Applicazioni della citometria a flusso nella microbiologia dei processi alimentari

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Workshop

Fast and fluo: high processing flow cytometry techniques for green biotech, the environment and the food chain

Tanti, piccoli e importanti: analizzare microrganismi e particelle con la citometria a flusso

Salone dei Convegni
ENEA Sede Lungotevere
Thaon di Revel, 76 -
Roma

15 APRILE 2019

9.00 - 13.30

Si prega di comunicare la partecipazione via
e-mail a:
sergio.lucretti@enea.it

Abstract

Diego Mora

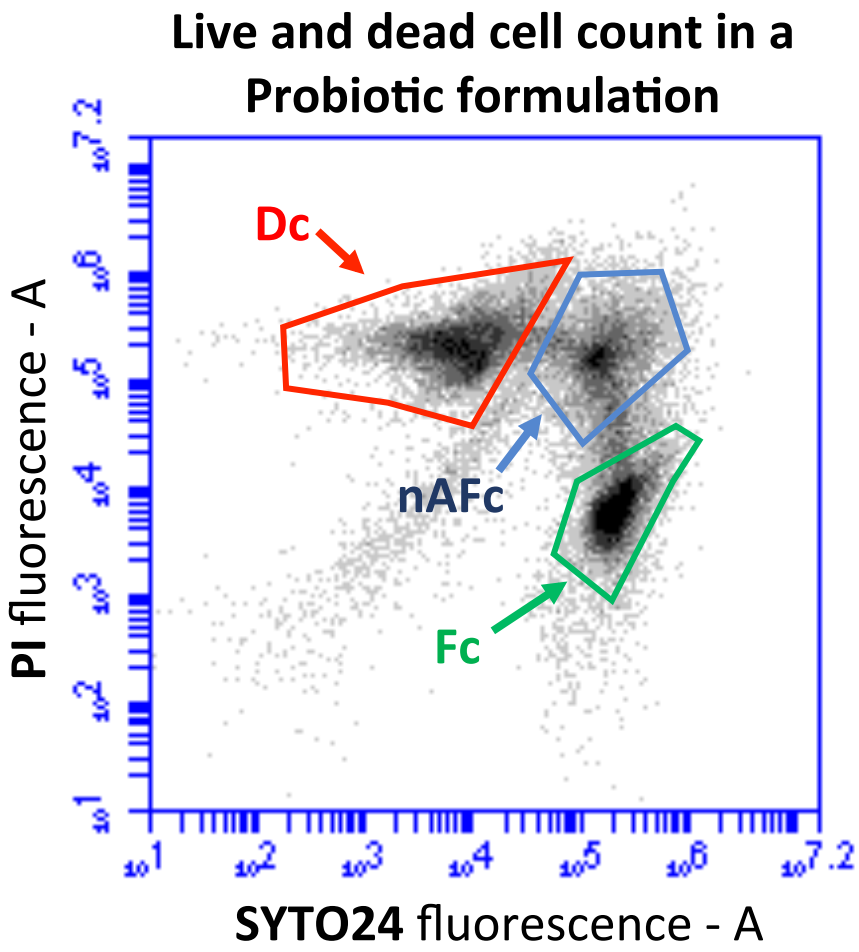
L'impiego della citometria a flusso nella microbiologia alimentare non si limita alla sola quantificazione delle cellule microbiche nonostante la corretta valutazione di questo parametro sia tutt'altro che scontata in ambito microbico dove le morfologie cellulari e le strutture di aggregazione possono essere molto complesse. Queste complessità si sommano alla difficoltà di stabilire la vitalità delle popolazioni microbiche in cui la componente vitale-ma-non-coltivabile può avere importanti risvolti sia in termini di qualità che di sicurezza nel caso siano coinvolti microorganismi patogeni. La citometria a flusso viene impiegata anche per valutare diversi parametri cellulari utili per descrivere aspetti della fisiologia microbica, quali il potenziale di membrana, l'integrità della membrana cellulare, il pH intracellulare e l'attività dei sistemi di efflusso coinvolti in meccanismi di resistenza a molecole ad attività antimicrobica, la capacità di aggregazione e interazione tra cellulare anche tra specie microbiche diverse. La presentazione sarà focalizzata sulle applicazioni in ambito microbiologico alimentare e probiotico delle applicazioni di citometria a flusso sopra elencate.

Flow cytometry applications in Food Microbiology

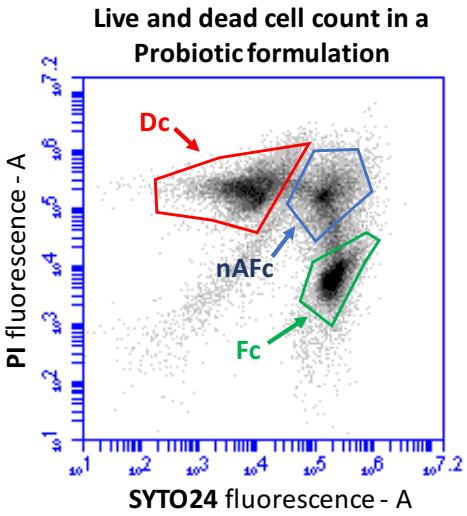
- Cell counting
- Cell physiology
- Cell-cell metabolic interactions
- Cell-sensitivity to toxic compounds
- Quality control on probiotic multi-strain formulation
- New protocols for strains isolation

- **Cell counting**

ISO 19344 IDF 232. 2015. Milk and milk products. Starter cultures, probiotics and fermented products; Quantification of lactic acid bacteria by flow cytometry;



Multi-paramtetric fast quantitative analysis, **no taxonomic information can be obtained by FCM**



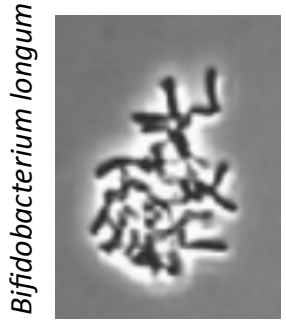
Criticisms …

not yet comparable to the plate counting …

viable count is often higher than plate counting …

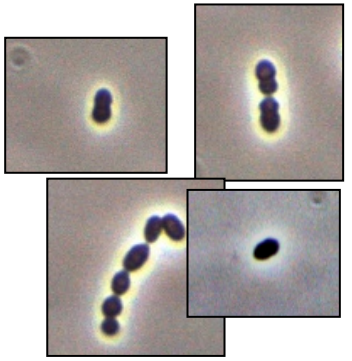
Probiotics and lactic acid bacteria cells grown as single-cell, chains, aggregates, pleomorphic cells.

Sample preparation is extremely critical and often species/strain-dependent

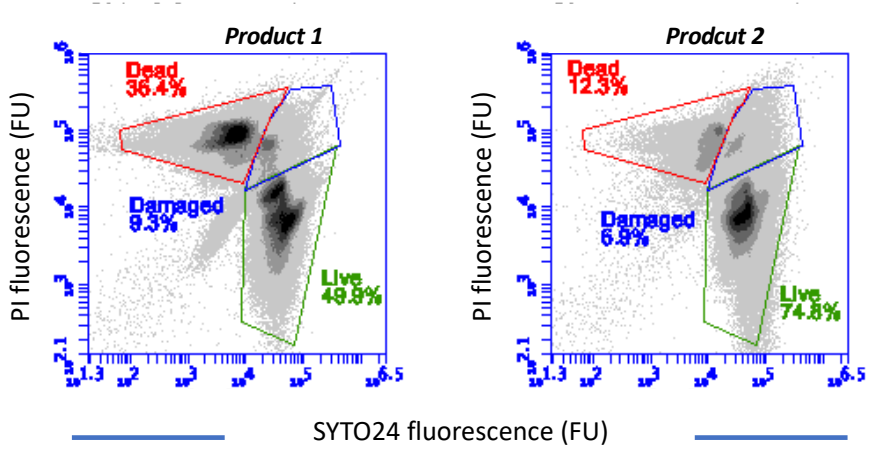


Bifidobacterium longum

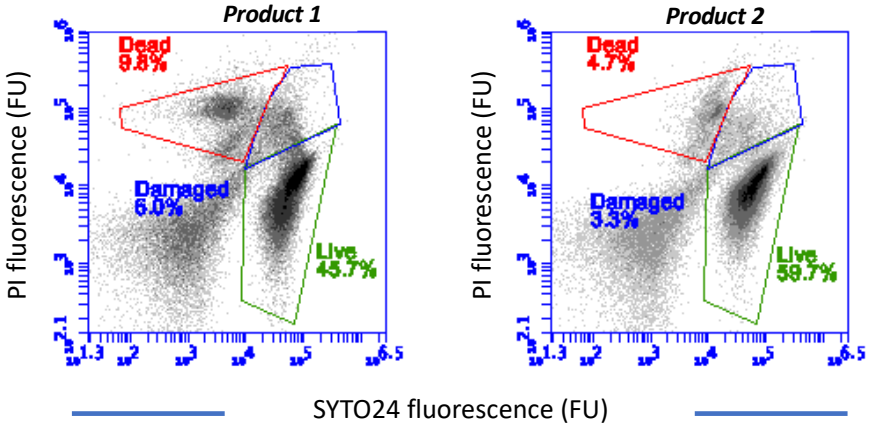
Bifidobacterium bifidum



Streptococcus thermophilus



Buffer A

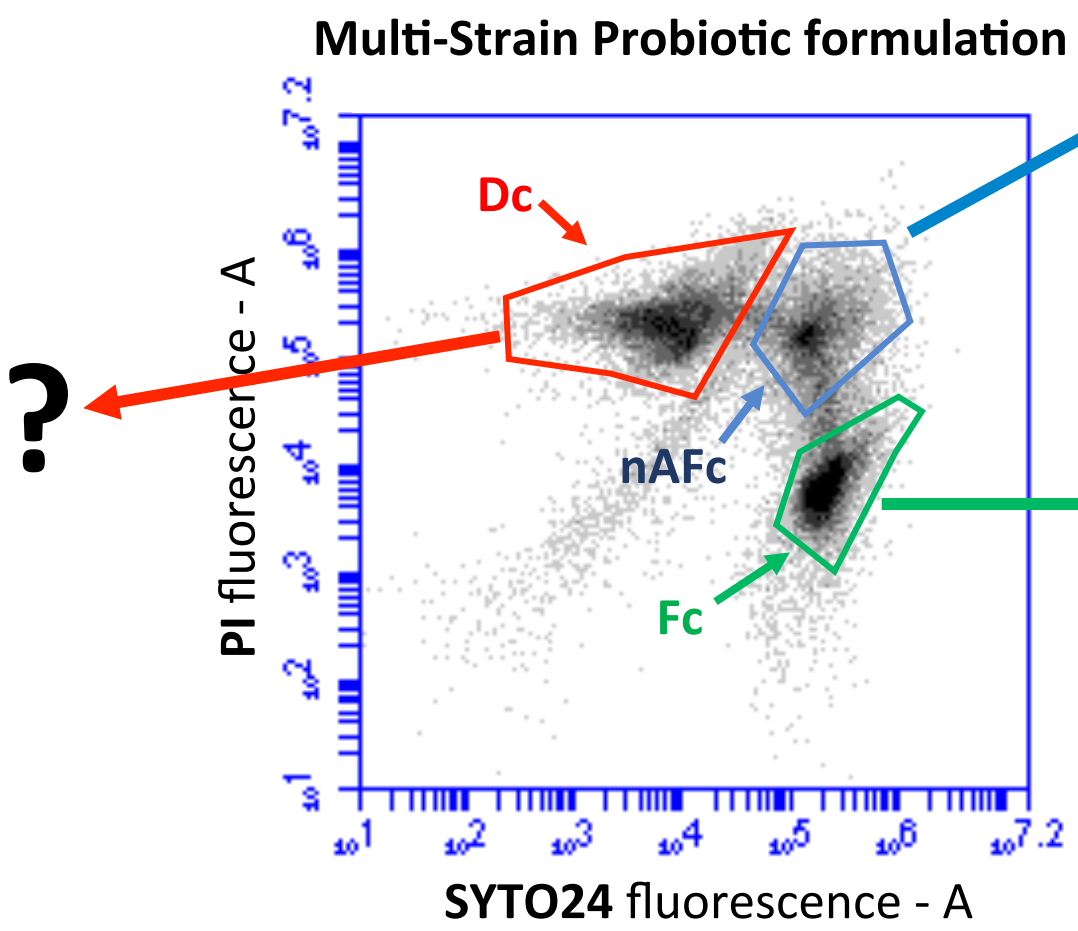


Buffer B

The choice of the buffer where cells are suspended strongly affects the % of dead and damaged cells

Buffer	Product/Lot n.	%nAFc
A	Product 1	**36%**
A	Product 2	**14%**
B	Product 1	**13%**
B	Product 2	**7.5%**

Live and dead cell count in a Multi-Strain Probiotic formulation

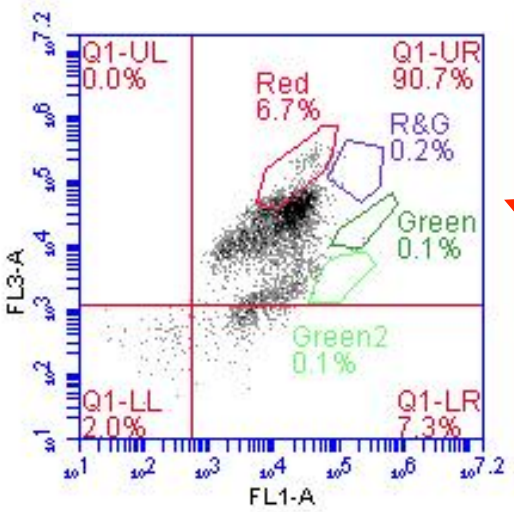


Unknown relative abundance of the species in each flow cytometry population

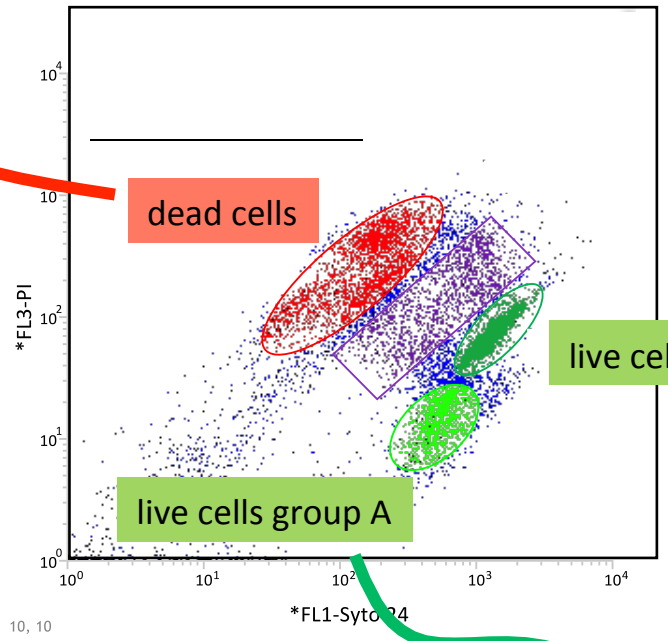
Multi-paramtetric fast quantitative analysis, no taxonomic information can be obtained by FCM

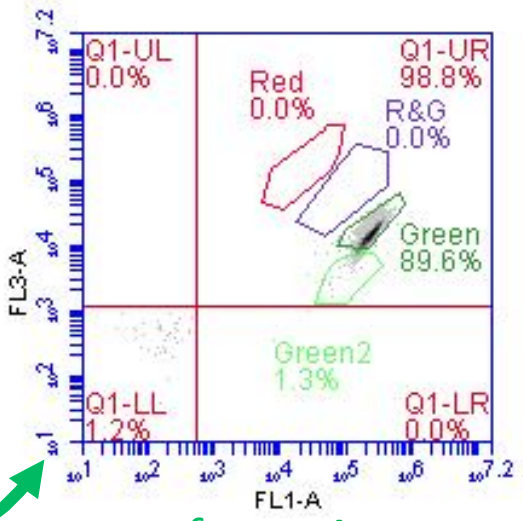
Flow cytometry data should be interpreted as follow:
Fc (active fluorescent units) should be considered live cells, *i.e.* able to growth;
Dc (damaged cells) should be considered injured cells not dead and potentially able to growth;
nAFc (non-active fluorescent cell) should be considered dead cells.

Fc and **nAFc** populations have been sorted

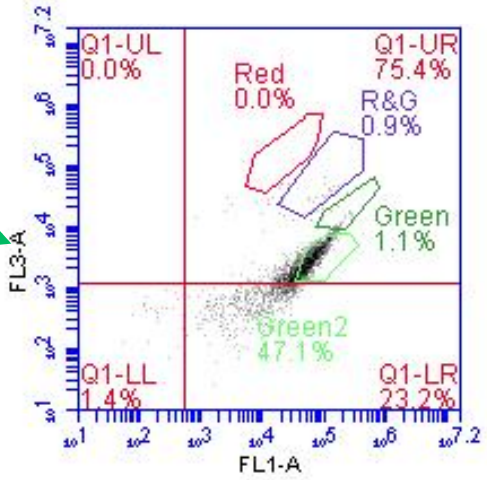


2,88 10^6 sorted events





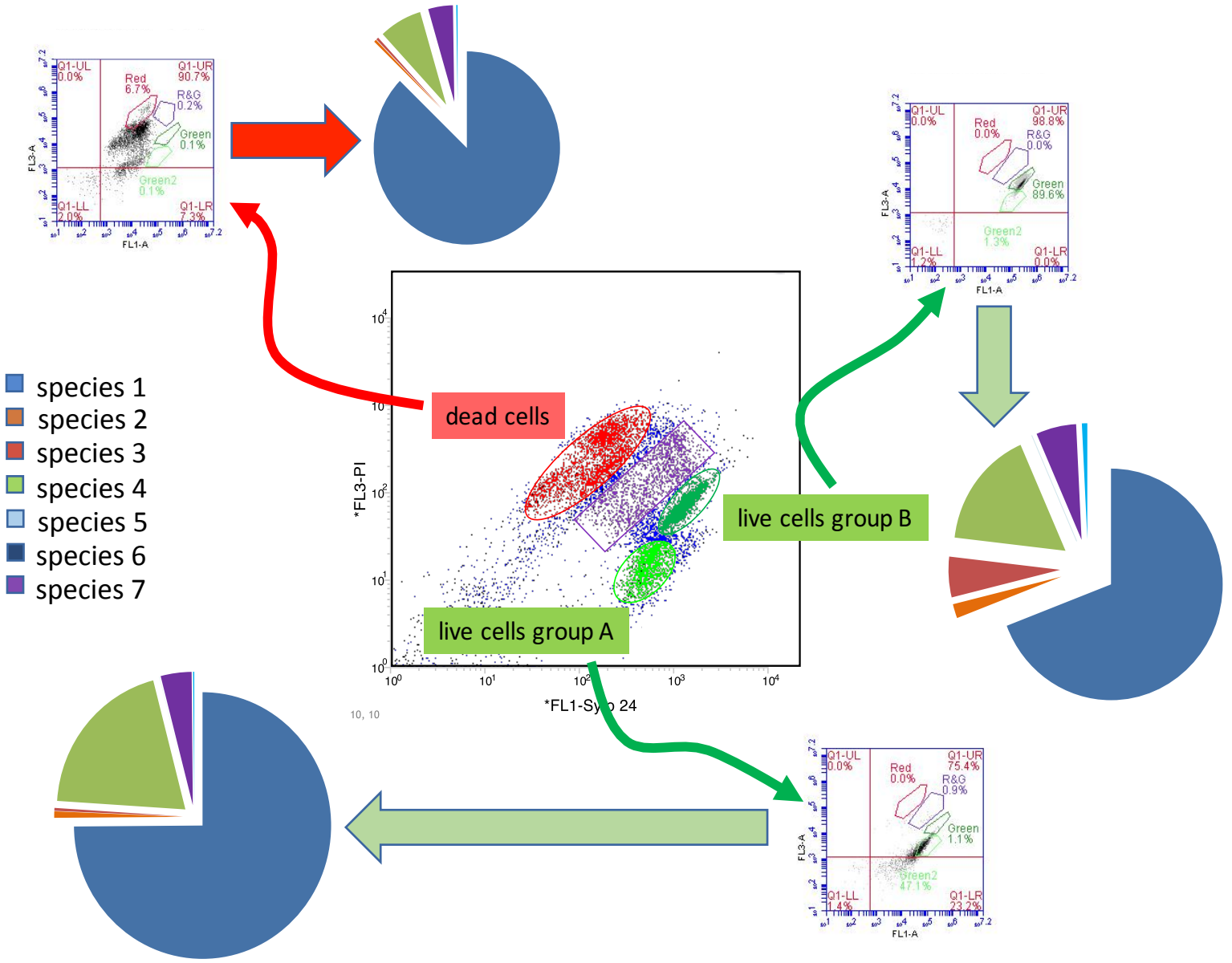
1,0 10^6 sorted events



6,6 10^5 sorted events

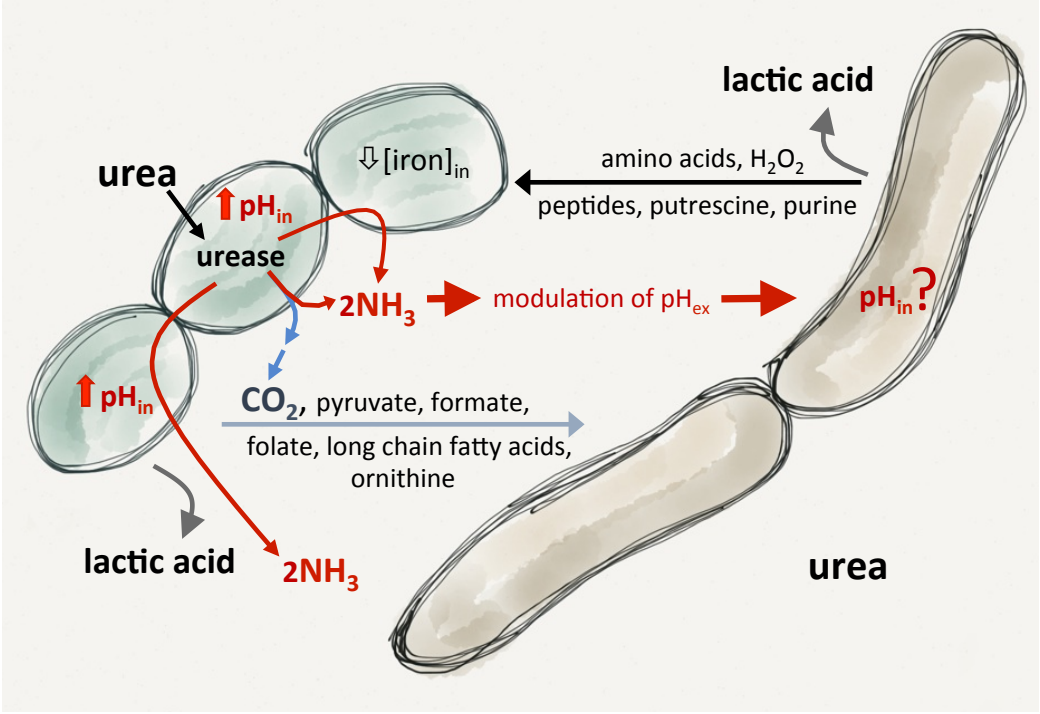
Cell sorted have been subjected to DNA extraction and qPCR assay for the quantification of the relative abundance of each species in the probiotic formulation

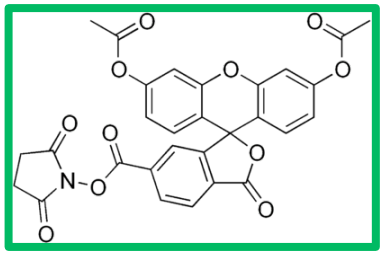
Quantification of the relative abundance of each species in each sorted population



- **Cell physiology**

Yogurt consortium
(*Streptococcus thermophilus-Lactobacillus delbrueckii*)

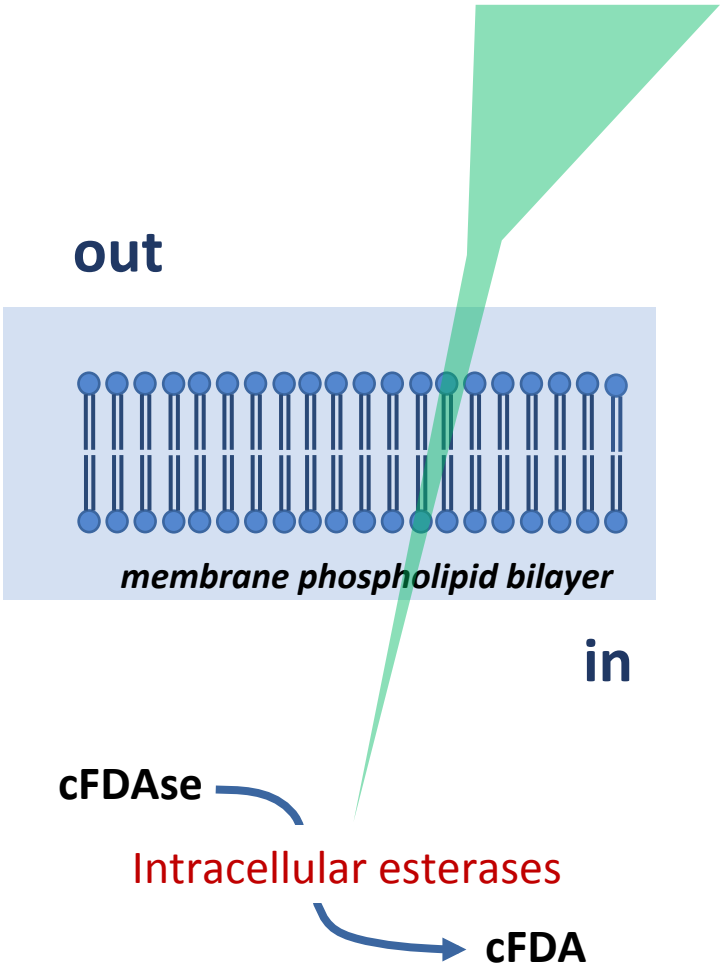




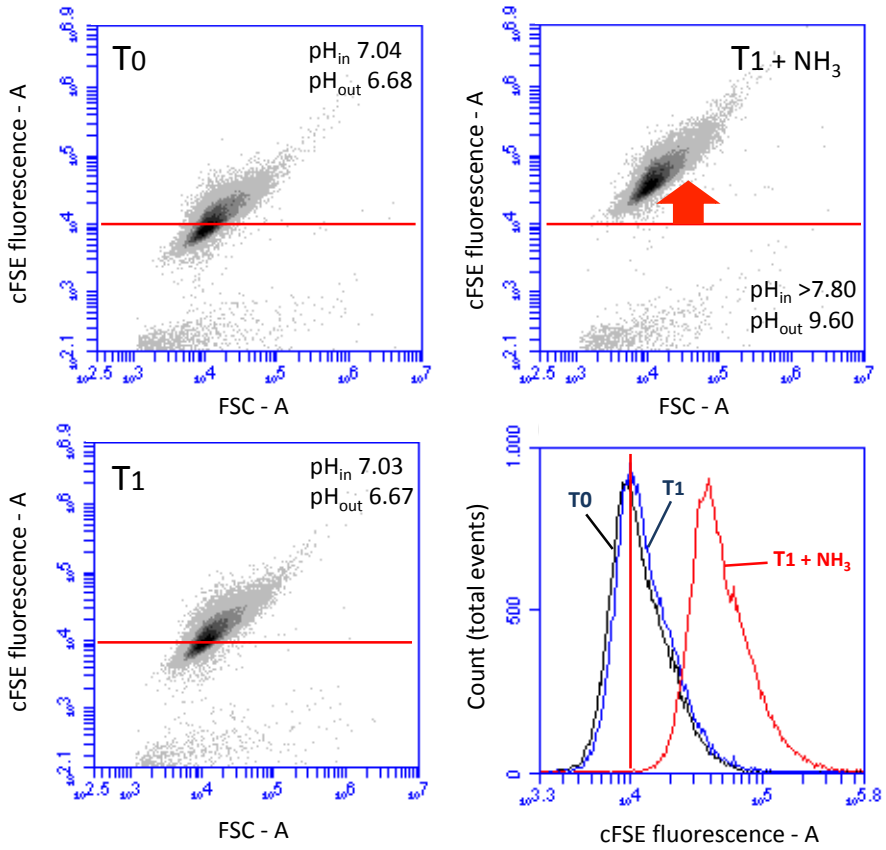
cFDAse

1st Only cells having active esterase were stained

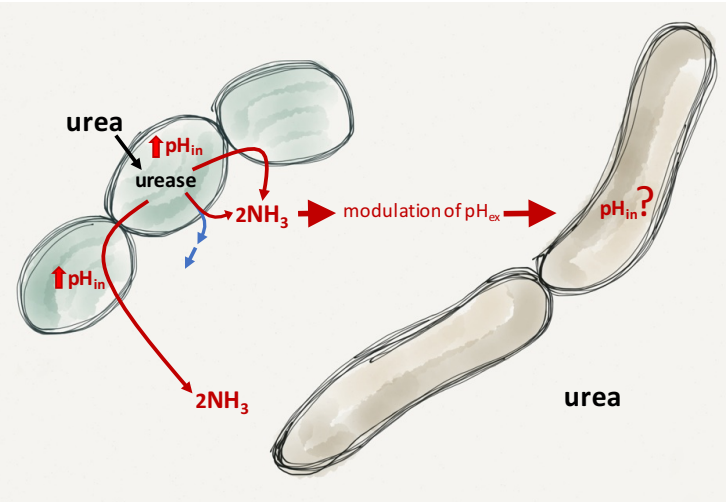
2nd Fluorescence intensity is **pH-dependent**

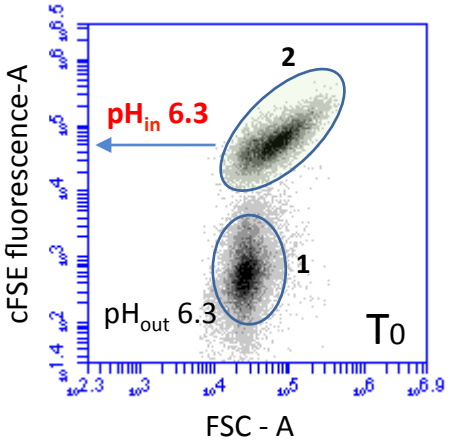


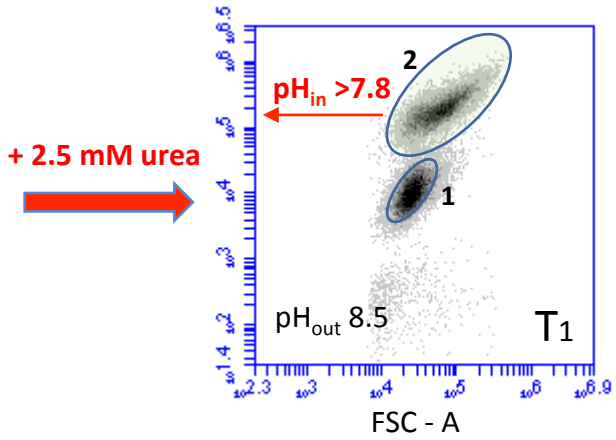
S. thermophilus intracellular pH variation



S. termophilus urease activity increase the intracellular pH of _L. delbrueckii_ in the yogurt consortium ...

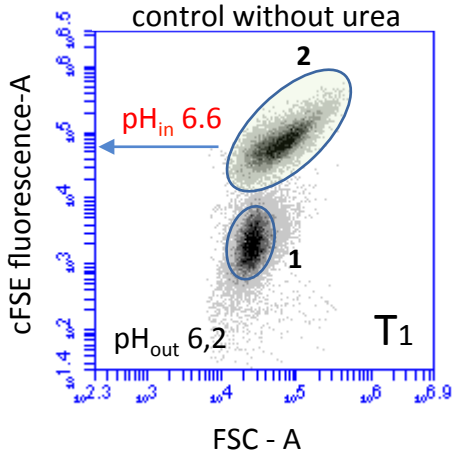






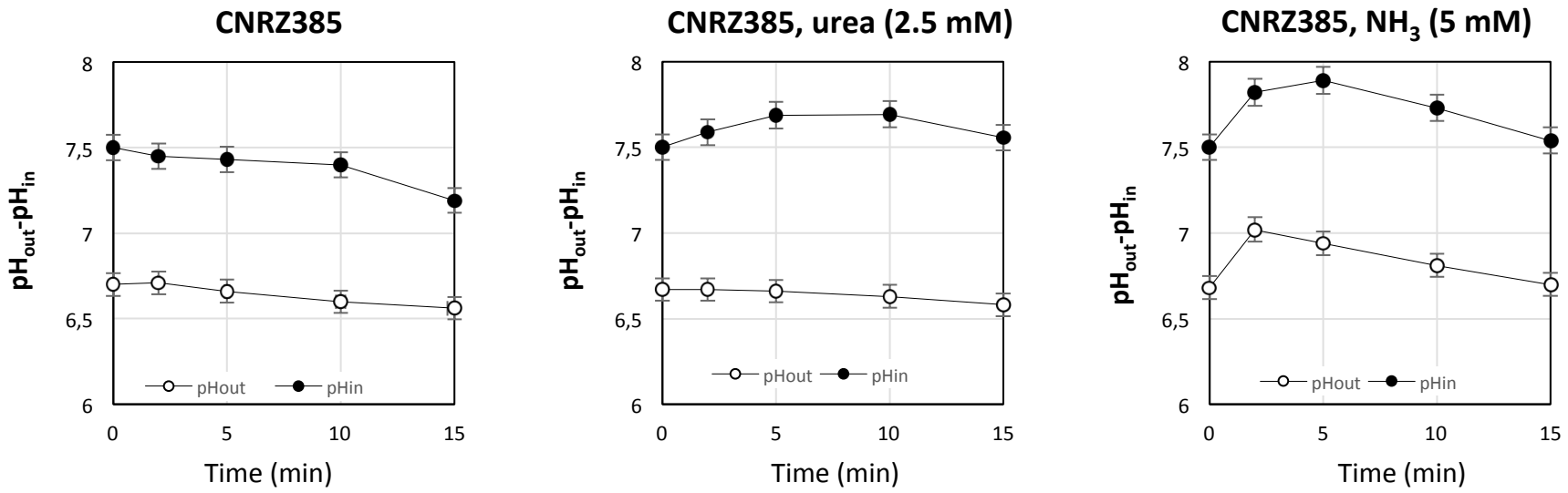
Gate 1 (_S. thermophilus_ – urease-positive not labelled)

Gate 2 (_L. delbrueckii_ – urease-negative cFDAse labelled)



… the flow-cytometry approach allowed the measurement of intracellular pH in *S. termophilus* and *L. delbrueckii* in milk due to urea hydrolysis or NH_3 alkalization

S. termophilus

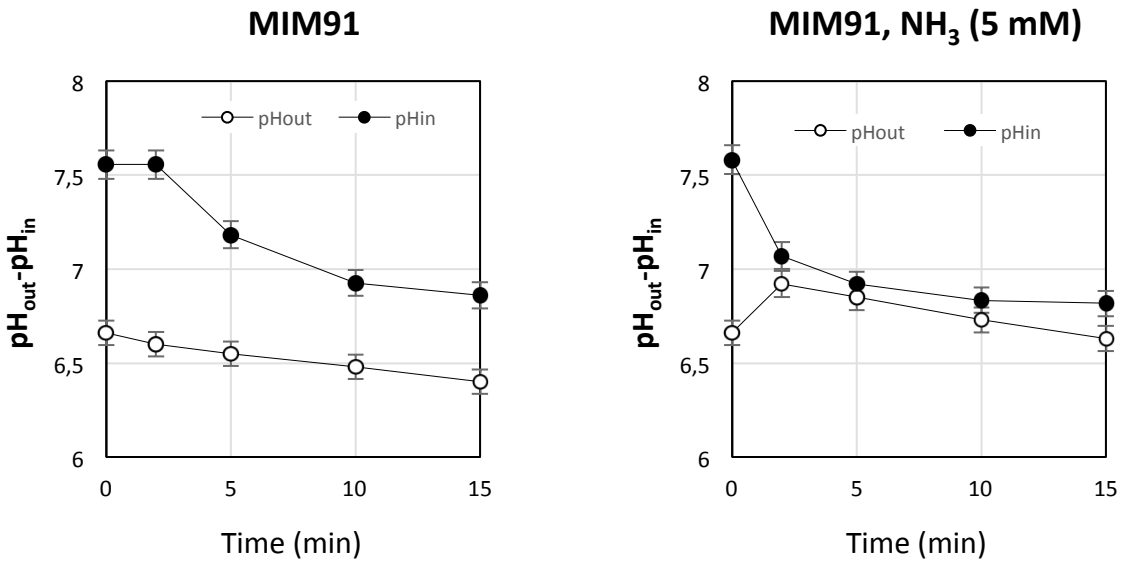


L-lactic acid (mM)

8.9 ± 0.2	**9.4 ± 0.1**	**9.7± 0.1**

… the flow-cytometry approach allowed the measurement of intracellular pH in *S. termophilus* and *L. delbrueckii* in milk due to urea hydrolysis or NH_3 alkalization

L. delbrueckii



D-lactic acid (mM)

7.9 ± 0.2

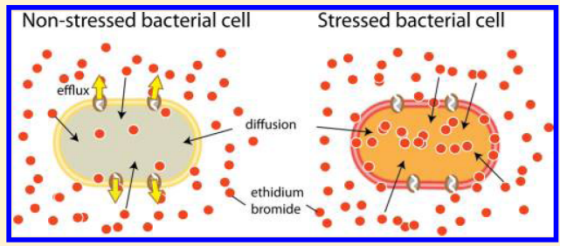
9.4 ± 0.1

- **Cell sensitivity to toxic compounds**

  - Efflux pump efficiency in *S. thermophilus* (Ethidium bromide as probe);
  - *Listeria monocytogenes* sensitivity to essential oils (SYBR Green I and PI);
  - Promysalin mechanism of action (SYBR Green I and PI);

- Efflux pump efficiency in *S. thermophilus* (Ethidium bromide as probe);

Ethidium bromide efflux

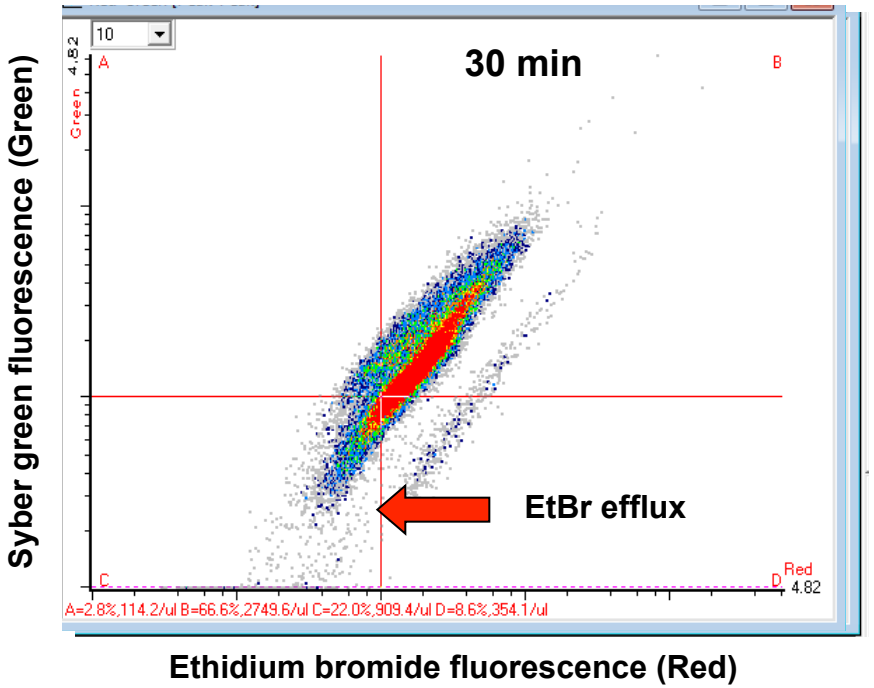


Ethidium bromide (**EtBr**) efflux was assessed by flow-cytometry.

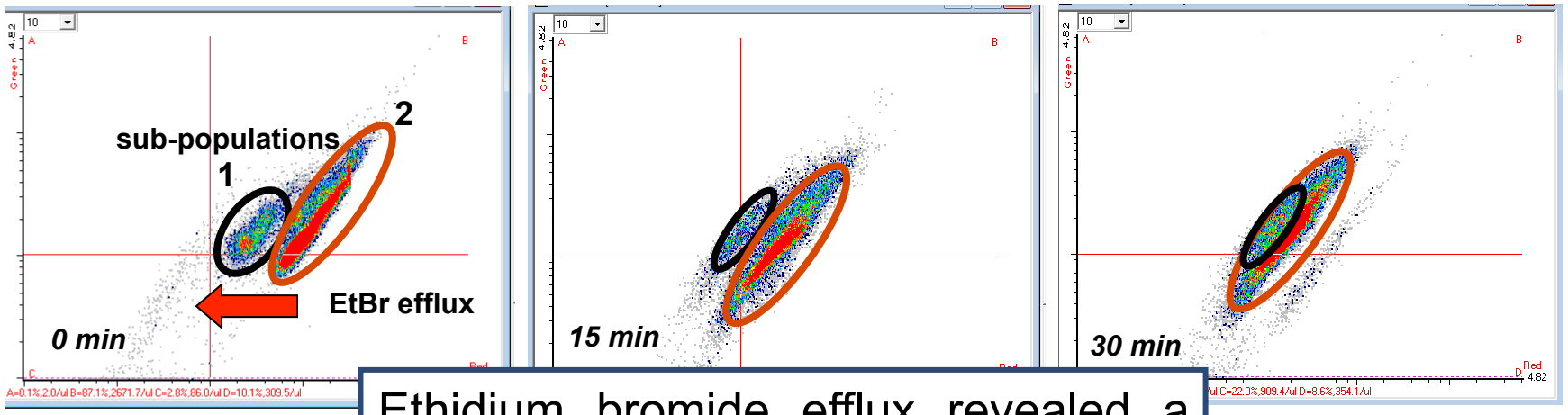
Cells were loaded with ErBr and the efflux was monitored by flow-cytometry energizing the cells with lactose.

The fluorescence signal of EtBr was increased staining the cells with sybergreen before the flow-cytometry analysis.

wild-type

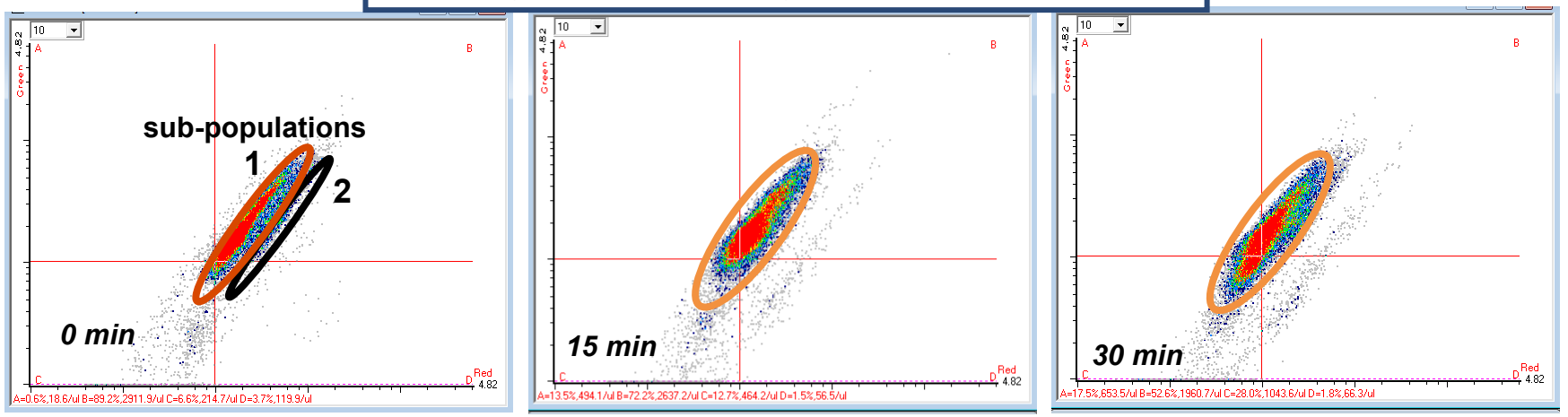


MIM20 (wild-type + empty vector)



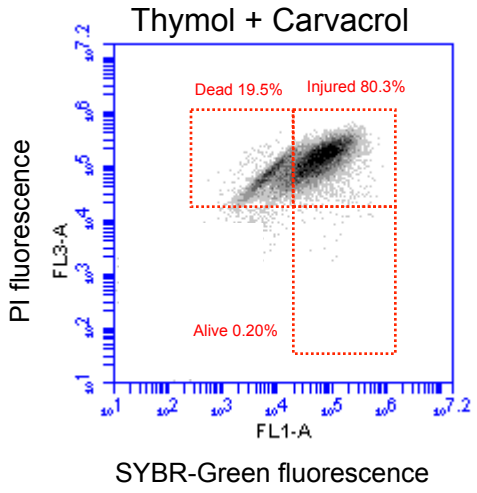
Ethidium bromide efflux revealed a heterogeneity of PmrB in a genetically homogenous background

MIM27 (overexpressio



Listeria monocytogenes sensitivity to essential oils

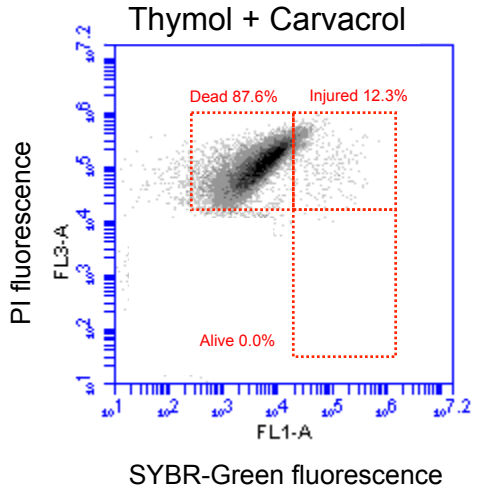
50°C



	Membrane permeability AFU FL3	Membrane potential AFU FL1
T0	988	8860
Control	1230	34766
Thymol	2869	39777
Carvacrol	3102	31171
Thymol + Carvacrol	17153	29127

- Viability vs Cultivability
- Recovery of VBNC cells

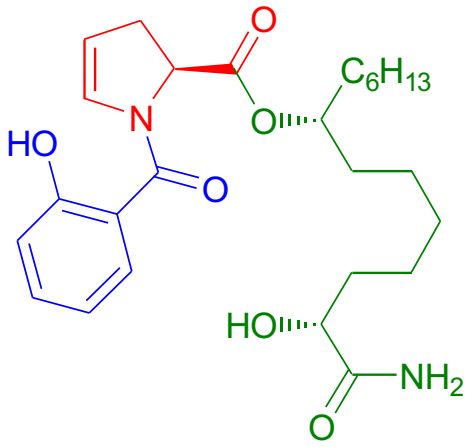
55°C



	Membrane permeability AFU FL3	Membrane potential AFU FL1
T0	988	8860
Control	1786	53636
Thymol	3675	34763
Carvacrol	9608	27404
Thymol + Carvacrol	18223	28015

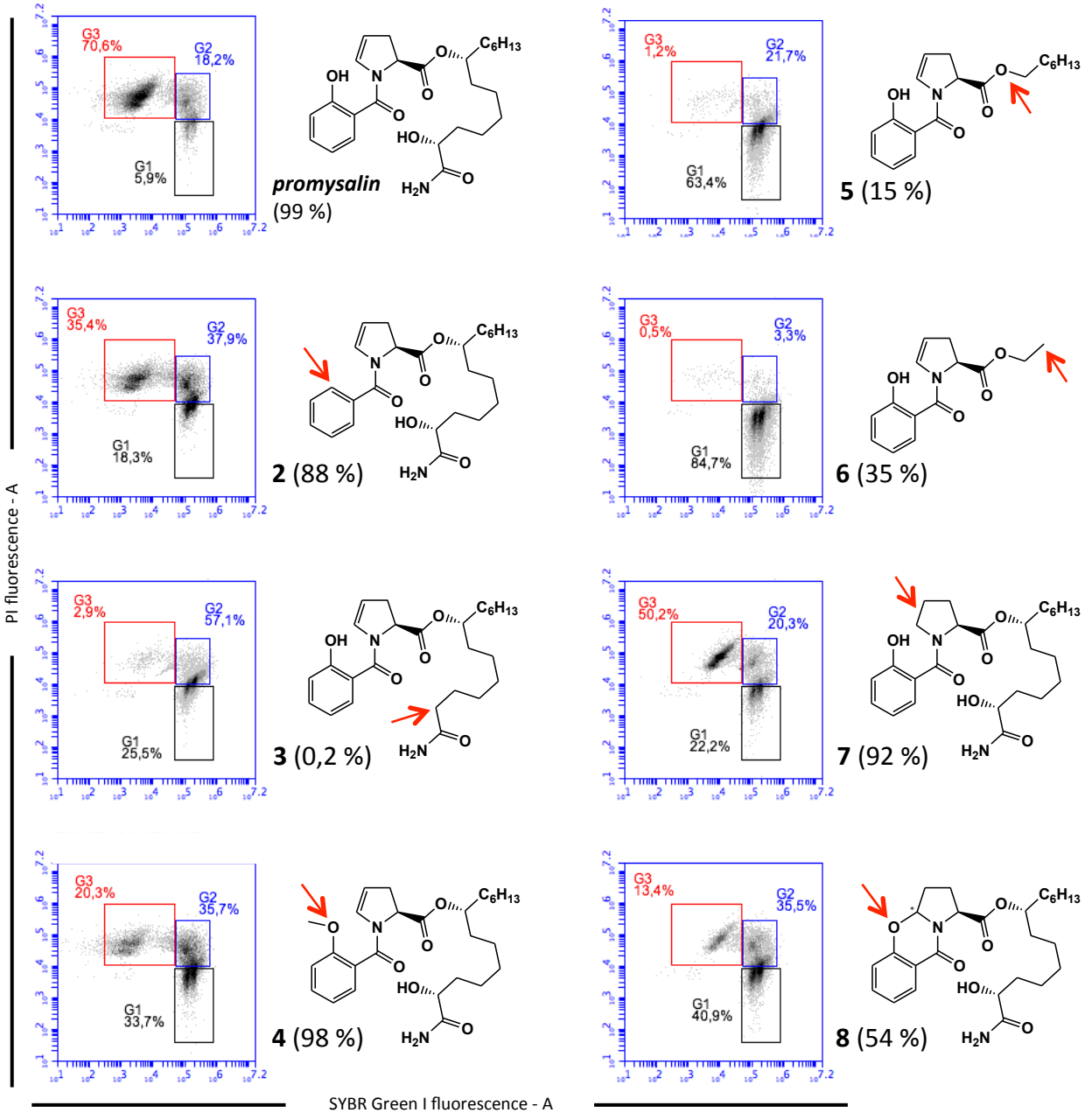
Exposition to Thymol 50 mg/l and/or Carvacrol 50 mg/l, at 50°C or 55°C for 30 min

- Promysalin mechanism of action (SYBR Green I and PI);



Promysalin is a salicylate-containing *Pseudomonas putida* antibiotic active against Gram-negative and Gram-positive bacteria

Chemical synthesis of promysalin derivatives revealed that the salicylate fragment, the dehydroproline moiety, and the myristamide chain are confirmed mandatory to maintain the Cell-Membrane damage



Kaduskar et al., 2017 Scientific Reports 7: *8861*

- **Quality control on probiotic multi-strain formulation**

i) **be taxonomically defined**;

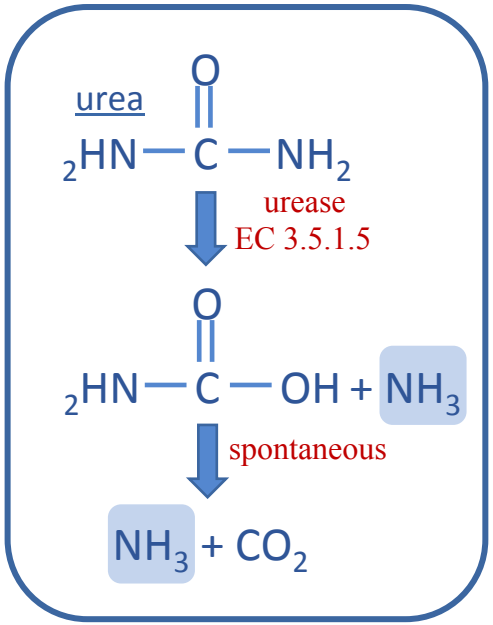
ii) **have a reproducible composition**;

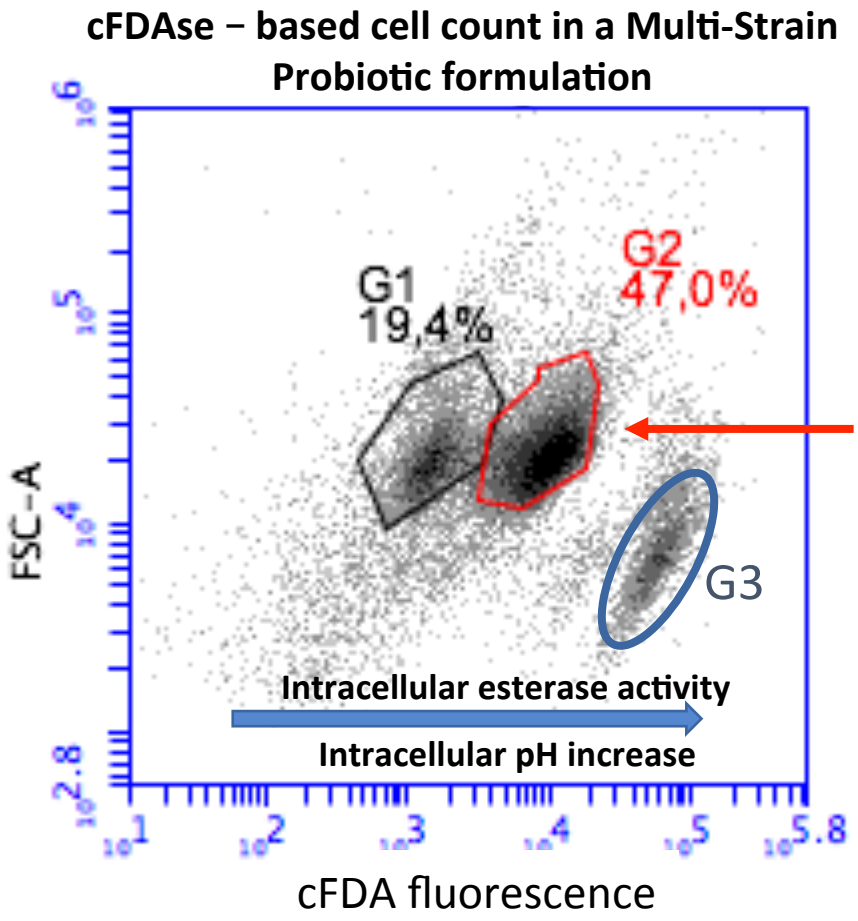
iii) **be safe, no transferrable Antibiotic-Resistance Genes**;

iv) **contain viable cells**;

v) and **ideally, should be controlled for probiotic molecular markers**;

Flow-cytometry based assay could help to evaluate probiotic marker … giving also a taxonomic information

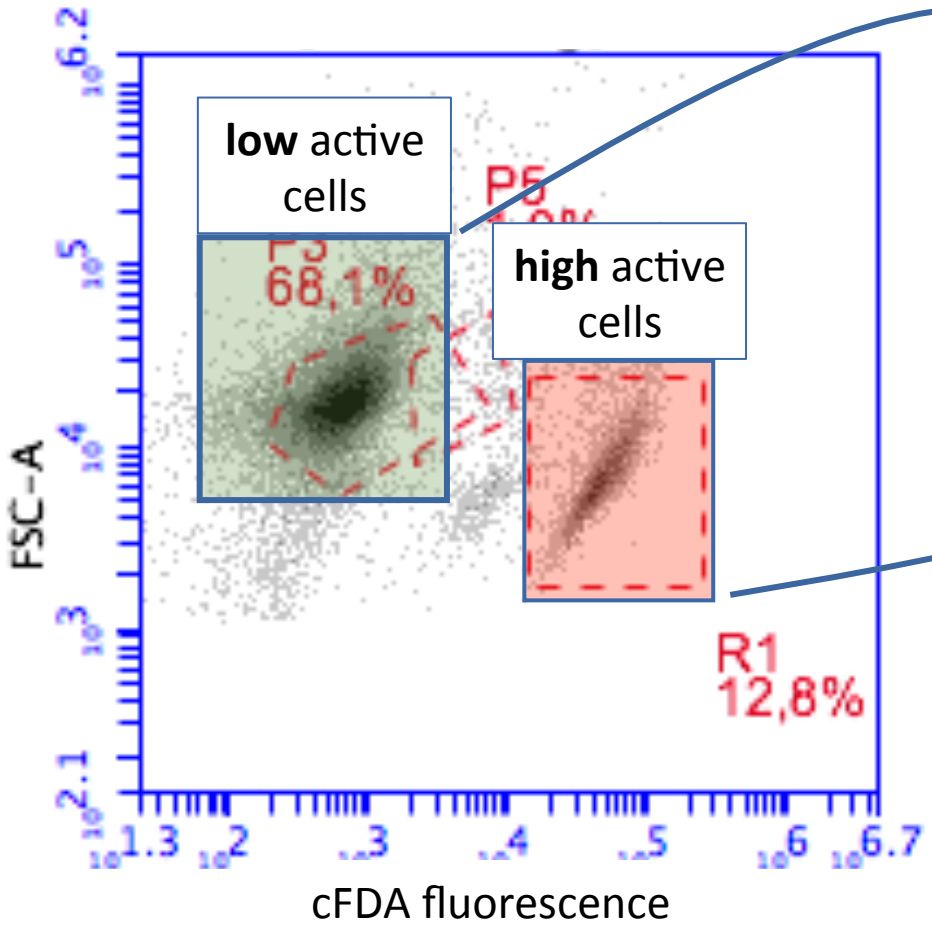


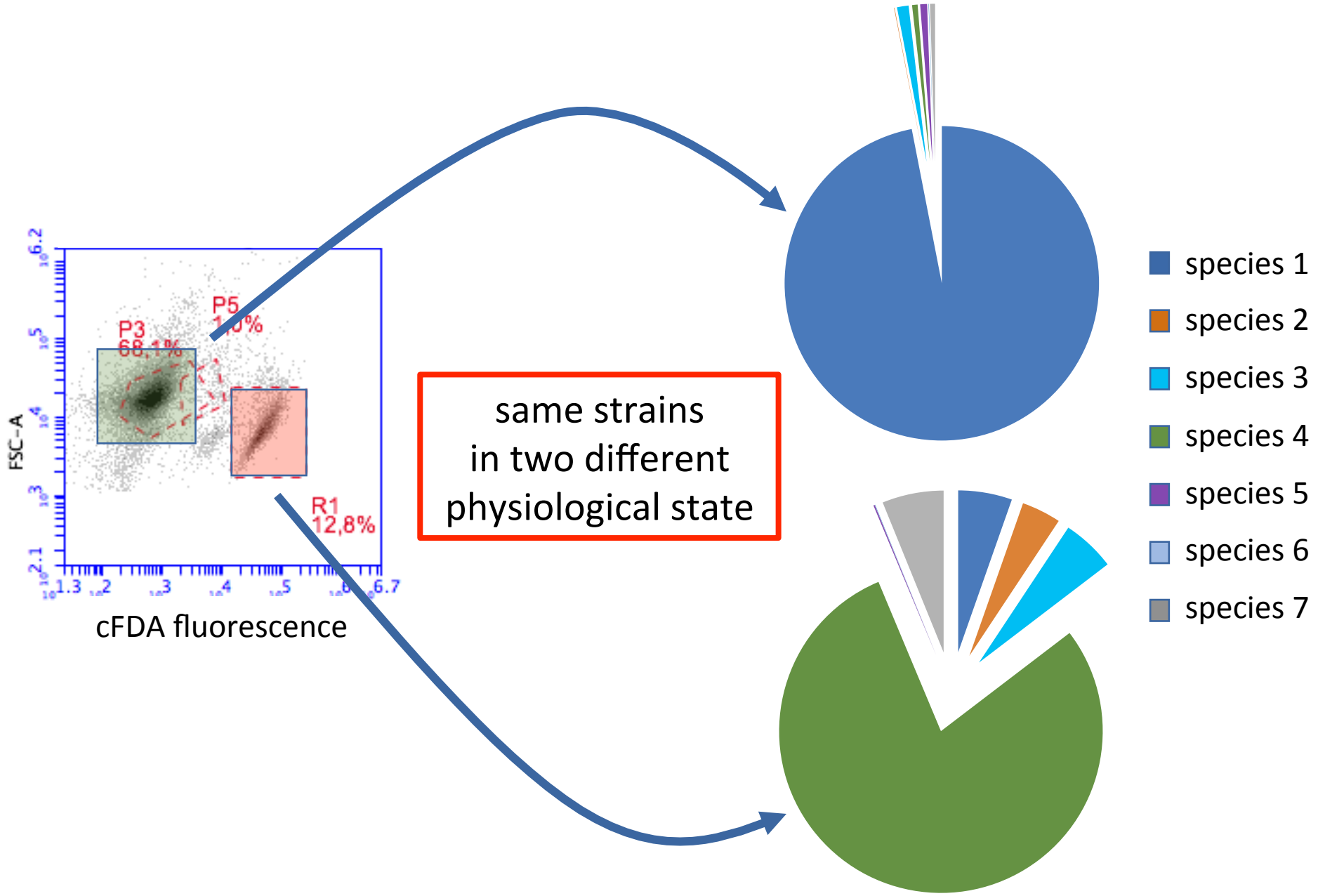


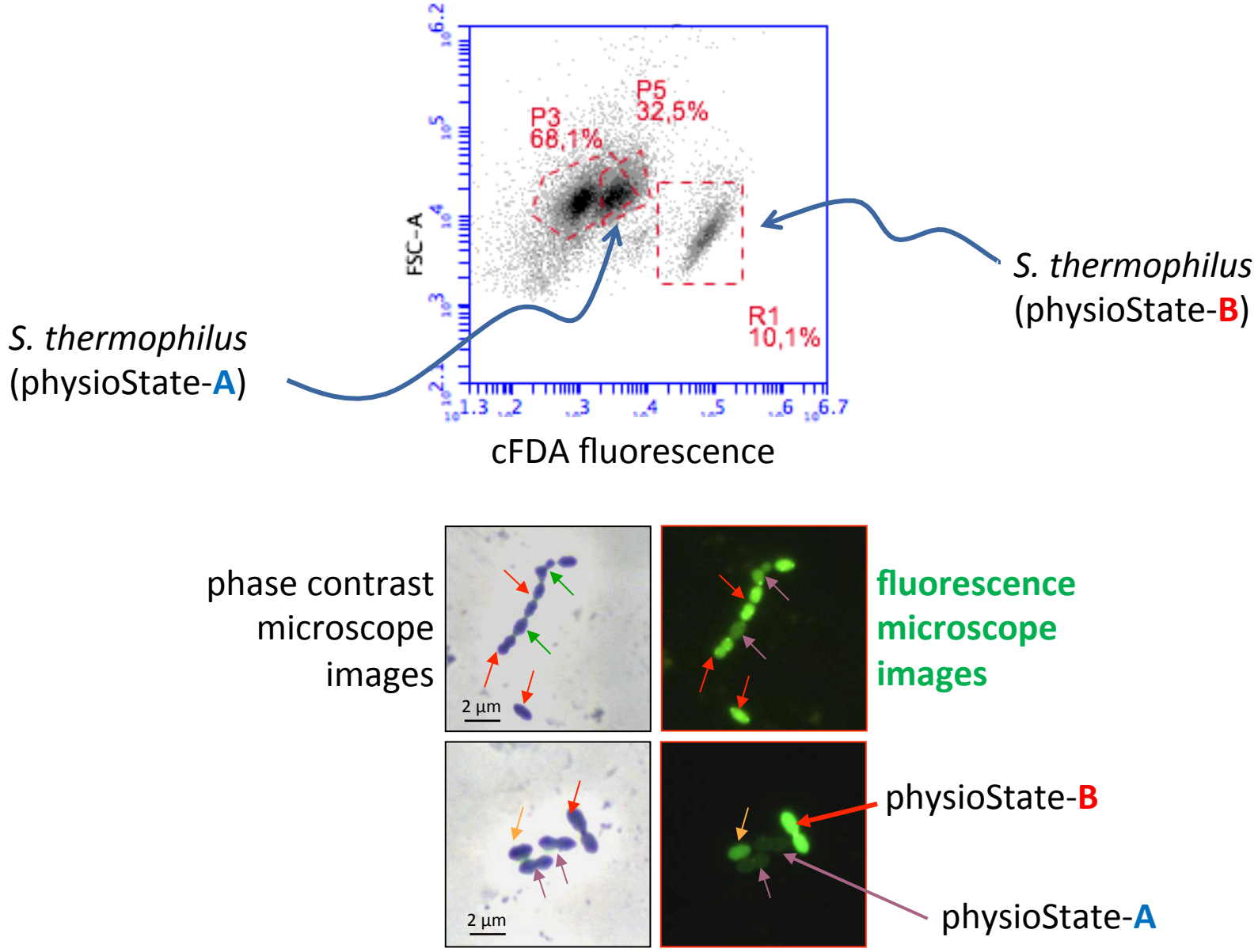
+ 10 mM Urea

urease-positive population

iii) **Further characterization of a multi-strain probiotic product**




P3
68,1%
P5
R1
12,8%
FSC-A
cFDA fluorescence
same strains
in two different
physiological state
species 1
species 2
species 3
species 4
species 5
species 6
species 7


P3
68,1%
P5
32,5%
R1
10,1%
FSC-A
cFDA fluorescence
S. thermophilus
(physioState-A)
S. thermophilus
(physioState-B)
phase contrast
microscope
images
fluorescence
microscope
images
2 µm
2 µm
physioState-B
physioState-A

- **New protocols for strains isolation**

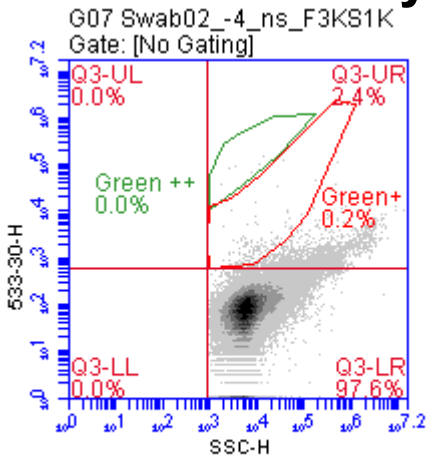
Isolation of lactic acid bacteria from vaginal swab samples,
to be further selected for probiotic applications

Dilution and plating
- is time consuming;
- several dilutions must be plated to allow single strain isolation;

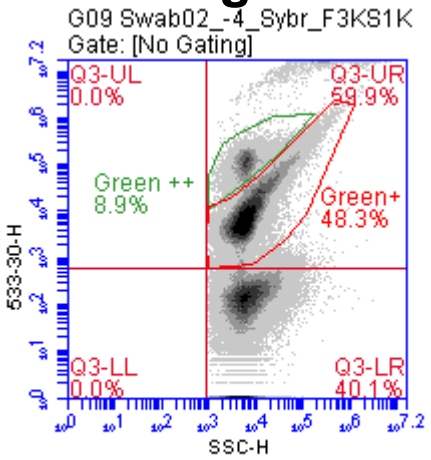
FACS-based strain isolation
- rapid;
- strain colony well separated;
- strains could be easily screened by colony morphology

SYBR Green I-stained vaginal Swab02 suspension

Flow cytometry and cell sorting

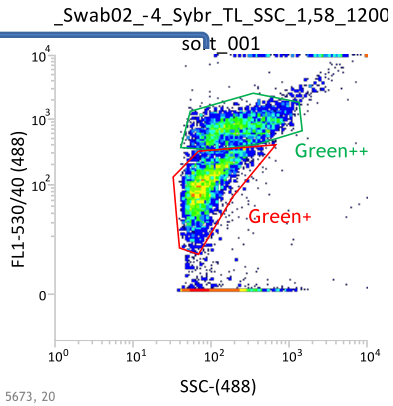


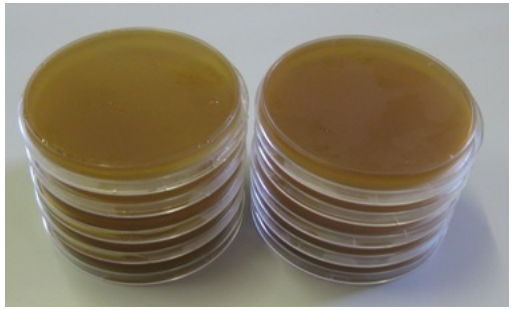
+SYBR Green I
37°C



Non-compensated

FACS gating





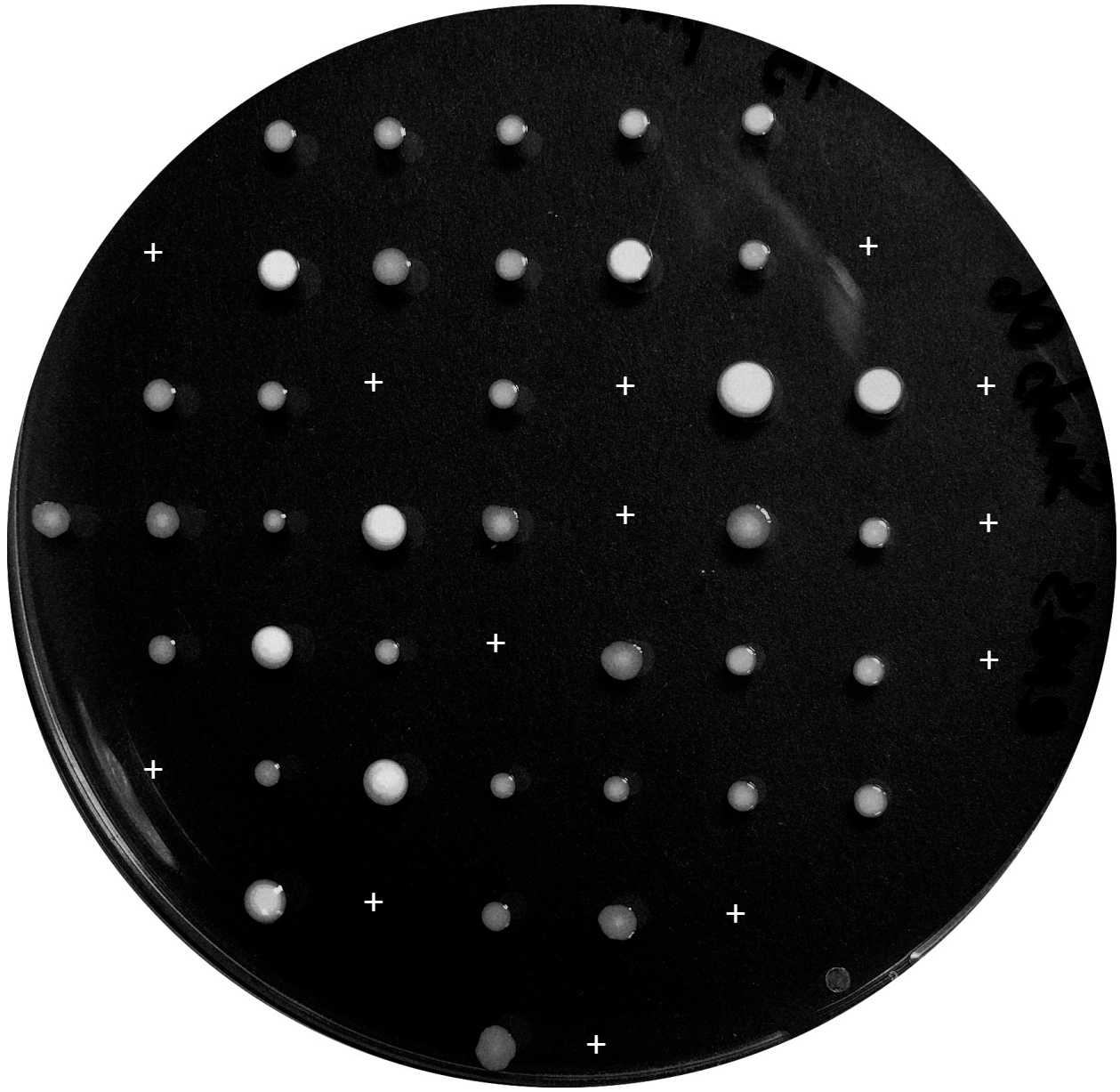
5673, 20

Statistics: 2018-10-11_12,12_Swab02_-4_Sybr_TL_SSC_1,58_1200evtss_0,50psi_pre-sort_001

Populations	Events	% Total	% Parent	SSC-(48... Median	SSC-(48... RCV	FL1-530... Median	FL1-530... RCV
All Events	10,000	100.00%	####	100	59.65	121	146.95
Swab01_SSC	9,620	96.20%	96.20%	98	57.49	118	146.91
Green++	2,894	28.94%	28.94%	216	76.39	720	39.38
Green+	4,482	44.82%	44.82%	81	40.39	97	71.18

BD Accuri C6 Plus: triggers on FSC-H (threshold level: 3,000) and SSC-H (threshold level: 1,000)
Workspace: FACSJazz_2018 → Mora_ActialFarmaceutica_Swabs_2018 → 2018-10-10&11_Swabs.ci
Density plots. Unstained: 74,838 evts/50 µL (1497 evts/µL); Stained: 145,741 evts/50 µL (2915 evts/µL)

BD FACSJazz: Trigger on SSC-H, threshold level: 1.58 (log)
Density plot. Stained: 10,000 evts



from October 2017

FACS-service

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DEPARTMENT OF FOOD ENVIRONMENTAL AND NUTRITIONAL SCIENCES

… thanks for your attention

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