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

USP Symposium, October 7th 2022
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Main topics

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

Representation of various methods for the determination of bacterial viability.
Plate count methods

![Diagram of plate count method](image)

1 ml

- 1 ml
- $10^{-1}$
- 9 ml
- $10^{-2}$
- 9 ml
- $10^{-3}$
- 9 ml
- $10^{-4}$
- 9 ml
- $10^{-5}$
- 9 ml
- $10^{-6}$
- 0.1 ml
- $10^{-7}$
- 0.1 ml
- 0.1 ml

Sample of *E. coli*
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 |
Flow Cytometry (FC) principle

ISO 19344
3 staining procedure:
• Membrane integrity
• Enzymatic activity
• Membrane polarization

Basically it “just” enumerate cells

https://www.creative-bioarray.com/support/principle-of-the-flow-cytometry.htm
Figure 2. (a) Simplified equivalent circuit model of *E. coli* bacteria. The model is composed of components representing the electrolyte resistance and capacitance (R<sub>m</sub>, C<sub>m</sub>), the resistance and capacitance of the cell membrane (R<sub>mem</sub>, C<sub>mem</sub>) and the resistance of the cell interior (R<sub>cyto</sub>). (b) Electric field penetration in polystyrene beads and in bacteria with different viability states at low and high frequencies.
Milk and milk products — Starter cultures, probiotics and fermented products — Quantification of lactic acid bacteria by flow cytometry

SAME method for ALL the lactic bacteria!
Propidium iodide (PI) penetrates only bacteria with damaged membranes with red fluorescence (x axis).

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

Probiotic Enumeration Example: Membrane Integrity

Forward Scatter (FS) = dimension
Side Scatter (SS) = granularity and morphology

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.
New insights in enumeration methodologies of probiotic cells in finished products

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Bacterial heterogeneity and population dynamic during product \textbf{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<sup>b</sup> | 5°C ±3°C                                | 12<sup>b</sup>-24<sup>a</sup> months   | 0, 3, 6, 12, 18, 24      |
| Long Term<sup>a,b</sup> | Zone II 25°C ± 2°C  
60% ± 5% RH  
Zone IVb 30°C ± 2°C/  
75% ± 5% RH | 12<sup>b</sup>-24<sup>a</sup> months | 0, 3, 6, 12, 18, 24      |
| Accelerated<sup>a,b</sup> | 40°C ± 2°C  
75% ± 5% RH | 12<sup>b</sup>-24<sup>a</sup> 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                              |
| 30 °C (303,15)       | 0.0703                         | 0.0109    | 6.449                             | 32.8                              |
| 40 °C (313,15)       | 0.7098                         | 0.1033    | 6.871                             | 3.2                               |

doi: 10.1016/j.mimet.2020.105993.
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.
b) **Measurand:** cryoprotected freeze-dried multi-strain probiotic supported in inulin  
(accepted for publication 27/09/22)
CFU and AFU trends at 5, 25, 30 and 40 °C for 12 months
## 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 $D_1$ (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
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

http://dx.doi.org/10.1016/j.mimet.2014.04.012
Is it possible to “see” VBNC and prove that they could revert to CFU (close the gap between AFU and CFU)?
c) Measurand: 400x10^9 AFU/g cryoprotected freeze-dried *Lactobacillus acidophilus* supported in maltodextrin

No growth in 30'

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

USP-NF: 30 min incubation

ISO 20128: 0 min incubation

10 replica in triplicate later ..

ISO

USP

Vs AFU

82%

Vs AFU

57%

330x10^9 CFU

30 min incubation

230x10^9 CFU

USP vs ISO +33%

Pour Plate Method

Spread Plate Method

Pipe the bacterial sample onto petri dish

Pour liquid nutrient agar

Pipe the bacterial sample onto surface of agar plate

Spread sample onto plate surface

Incubated
The industrial production of probiotics is stressful for the bacterial cell
Generally probiotic food supplements are characterized by a heterogeneous bacterial population. Can we expect that some biological functions are better represented by TFU and AFU than CFU?
AFU and TFU are representative of cells number and ... their cell wall.
Peptidoglycan as immune-modulators

CFU could underestimate the effective quantity of functional probiotic cells
“It is the theory (method) which decides what can be observed”
