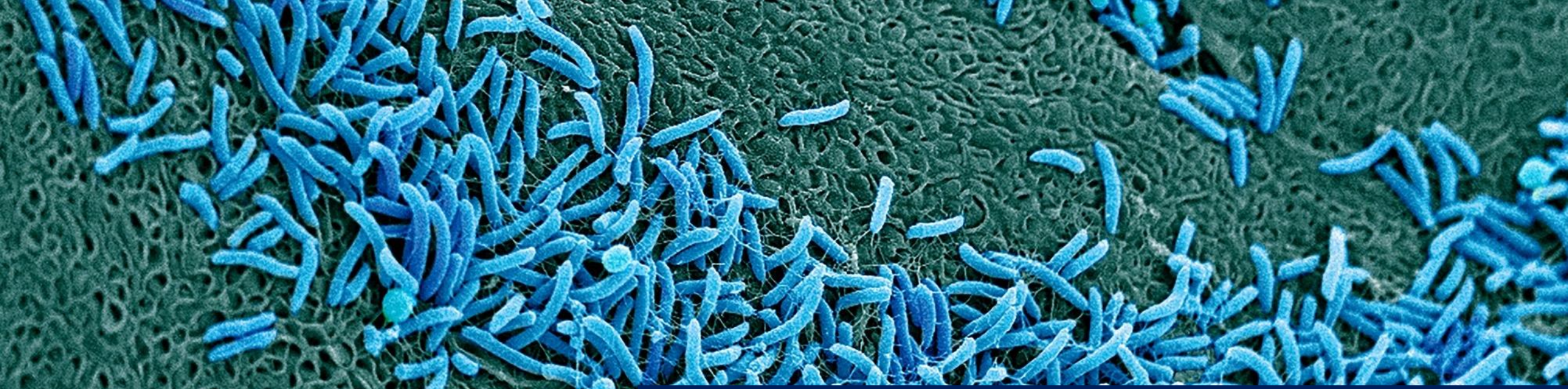


USP Symposium, October 7th 2022

Assessment of shelf-life and metabolic viability of a multi-strain synbiotic using standard and innovative enumeration technologies

PROBIOTICAL

Dr. Marco Pane

m.pane@probiotical.com

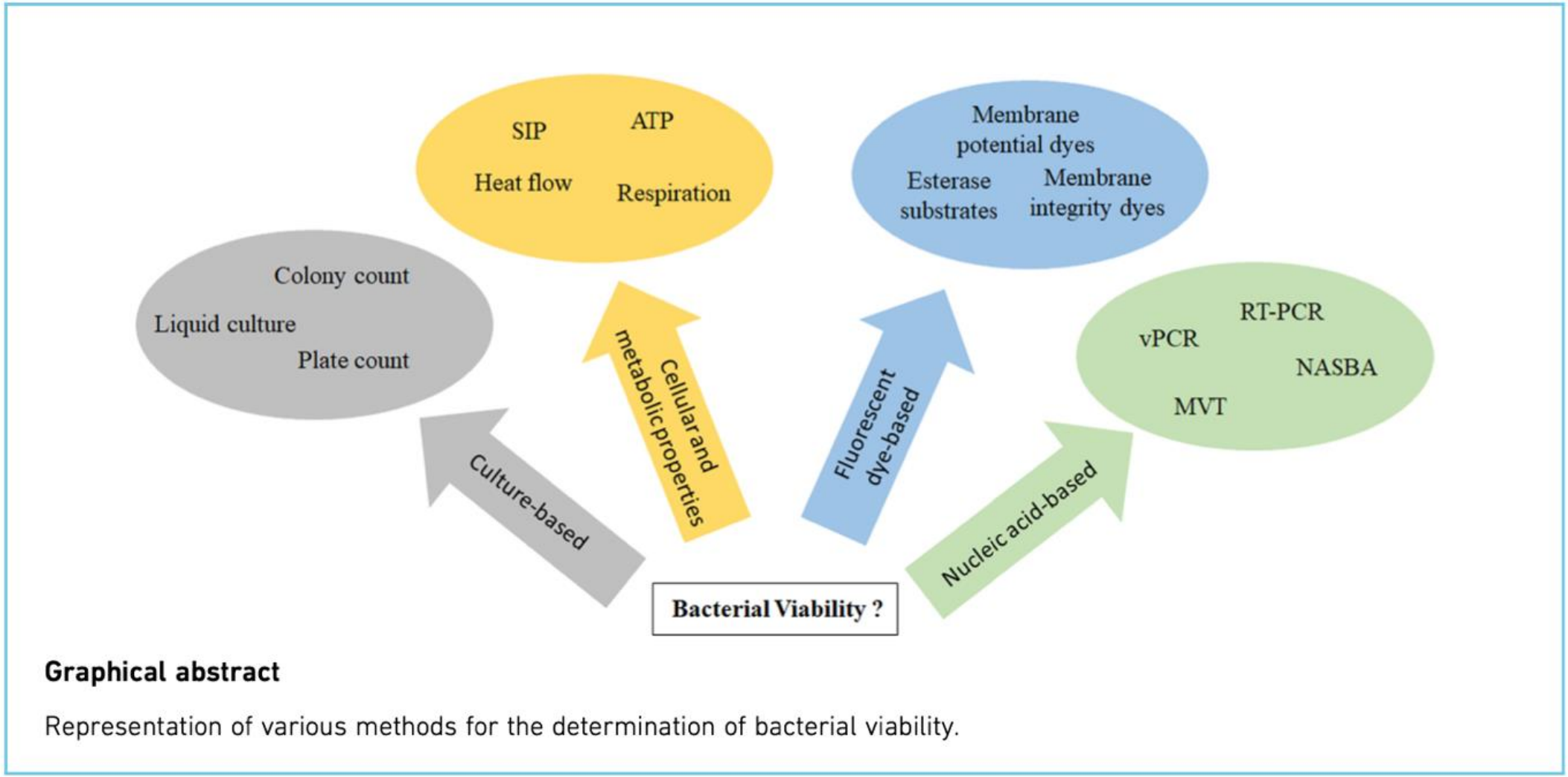
- *R&D Head Probiotical Research*
- *IPA member Scientific and Manufacturing Committee*
- *Board Director Pharmabiotic Research Institute*
- *Expert consultant Probiotic Committe US Pharmacopoeia*

- *Master degree in Food Techology*
- *PhD (ongoing) in Food, Health and Longevity*

PROBIOTICAL

Main topics

- General principles of Plate Count (PC) and Flow Cytometry (FC) for bacterial enumeration
- Long-term Real-life stability study Probiotic Food Supplment using PC, FC and metabolic assesment
- Case study on VBNC (Viable But Not Cultivable)

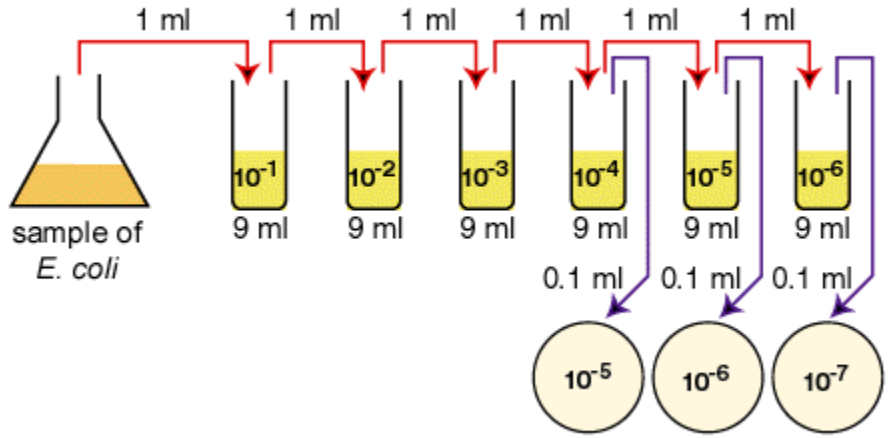


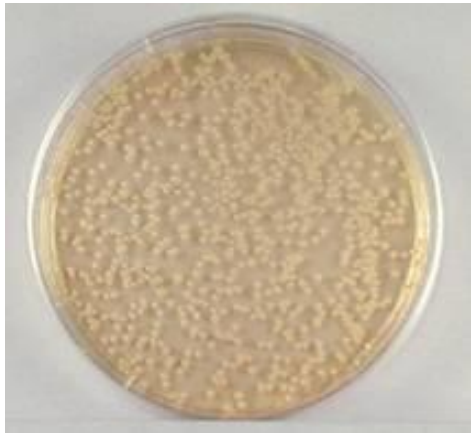
Graphical abstract

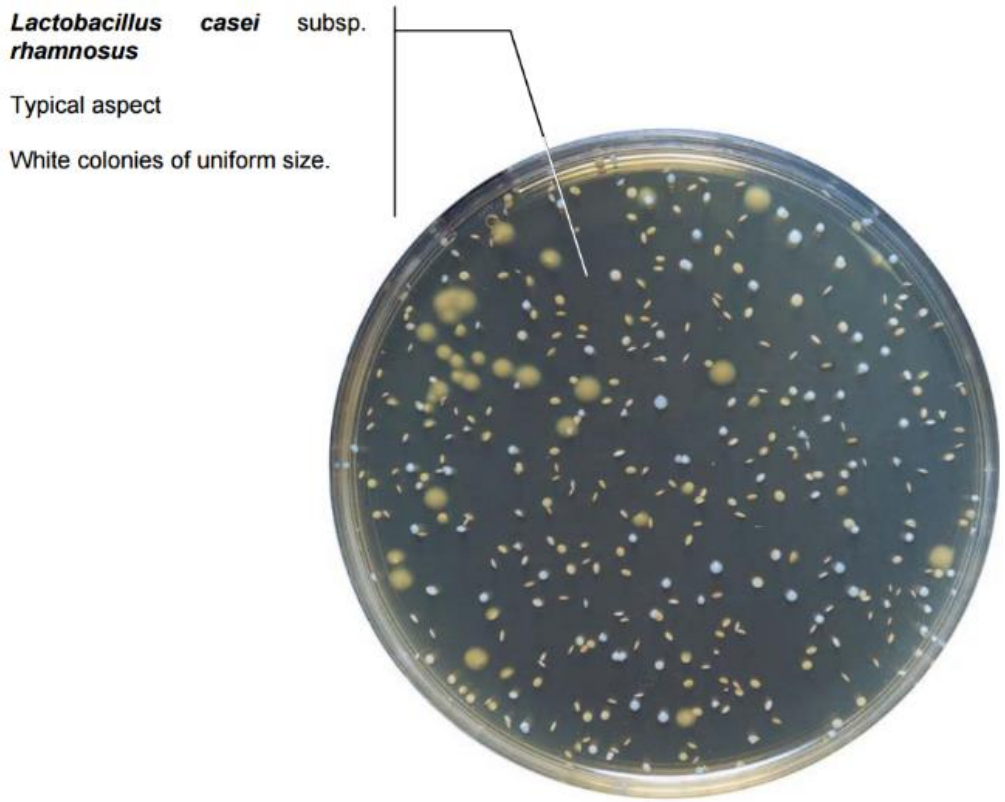
Representation of various methods for the determination of bacterial viability.

Doi:10.1099/mic.0.000786

Plate count methods







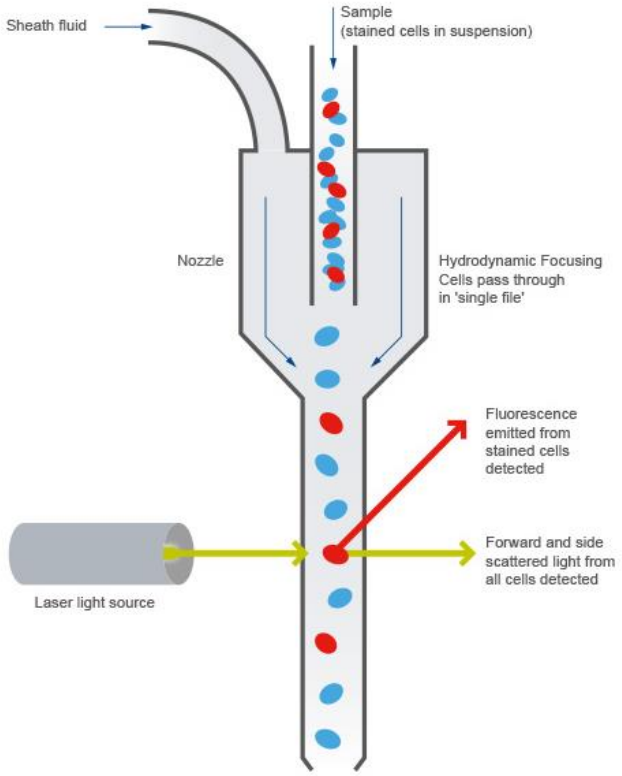
The plate count method is based on the premise that a single bacterium can growth and divide to give an entire colony

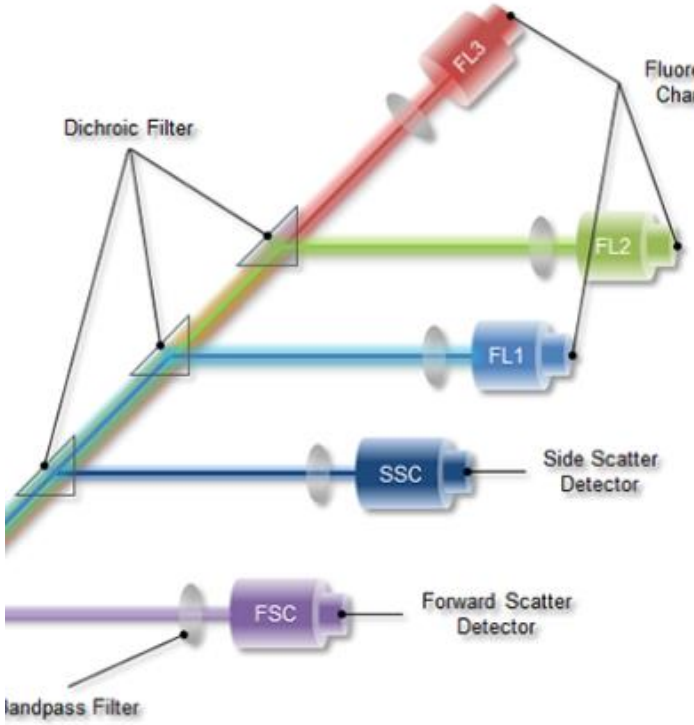
Interpretation of plate count methods

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

Flow Cytometry (FC) principle





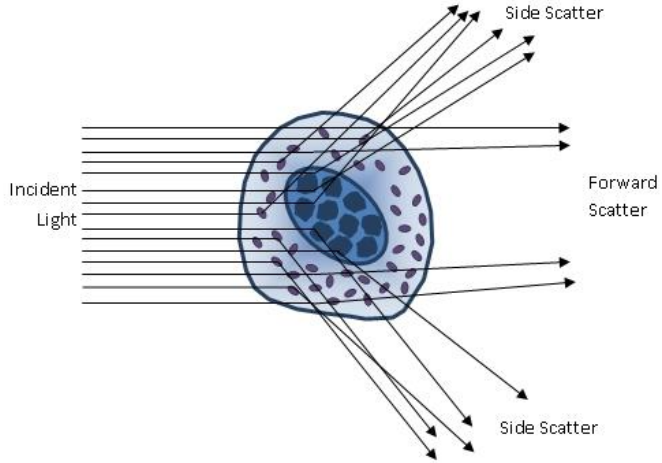
Fluorescence

ISO 19344

3 staining procedure:

- **Membrane integrity**
- Enzymatic activity
- Membrane polarization

SC, FS



Basically it "just" enumerate cells

https://www.creative-bioarray.com/support/principle-of-the-flow-cytometry.htm

Impedance Flow Cytometry

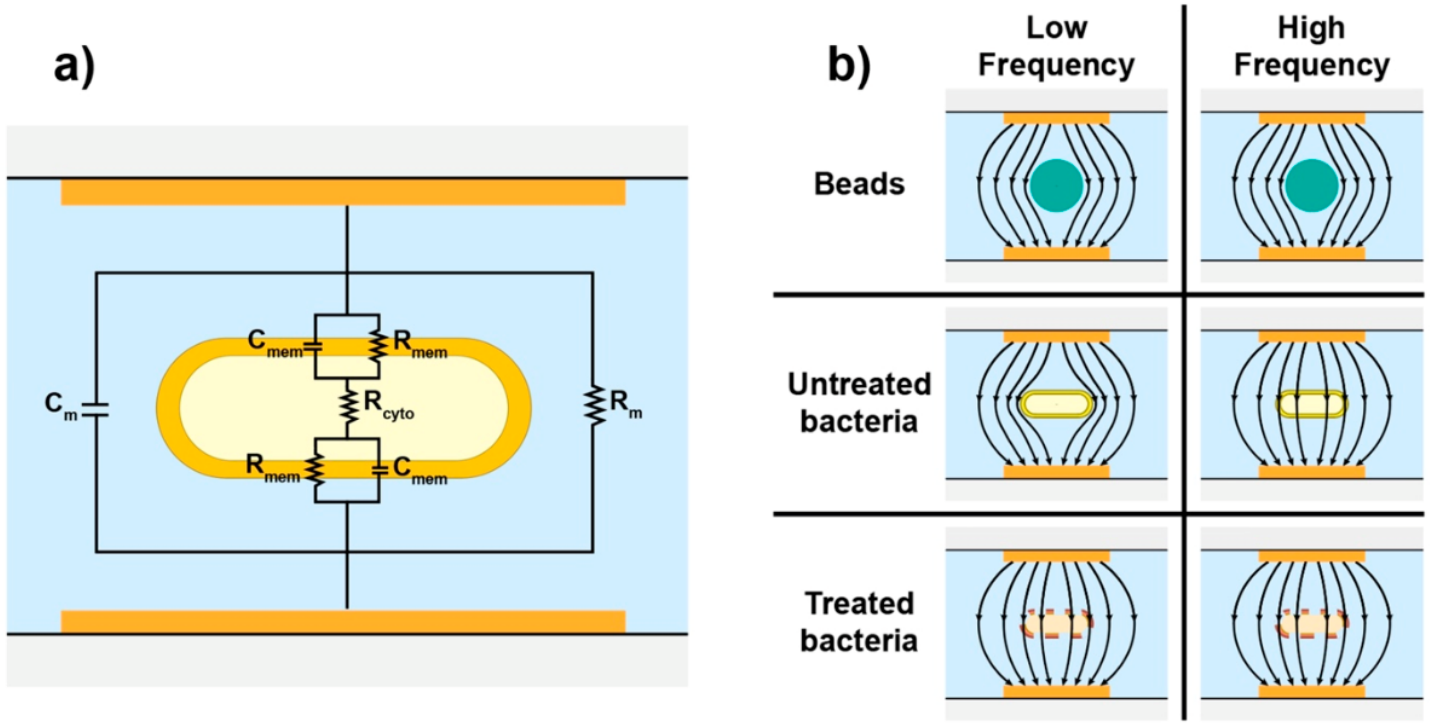


Figure 2. (**a**) Simplified equivalent circuit model of *E. coli* bacteria. The model is composed of components representing the electrolyte resistance and capacitance (R_m, C_m), the resistance and capacitance of the cell membrane (R_{mem}, C_{mem}) and the resistance of the cell interior (R_{cyto}). (**b**) Electric field penetration in polystyrene beads and in bacteria with different viability states at low and high frequencies.

doi:10.3390/s20216339

PROBIOTICAL

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 model
- **10** samples (2 different batch)
  - **8** pure starter culture
  - **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!**

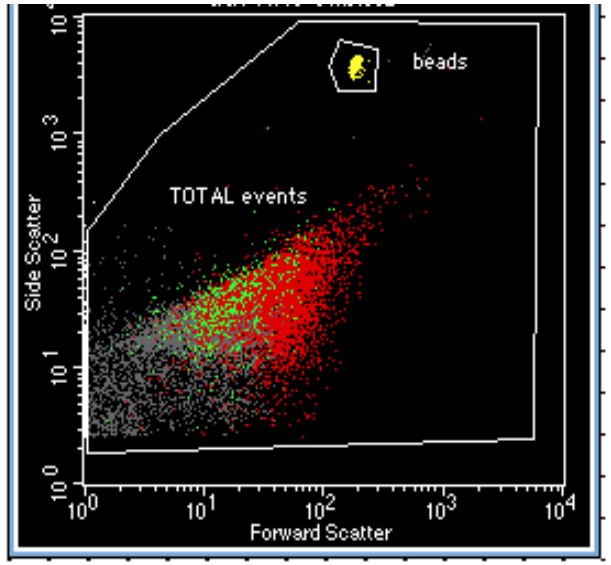
https://www.iso.org/standard/64658.html

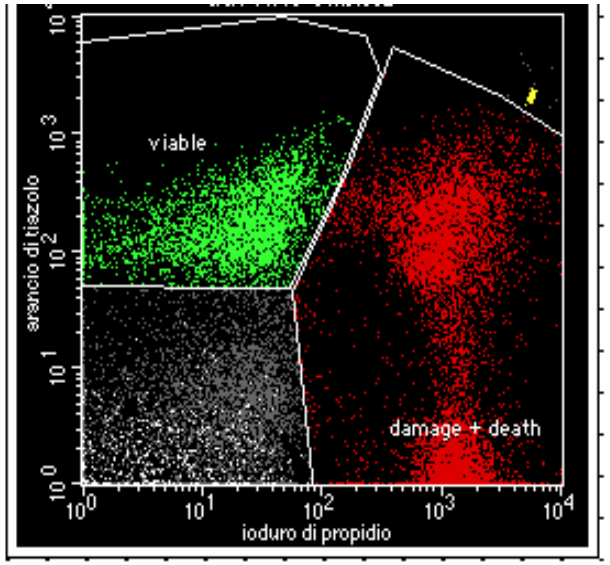
PROBIOTICAL

Probiotic Enumeration Example: Membrane Integrity

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)

AFU: Active Fluorescent Unit (viable cells)

TFU: Total Fluorescent Unit

(total number of cells: viable + damaged + dead)

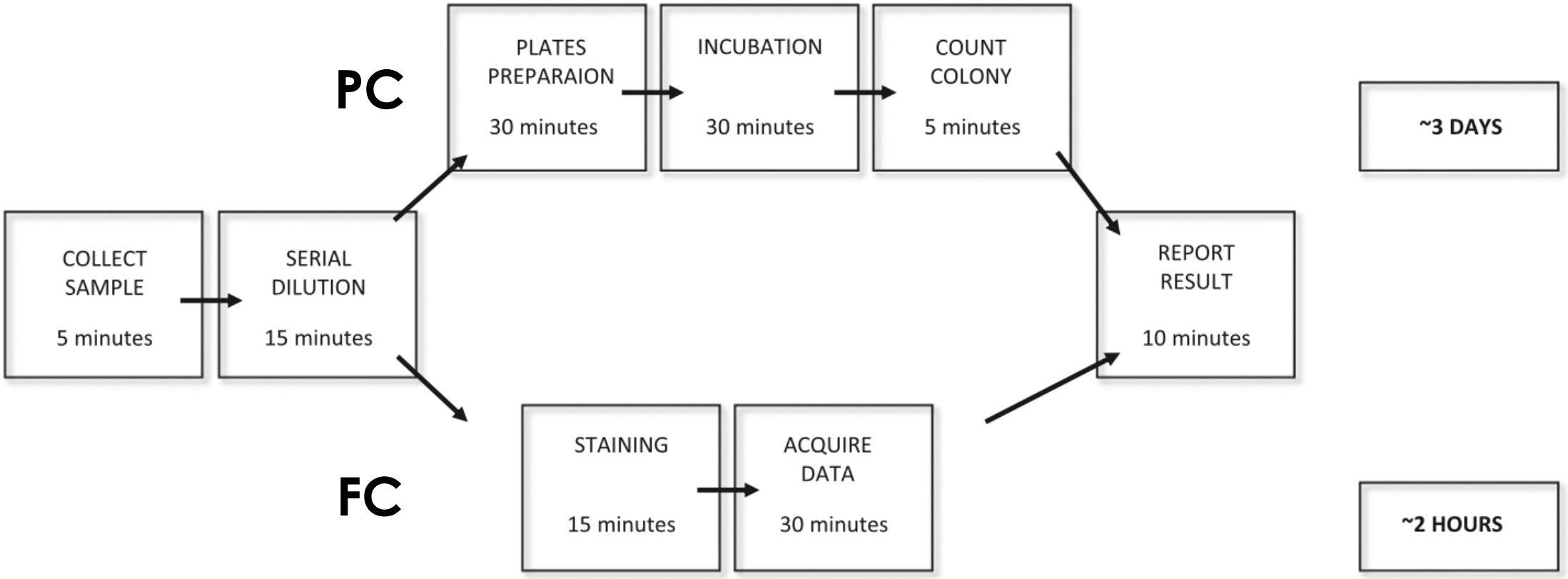
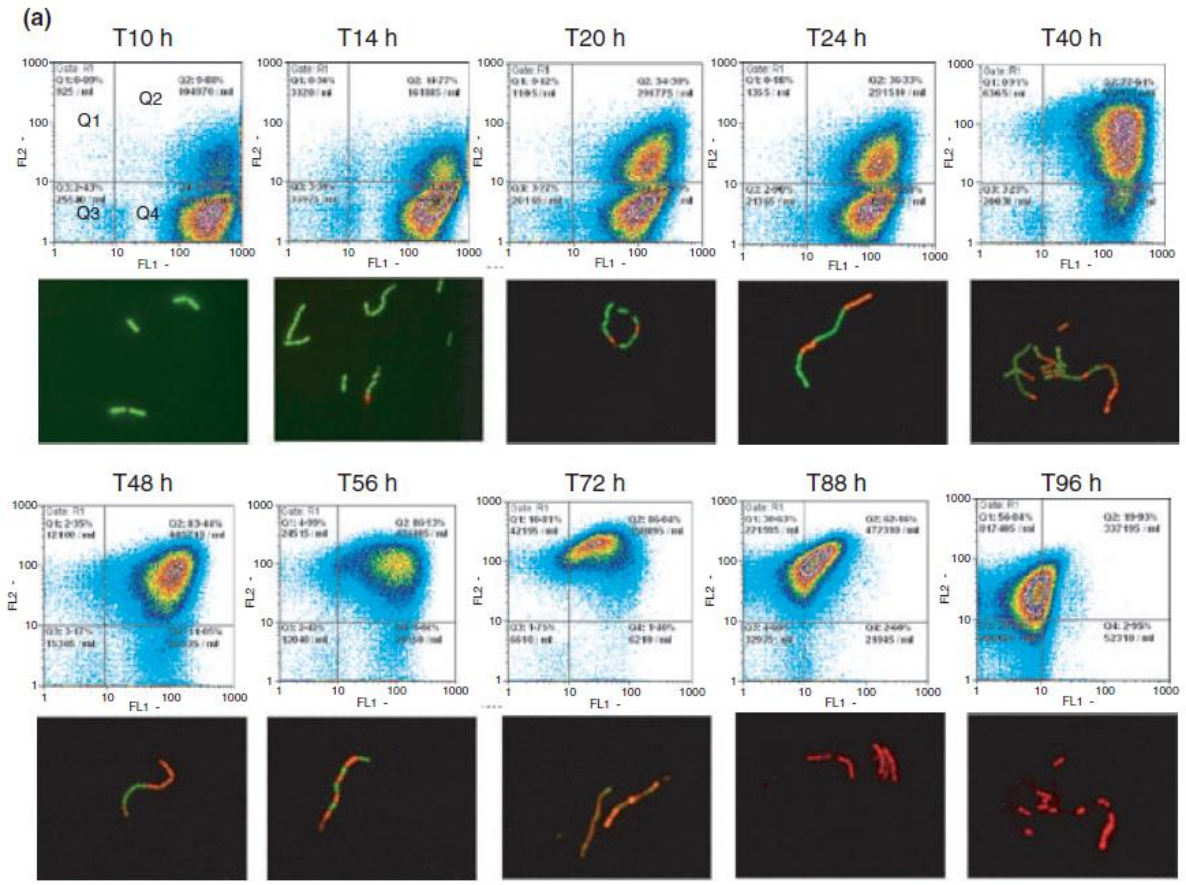
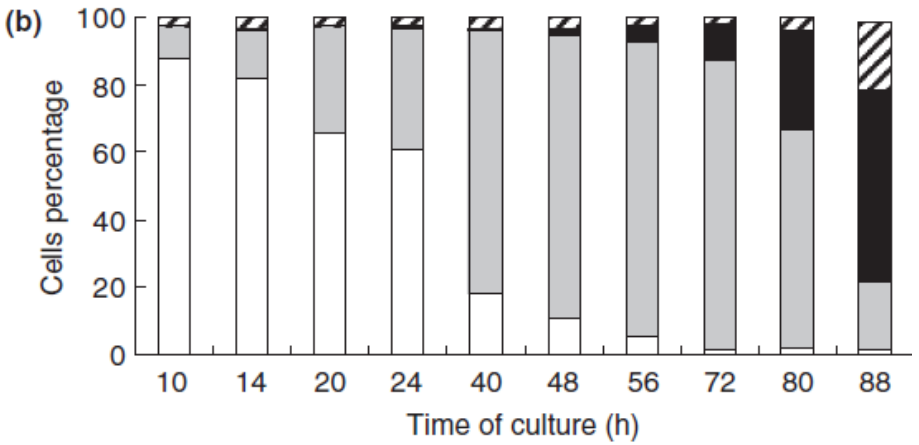


Fig. 1. Work time comparison PC method and FCM method. The plate method requires more time from researcher such as preparation of sterile media, plates and incubation time compared to FCM method which only requires a one-time optimization of the protocol and then only sample staining is required each time.

doi.org/10.1016/j.ifset.2020.102598

Bacterial population dynamic during **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.

http://dx.doi.org/10.1590/S0036-46652015000100003

PROBIOTICAL

Contents lists available at ScienceDirect

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

New insights in enumeration methodologies of probiotic cells in finished products

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Bacterial heterogeneity and population dynamic during product **STABILITY:**

the use of predictive microbiology

Table 1. Storage conditions according to International Conference on Harmonization (ICH) pharma guidelines Q1A for the development of new drug products. RH: Relative Humidity.

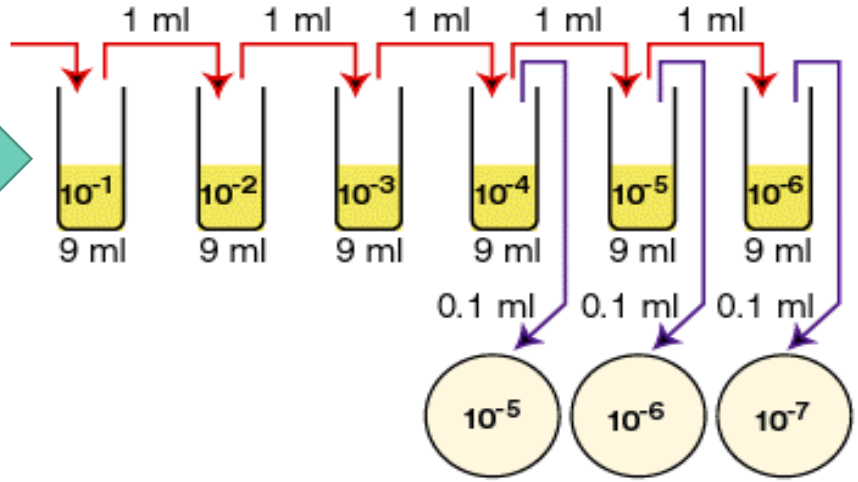
Study	Storage Condition	Minimum time needed for data submission	Control points (months)
Refrigerated condition[b]	5°C ±3°C	12[b]-24[a] months	0, 3, 6, 12, 18, 24
Long Term[a-b]	Zone II 25°C ± 2°C 60% ± 5% RH Zone IVb 30°C ± 2°C/ 75% ± 5% RH	12[b]-24[a] months	0, 3, 6, 12, 18, 24
Accelerated[a-b]	40°C ± 2°C 75% ± 5% RH	12[b]-24[a] months	0, 1, 2, 3, 6, 18, 24

a. First experiment (CFU vs AFU)
b. Second experiment (CFV vs AFU vs TFU vs pH)

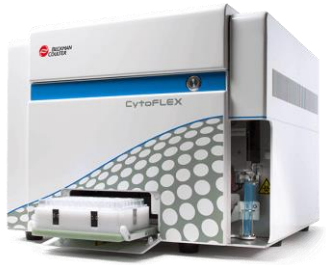
a) Measurand: cryoprotected freeze-dried single strain probiotic (*L. rhamnosus*) supported in maltodextrin

3 FREEZE DRYED INDUSTRIAL BATCH PRODUCTS

Enumeration



CFU: Colony Forming Unit



AFU: Active Fluorescent Unit Membrane Integrity
TFU: Total Fluorescent Unit

Long-term stability with PC and FC

Fig. 1. Effect of storage condition at 25 °C on bacterial stability. Plot of ln(N_t/N_0) versus time (mean of 3 values) (dotted line: FC, straight line PC).

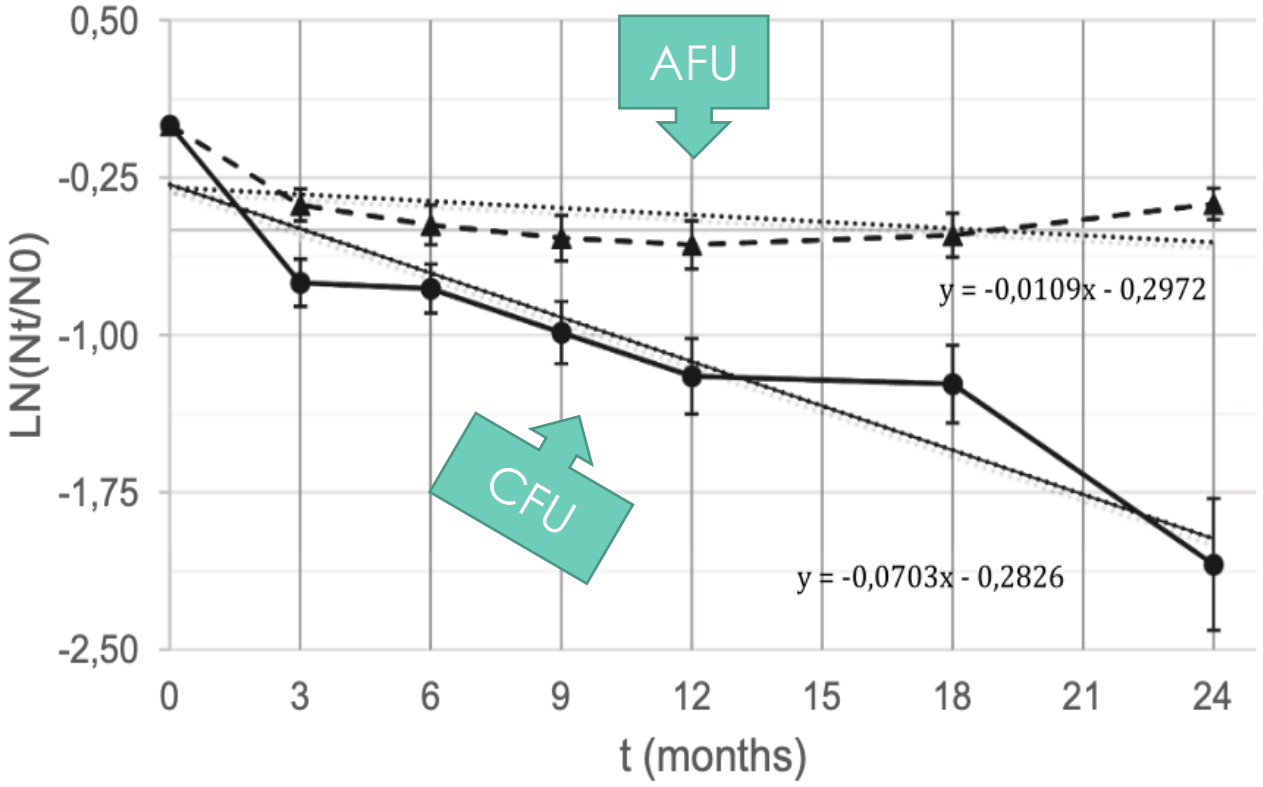
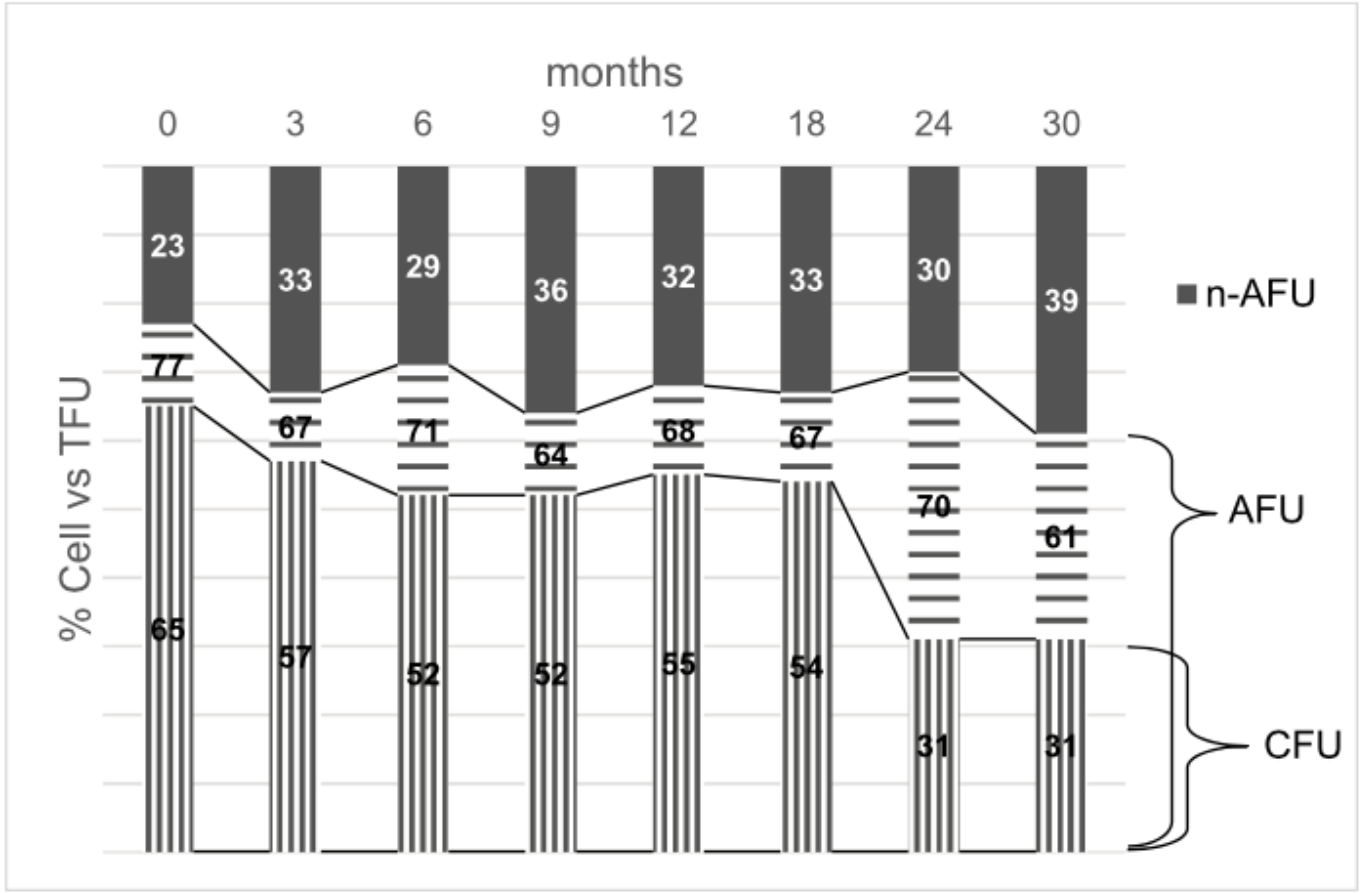


Table 3
Cultivability and membrane integrity destruction rate (k) and decimal reduction time (D_1) of probiotic samples at different temperatures.

Temperature °C (T K)	Destruction rate k (months^{-1})		k_{PC}/k_{FC}	Decimal reduction time D_1 (months)	
	PC	FC		PC	FC
25 °C (298,15)	0.0386	0.0049	7.877	59.7	469.9
30 °C (303,15)	0.0703	0.0109	6.449	32.8	211.2
40 °C (313,15)	0.7098	0.1033	6.871	3.2	22.3

doi: 10.1016/j.mimet.2020.105993.



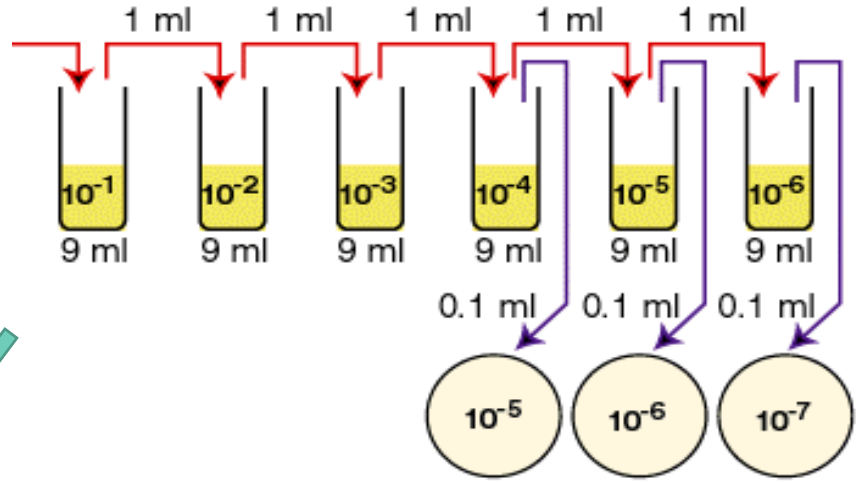
AFU-CFU = VBNC ?

Fig. 5. Bacterial heterogeneity distribution over time at 25 °C expressed as the % of cells (CFU, AFU and n-AFU) vs TFU (100% of the population at each time point). Relative % of AFU is the sum of AFU + CFU values; for example, t0 is represented by 65% CFU vs TFU, 77% AFU vs TFU and 23 % n-AFU vs TFU). First line from the top is representative of the AFU trend vs time. Second line from the top is representative of CFU trends vs time.

doi: 10.1016/j.mimet.2020.105993.

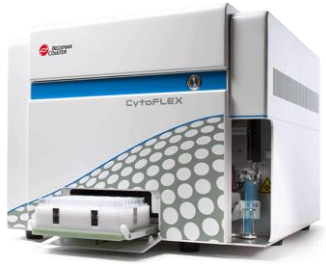
b) Measurand: cryoprotected freeze-dried multi-strain probiotic supported in inulin (accepted for publication 27/09/22)

FREEZE DRYED
INDUSTRIAL BATCH
PRODUCT

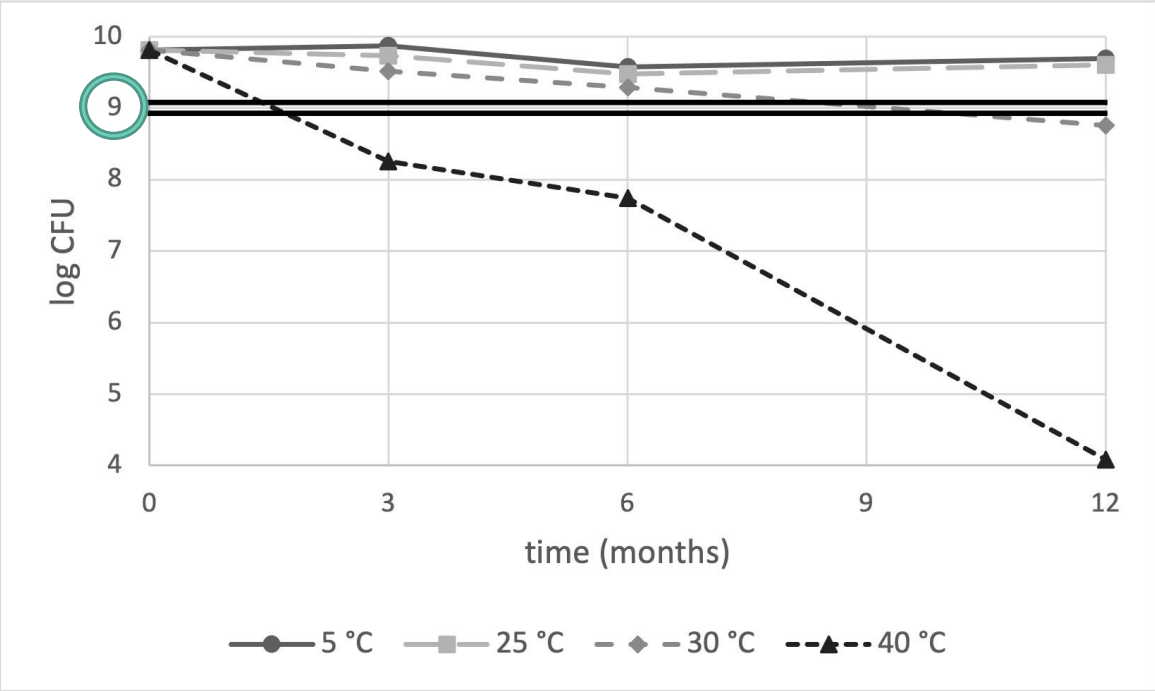


pH

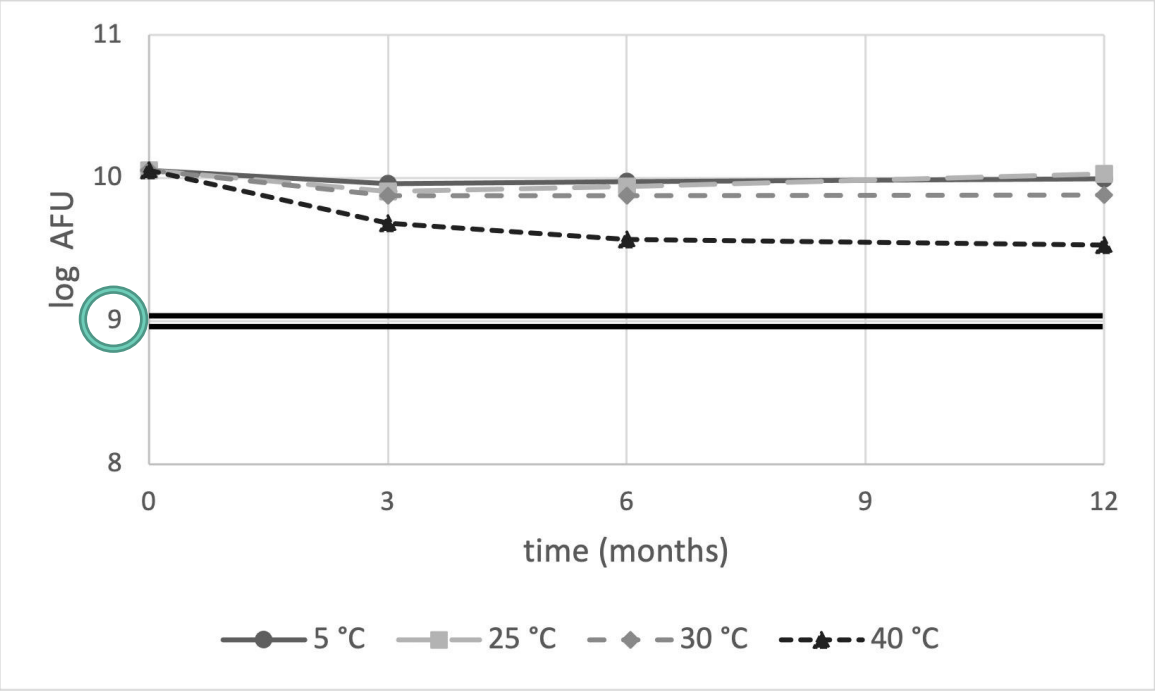
CFU
AFU
TFU



CFU and AFU trends at 5, 25, 30 and 40 °C for 12 months



CFU



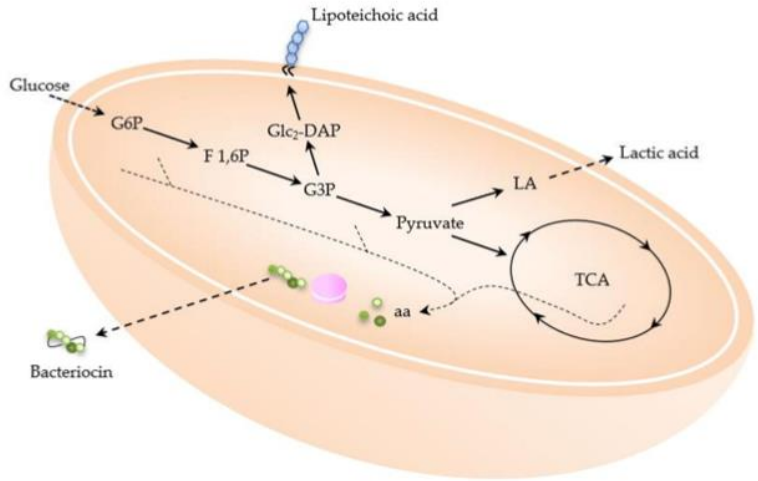
AFU

Destruction rate and Decimal Reduction Time

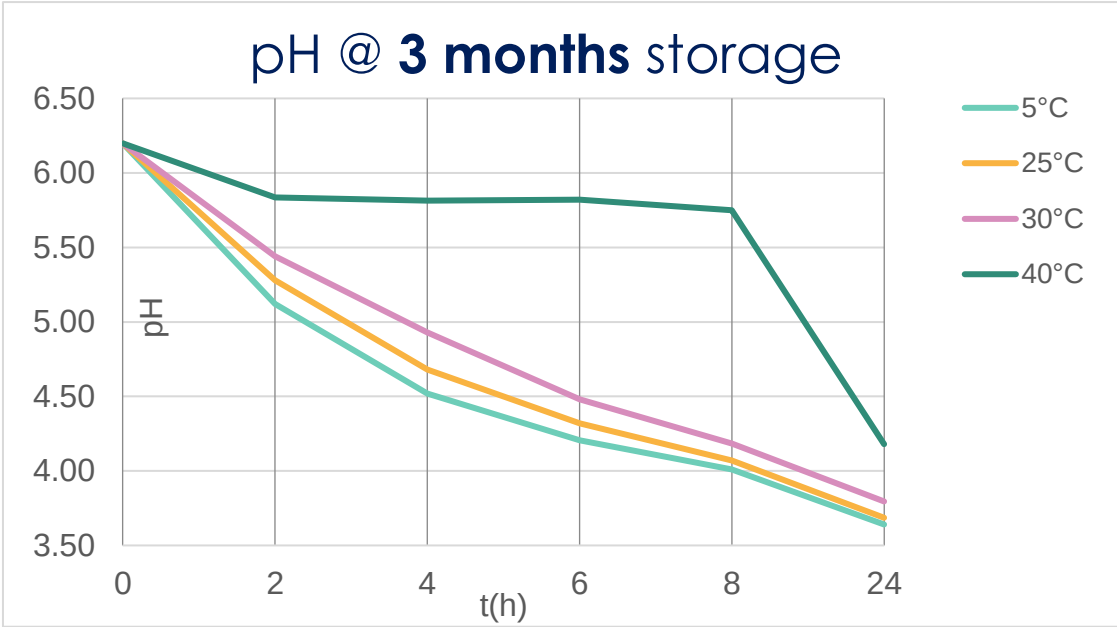
Table 2. Bacterial/probiotic cultivability and membrane integrity destruction rate (k) and decimal reduction time (D_1) of synbiotic samples at different temperatures.

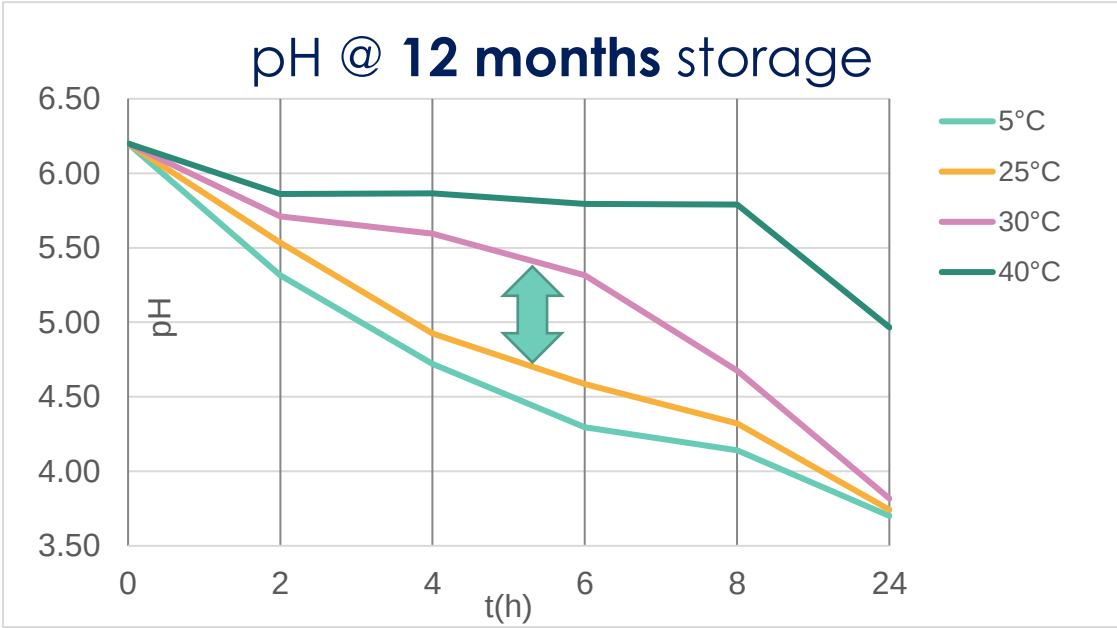
Temperature	Destruction rate k (months-1)			Decimal reduction time D1 (months)		
	CFU	AFU	TFU	CFU	AFU	TFU
5 °C (278.15 °K)	0.0247	0.0025	0.0020	93.2	921.0	1151.3
25 °C (298.15 °K)	0.0468	0.0034	0.0025	49.2	677.2	932.0
30 °C (303.15 °K)	0.1844	0.0161	0.0141	12.5	143.0	163.3
40 °C (313.15 °K)	0.6983	0.1417	0.1212	3.3	16.2	19.0

CFU= Colony Forming Units; AFU= Active Fluorescent Units; TFU= Total Fluorescent Units



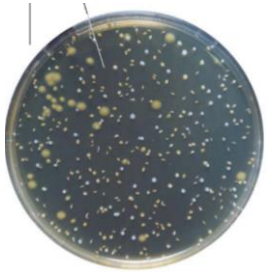
pH as a metabolism proxy thanks to lactic acid production



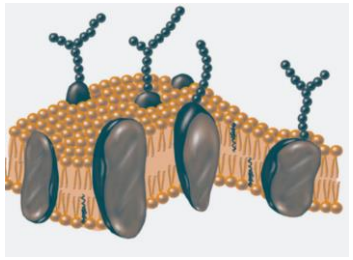


Decay Kinetics of TFU, AFU, CFU and pH @12 months

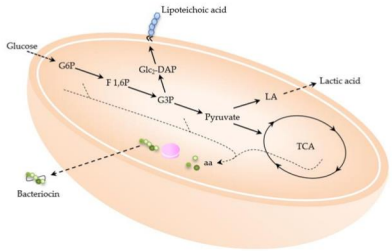
CFU

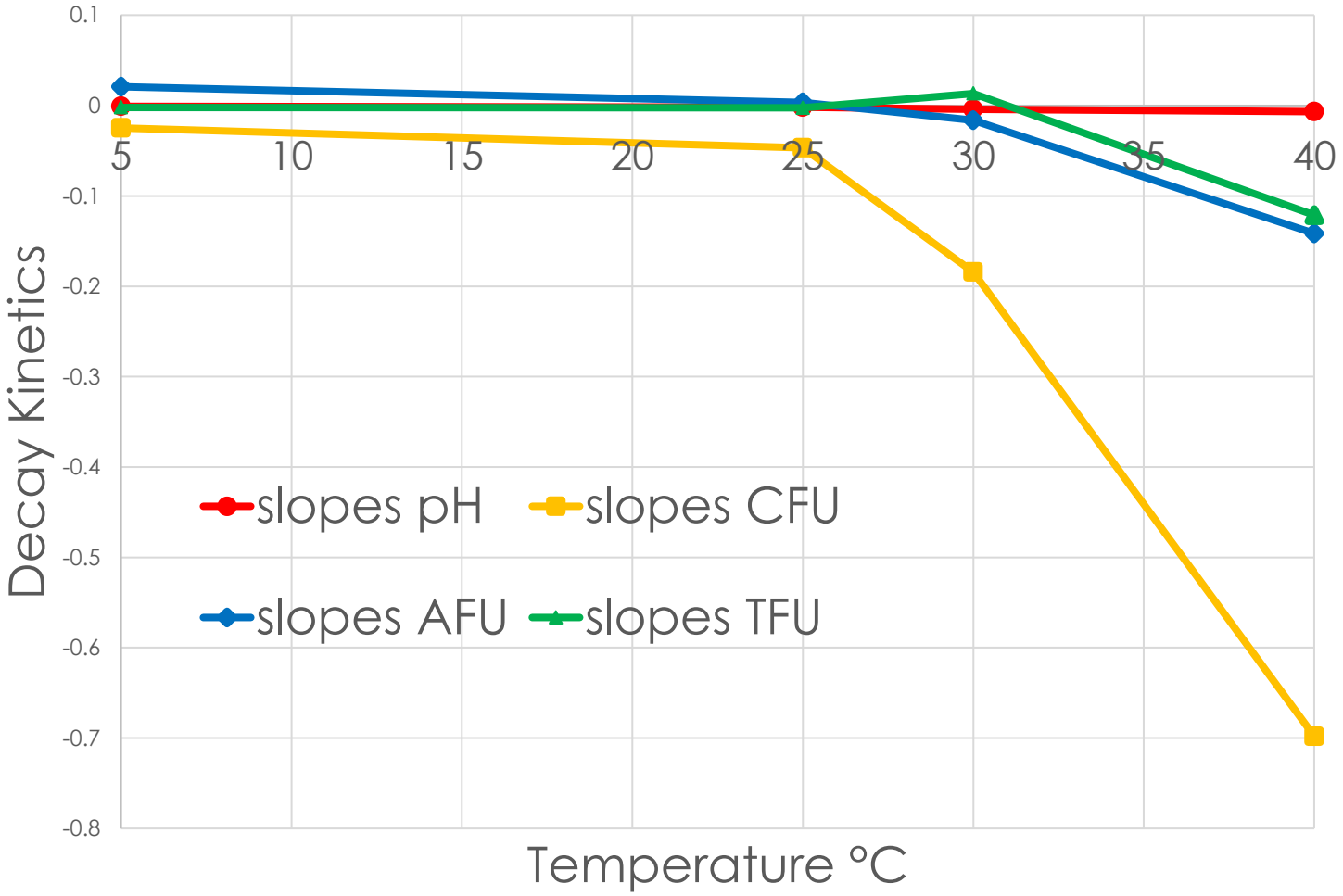


AFU
TFU



pH





Viable But Non-Cultivable (VBNC) cells

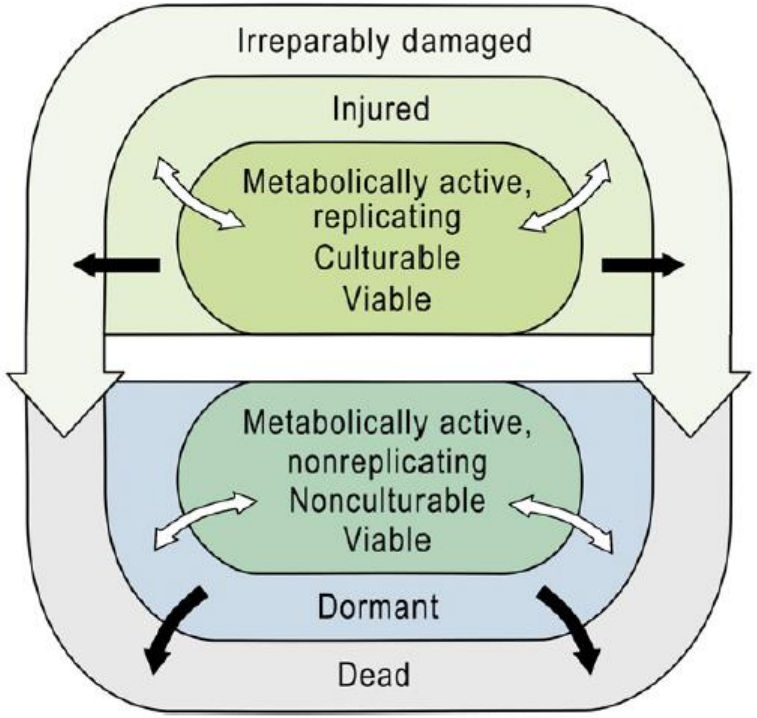


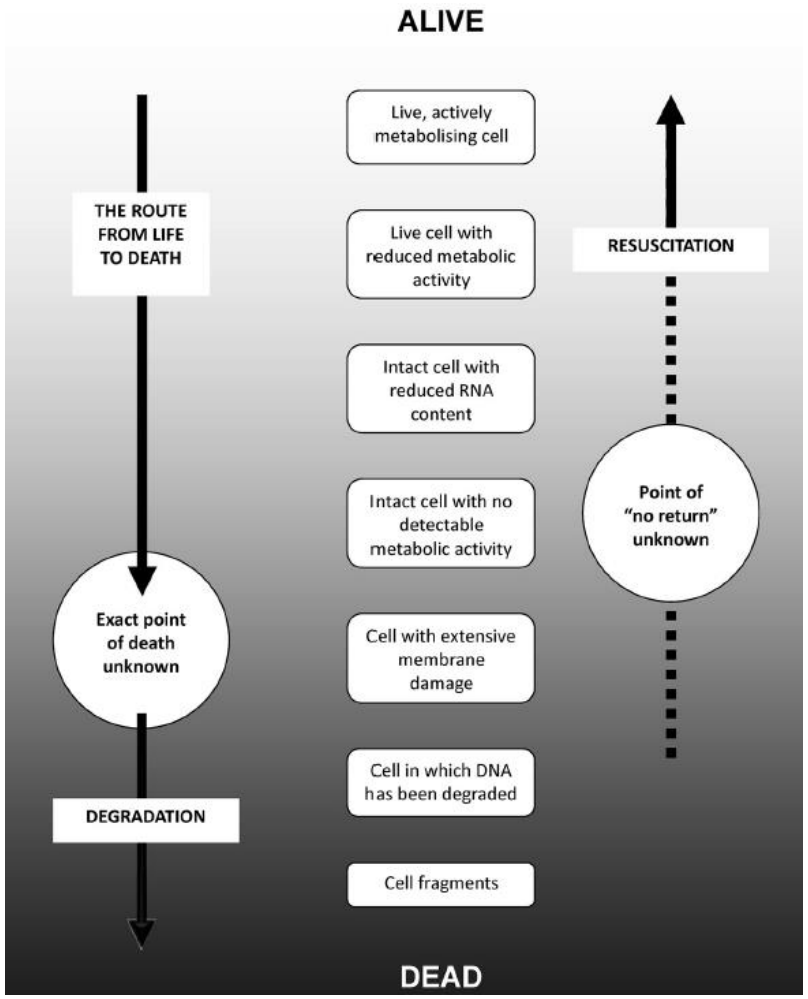
Fig. 1. A concept map for probiotic strains that describes metabolically active, replicating/culturable/viable states and the transitions that are possible. The arrow on the perimeter and the black one-way arrows indicate that once a cell is non-viable/dead it does not return to a viable state.

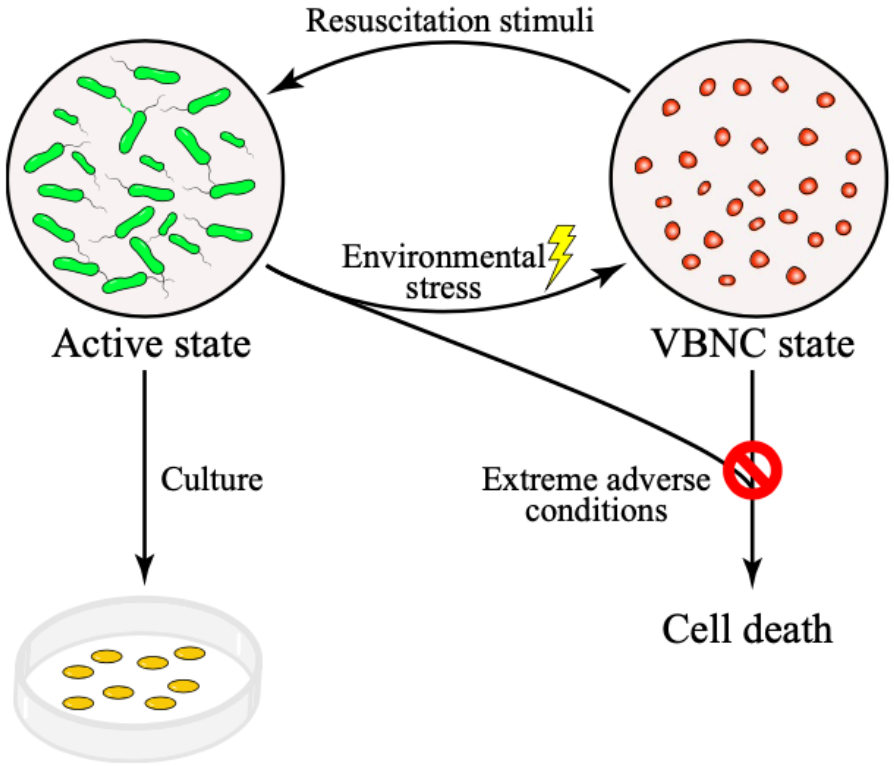
*"**Microbes exists in a variety of growth phases and metabolic states** depending on environmental conditions and stressor, and **only a subset of these involve active replication**. The convention that viable microbes must be capable of forming colonies excludes not only dead or irreparably damaged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

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

PROBIOTICAL

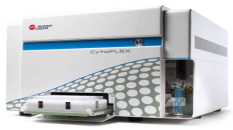
Is it possible to “see” VBNC and prove that they could revert to CFU (close the gap between AFU and CFU)?





https://doi.org/10.1007/s42995-020-00041-3

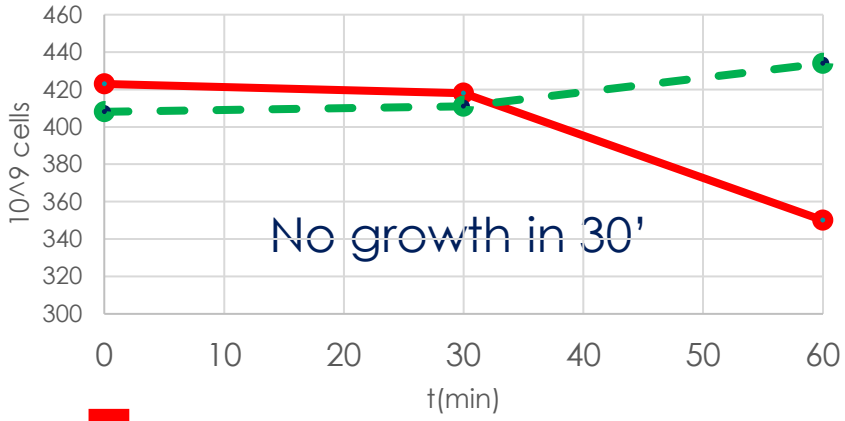
c) Measurand: 400x10 ^9 AFU/g cryoprotected freeze-dried *Lactobacillus acidophilus supported* in maltodextrin



SF AFU

MRS AFU

TFU and AFU @30 and 60 minutes in FS (ISO) and MRS (USP-NF)



Vs AFU 82%

USP vs ISO +33%

Vs AFU 57%

330x10^9CFU

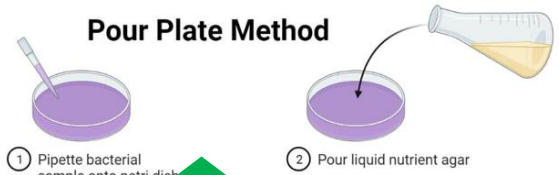
230x10^9CFU

USP

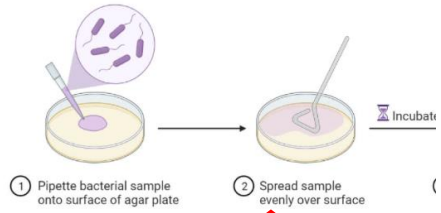
10 replica in triplicate later ..

ISO

Pour Plate Method



Spread Plate Method

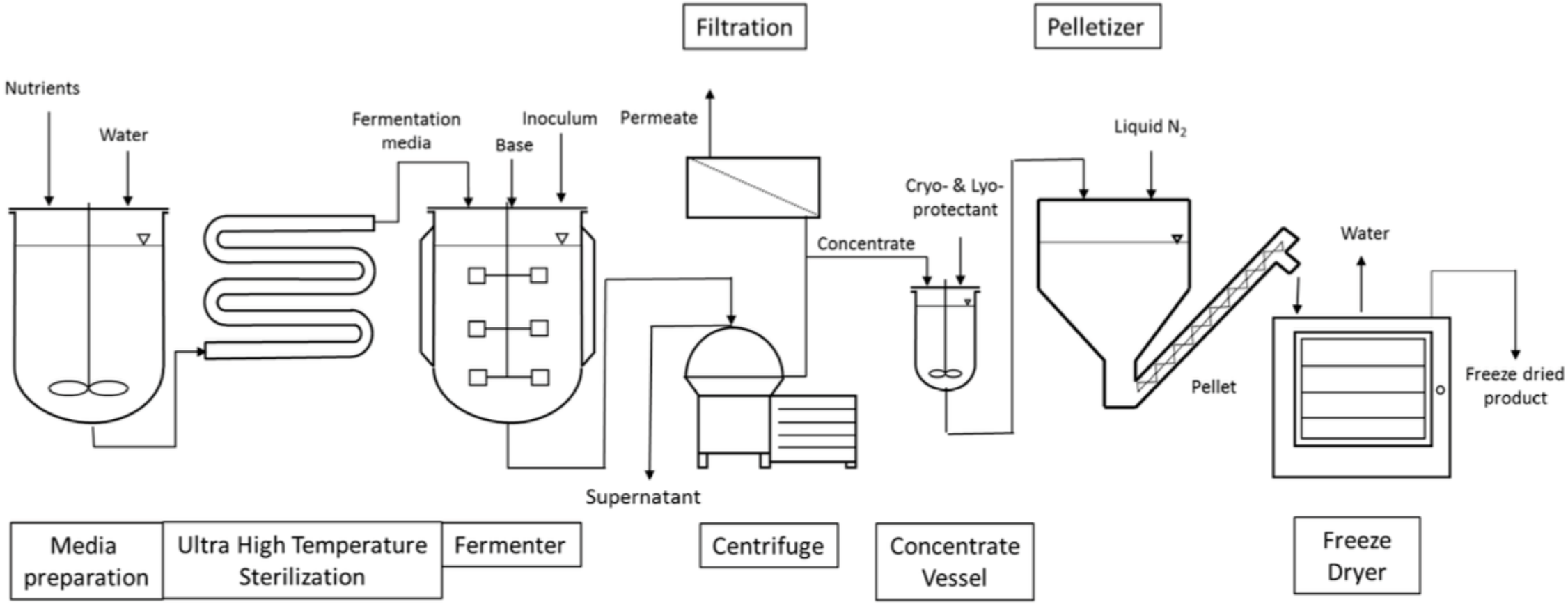


USP-NF: 30 min incubation

ISO 20128: 0 min incubation

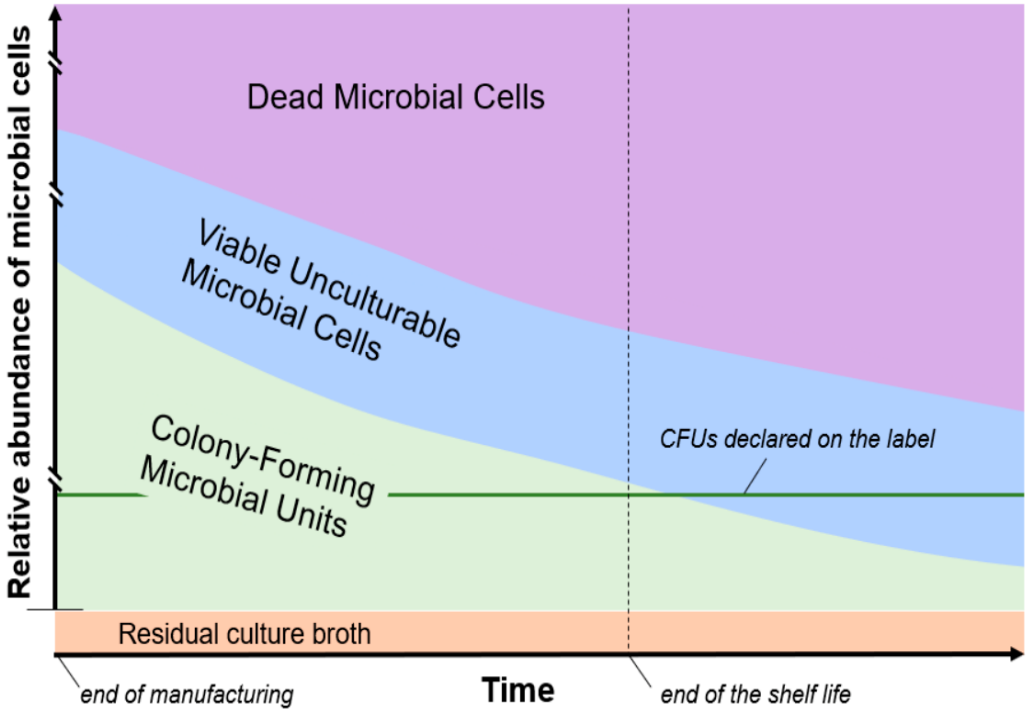
PROBIOTICAL

The industrial production of probiotics is stressful for the bacterial cell



doi:10.3390/microorganisms7030083

Generally probiotic food supplements are characterized by a heterogeneous bacterial population



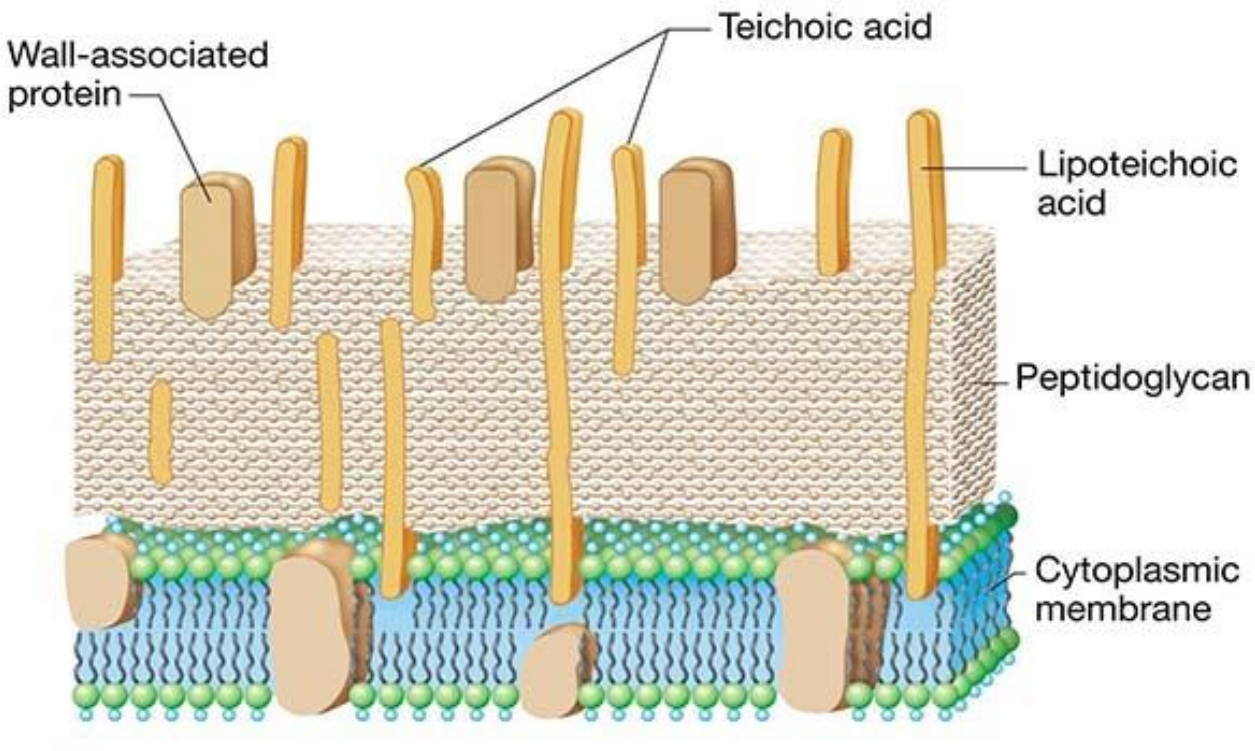
TFU

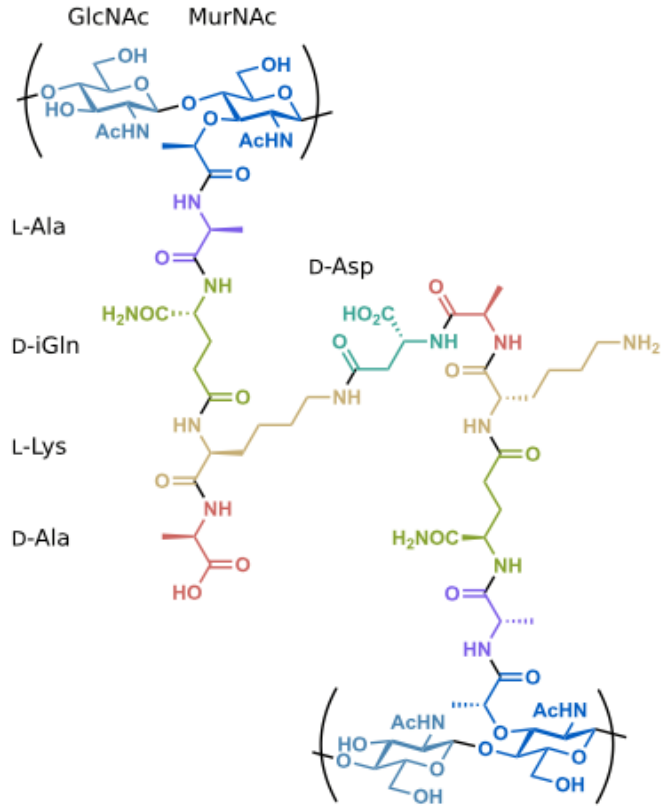
VBNC ≤ AFU

Can we expect that some biological function are better represented by TFU and AFU than CFU ?

doi:10.3390/microorganisms8081177

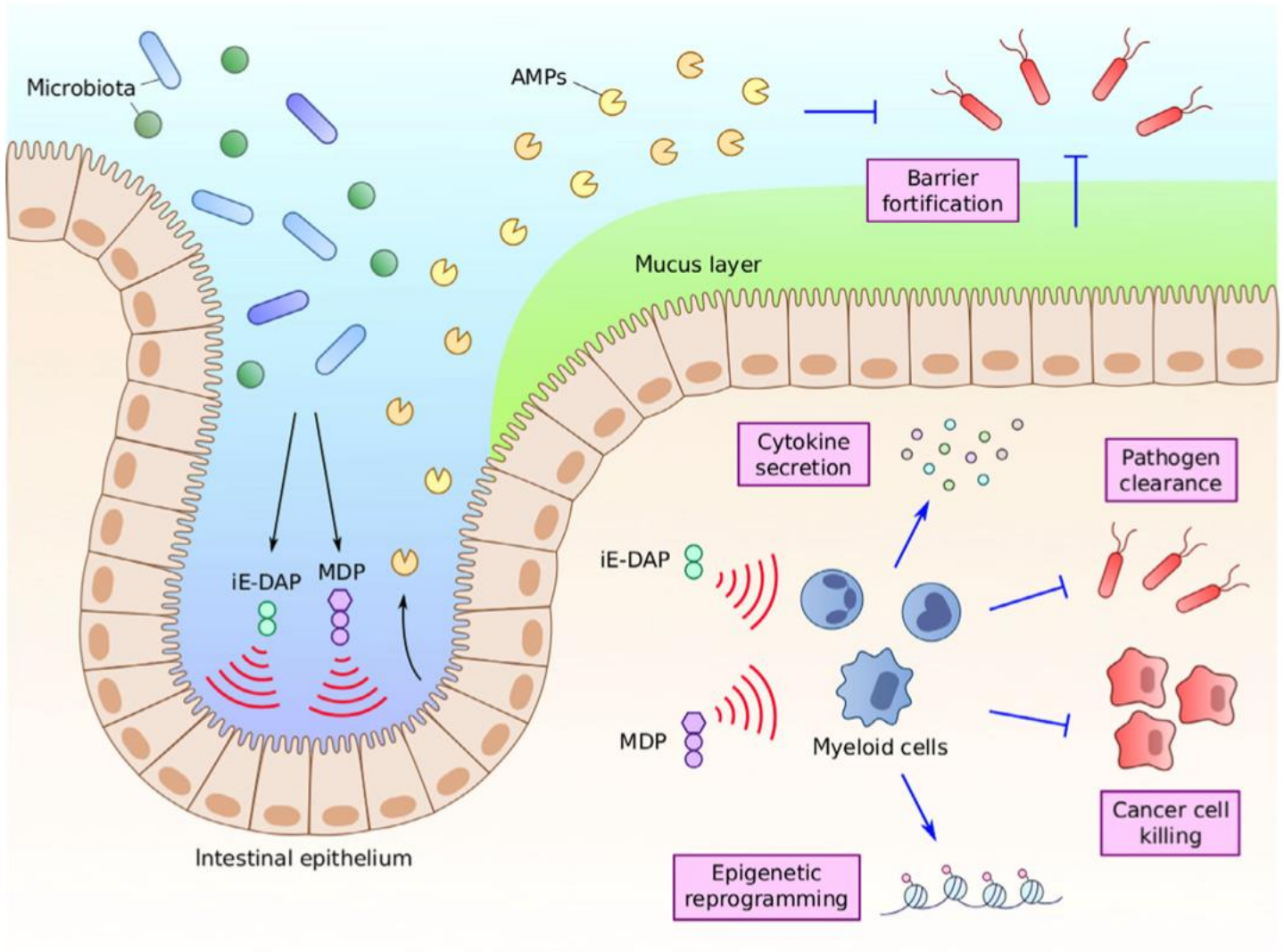
AFU and TFU are representative of cells number and ... their cell wall.





doi: 10.1002/cti2.1095

Peptidoglycan as immune-modulators



CFU could understimate the effective quantity of functional probiotic cells

doi: 10.1002/cti2.1095

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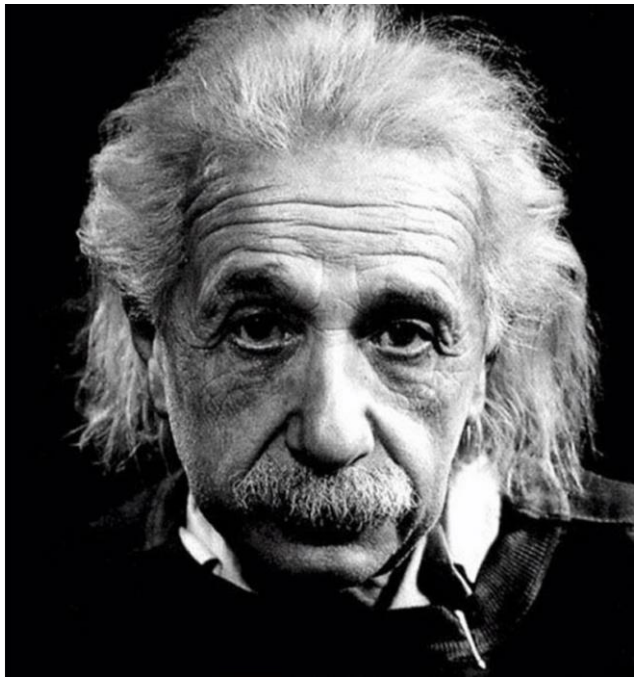

"It is the theory (method) which decides what can be observed"

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