-PMA principle. PMA penetrates into dead cells and intercalates with DNA after photo-activation. Thus only the DNA from live cells can be amplified and quantified in qPCR assay.

V-PCR– WHAT'S DONE?

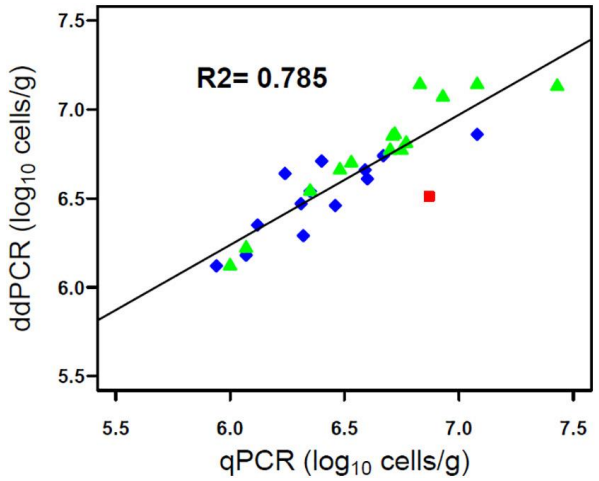
Beneficial microbes/probiotics	Matrix	References
Lb. acidophilus LA-5 *B. animalis ssp. lactis* BB-12 *L. acidophilus* La-14 *B. animalis subsp. lactis* Bi-07 *L. rhamnosus GG*	Lyophilised product	Kramer et al., 2009 Kiefer et al., 2020 Shehata and Newmaster; 2021
L. gasseri K7	Calcium alginate beads	Oketič et al., 2015
S. thermophilus, *Lb. delbrueckii subsp. bulgaricus* *Lb. casei subsp. casei* *Lb. acidophilus* *B. lactis*	Fermented milk products	Garcıa-Cayuela et al., 2009 Meng et al., 2010
Lactococcus sp. *B. animalis subsp. lactis* BB-12, *Lb rhamnosus* RO011, *Lb helveticus* RO052 *L. acidophilus, L. casei, L. paracasei and B. animalis subsp. lactis*	Cheddar cheese	Desfosses-Foucault et al., 2012 Ganesan et al., 2014
L. acidophilus La-5 *B. animalis* Bb-12	In vitro gastrointestinal resistance assay	Matias et al., 2016
B. bifidum *B. breve strain* Yakult *B. animalis subsp. lactis* Bb-12 *B. bifidum* BF-1 *L. paracasei* *L. rhamnosus*	Rat faeces Human faeces Piglet faeces	Lv et al., 2015 Fujimoto et al., 2010 Palaria et al., 2012 Fujimoto et al., 2013 Gobert et al., 2018

Blue = v-ddPCR

DROPLET DIGITAL PCR ASSOCIATED TO PMA TO IMPROVE QUANTIFICATION THRESHOLD OF PROBIOTIC SURVIVAL IN FECAL SAMPLES



.... qPCR LLOQ
--- ddPCR LLOQ



PMA-PCR APPROACH BASED ON DD-PCR LOWER THRESHOLD OF VIABLE CELL QUANTIFICATION FROM 5.7 TO 4.4 LOG CELLS/G

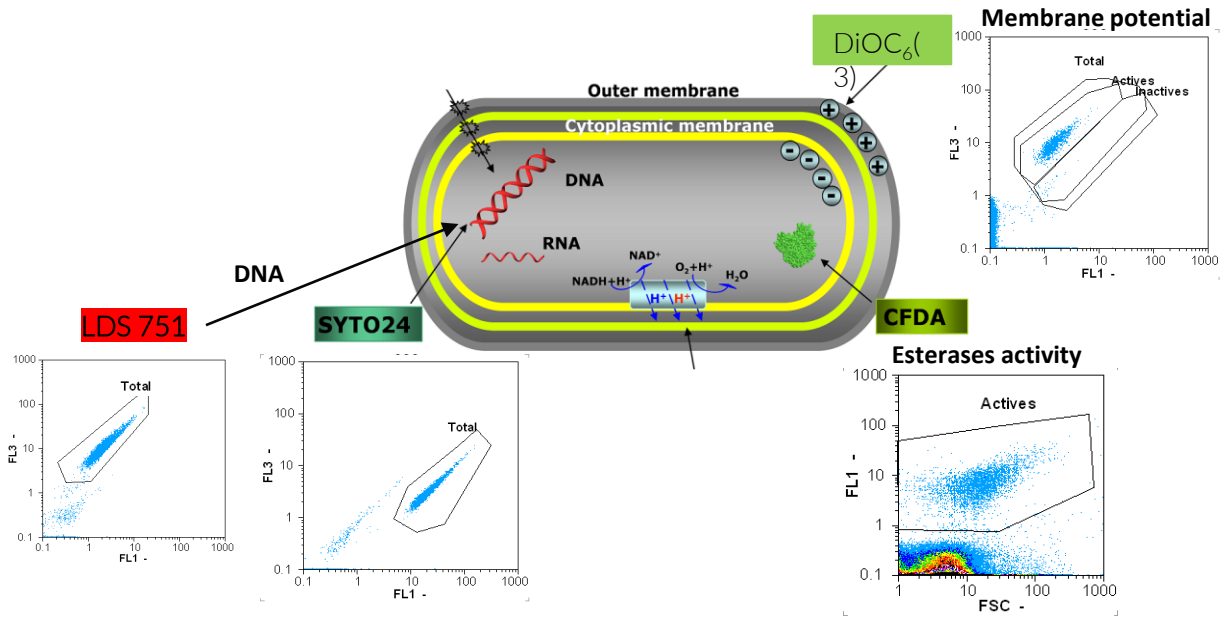
=> IMPROVEMENT OF 1.3 LOG !

(Gobert *et al.*, 2018)

FLOW CYTOMETRY AND PROBIOTIC ENUMERATION

A rapid, accurate and universal tool for microorganism detection, enumeration and characterization.

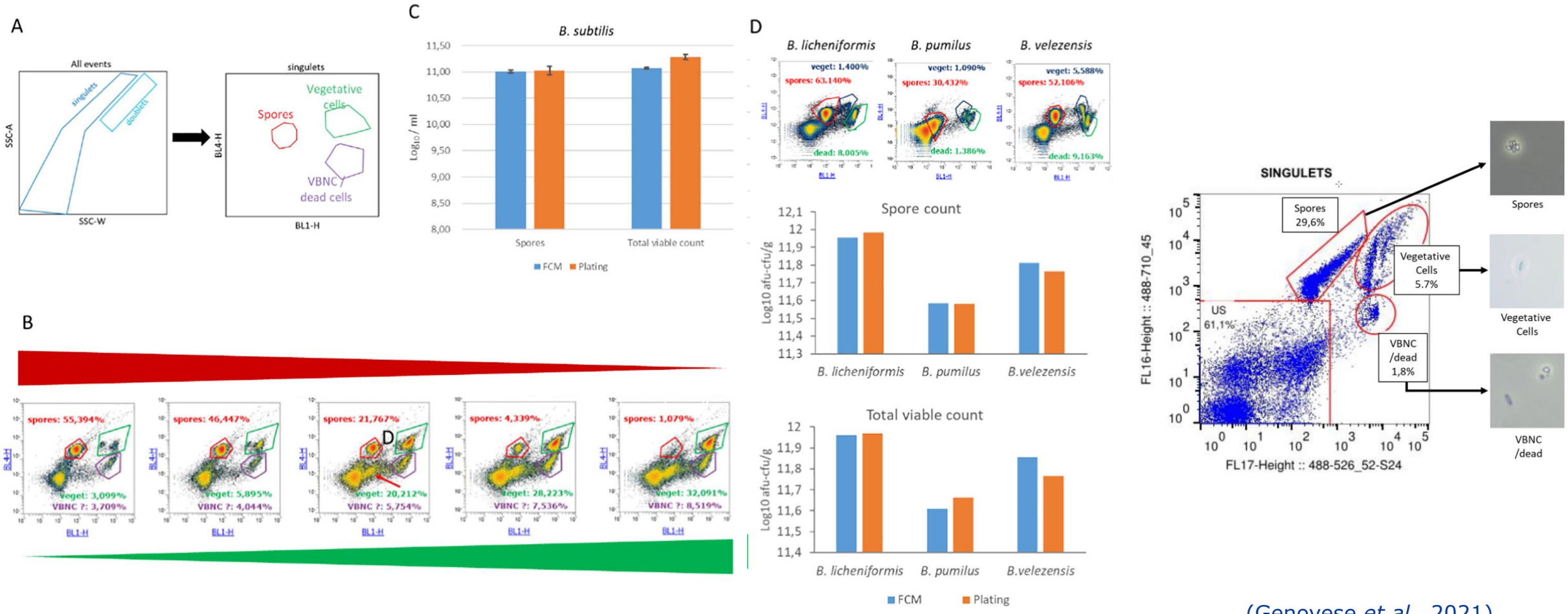
- FCM is a way to enumerate in real time microorganism in samples
- FCM is a way to discriminate viable (including VBNC) and dead cells



Beneficial microbes/probiotics	Matrix	References
Lactobacillus species *Bifidobacterium species* *Bacillus subtilis CU1* *B. coagulans MTCC 5856*	Food supplement	Michelutti et al., 2020 Genovese et al., 2021 Majeed et al., 2018
L. rhamnosus *B. bifidum R0071,* *B. longum ssp. infantis R0033,* *B. longum ssp. longum R0175,* *L. helveticus R0052* *L. rhamnosus R0011* *L. rhamnosus GG*	Freeze-dried product	Foglia et al., 2020 Chiron et al., 2018 Pane et al., 2018
Pediococcus acidilactici, *P. pentosaceus,* *L. plantarum* *B. subtilis*	Animal feed	Gorsuch et al., 2019
L. rhamnosus R0011	Chocolate matrix	Raymond and Champagne, 2015
L. plantarum WCFS 1 *B. animalis ssp. lactis*	Milk, Dairy starters,	Bunthof and Abee, 2002 Geng et al., 2014

PROBIOTIC SPORES ENUMERATION BY FLOW CYTOMETRY

Double staining LDS751 + Syto24 was able to differentiate three subpopulations: spores, vegetative cells and VBNC or dead cells.



(Genovese *et al.*, 2021)

VIABILITY OF YEAST IN BREADMAKING PROCESS

We report a fast and robust flow cytometry analysis using double staining (LDS751/DiBAC4) to analyze yeast viability in bread dough during baking.

Contents lists available at ScienceDirect

Journal of Microbiological Methods

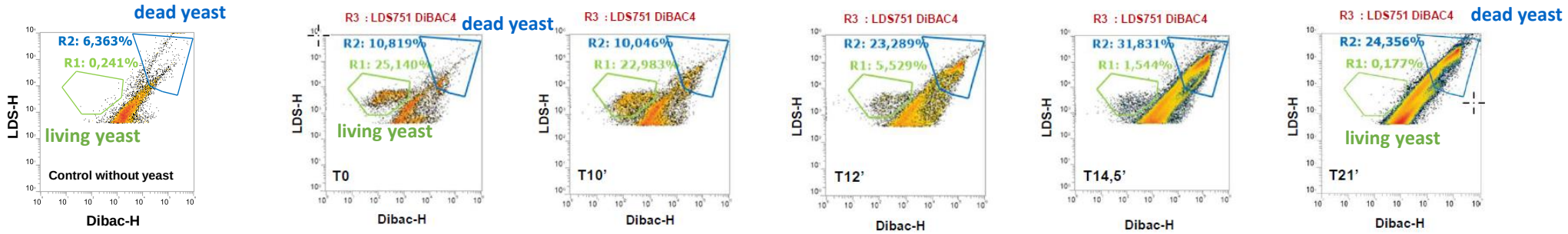
journal homepage: www.elsevier.com/locate/jmicmeth

Viability of *Saccharomyces Cerevisiae* during baking of bread dough by flow cytometry

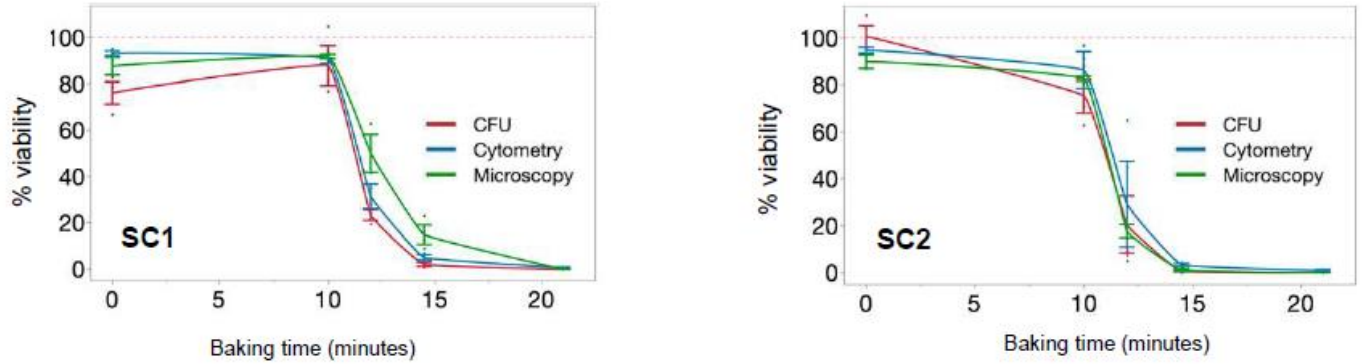
Florie Doppler [a], Laurie Jelonkiewicz [a], Mohammad N. Rezaei [b], Corinne Lesens [b], Renaud Toussaint [a,1], Mickael Durand-Dubief [c,*,1]

[a] Life Science, Lesaffre Institute of Science & Technology, Lesaffre, 59700 Marcq-en-Baroeul, France
[b] Baking Science, Lesaffre Institute of Science & Technology, Lesaffre, 59700 Marcq-en-Baroeul, France
[c] Discovery Lead Nutrition & Health, Lesaffre Institute of Science & Technology, Lesaffre, 59700 Marcq-en-Baroeul, France

Setting up the detection viable yeast population in dough during the baking process using flow cytometry

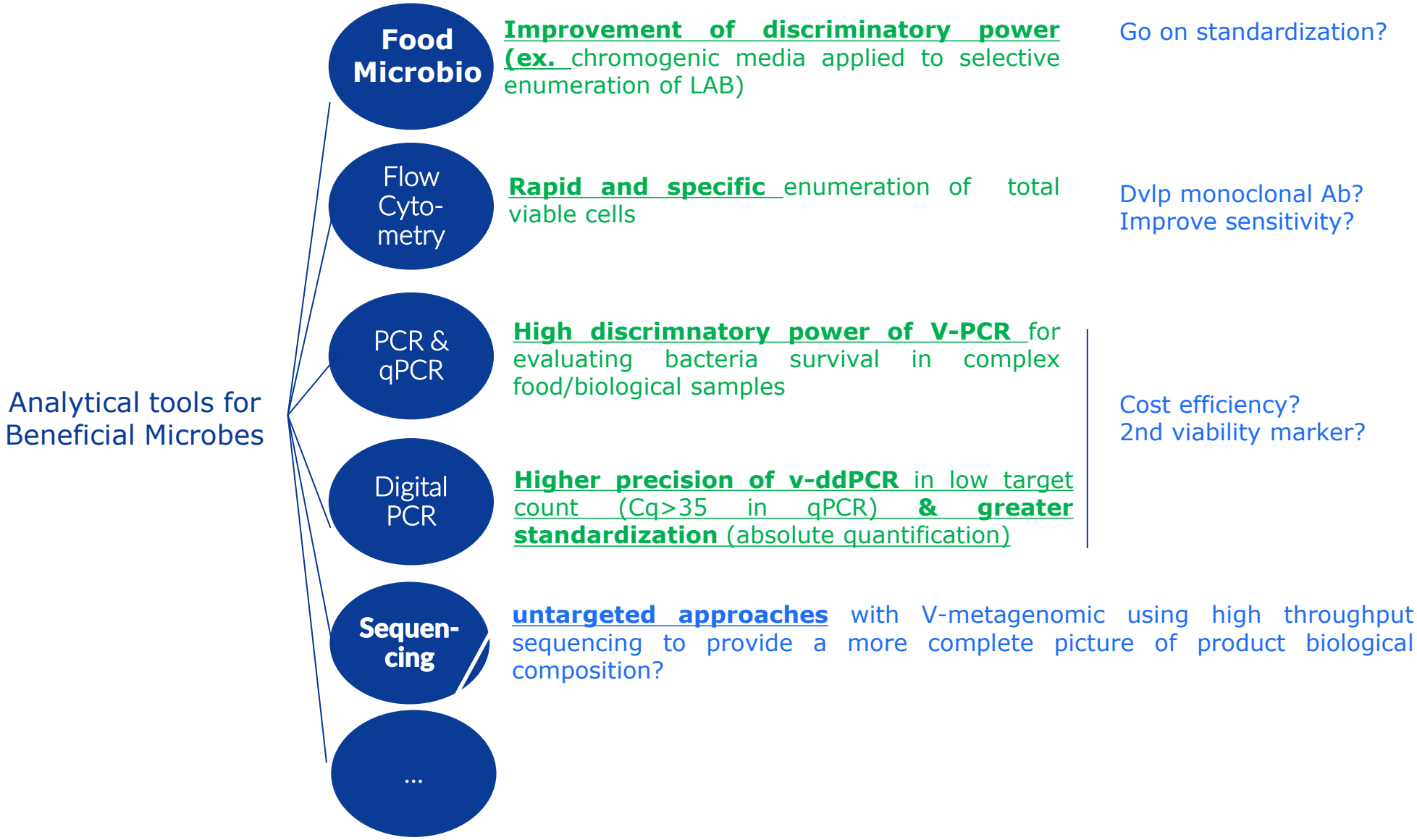


Comparison of different technique of cell viability (%) for two yeast strains in dough at different baking time



(Doppler *et al.*, 2022)

CONCLUSION AND PERSPECTIVES



Thanks for your attention