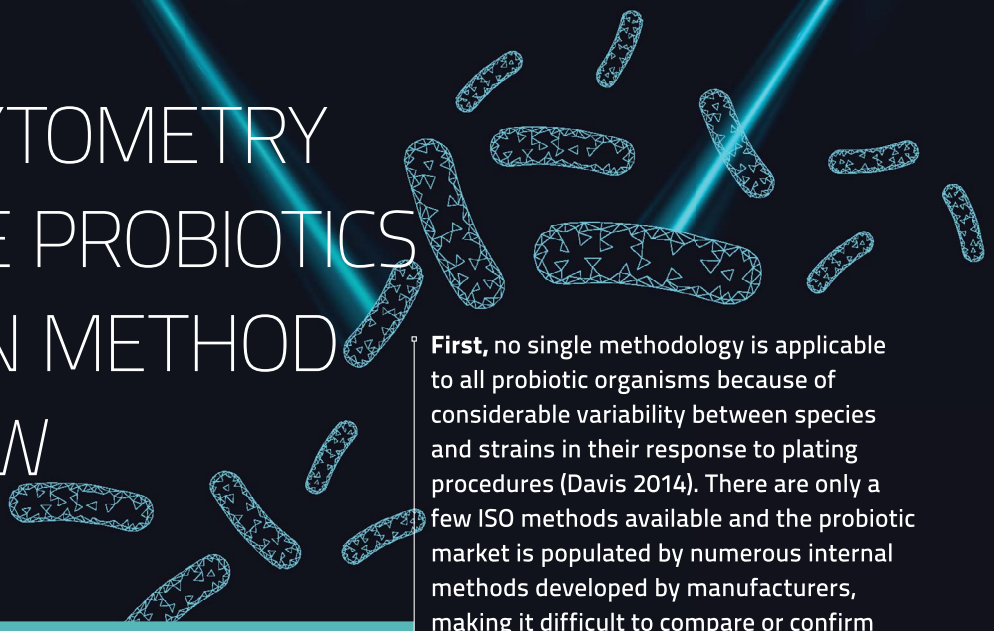
WHY FLOW CYTOMETRY COULD BE THE PROBIOTICS ENUMERATION METHOD OF TOMORROW



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Definition of Probiotics and Analytical Implications

Probiotic products are booming all over the World, in a diversity of foods from yogurt to kefir, kimchi, kombucha, sauerkraut and enriched products as well as dietary supplements, with a global market expected to exceed 85 billion USD by 2026 (Research and Markets, Probiotics – Global Market Outlook 2017-2026). Several definitions of the term "probiotic" have been used over the years, but the one from the Food and Agriculture Organization of the United Nations/World Health Organization is the one broadly used and recognized: "live microorganisms, which when administered in adequate amounts confer a health benefit to the host" (FAO/WHO 2001).

Inherently to the definition of probiotics are therefore the concepts of quantity and viability. Since over a century, bacterial viability is assessed by Plate Count (PC) methods, which evaluate bacterial cells' ability to proliferate into detectable colonies on agar media and specify the results in Colony Forming Units (CFU). PC is currently considered the "gold standard" for analytical quantification of probiotics and most recognized standards such as the International Organization for Standardization (ISO), the International Dairy Federation (IDF) and the United States Pharmacopeia (USP) apply PC methods for bacterial enumeration of beneficial bacteria as well as contaminants (ISO 29981, ISO 20128, USP<2021>;<2022>).

Aside from being widely accepted, the benefits of PC are mainly about technical simplicity and ease of implementation, since classical microbiology protocols do not require specialized equipment or scientific skills to be executed. Moreover, the resulting CFU undoubtedly arise from viable bacteria in the sample, as bacterial proliferation is a prerequisite for colony formation. In addition, clinical studies investigating the effects of probiotics generally apply CFU as a dose measure. To our knowledge, Jager *et al.* (2016) published the first clinical study on probiotics reporting the dose both in CFU and AFU (Active Fluorescent Unit, unit described here-under). A limit of Flow Cytometry (FCM) is that correlation factors between AFU and CFU are not yet fully apprehended, which makes difficult the extrapolation of studies published reporting CFU into products measured in AFU.

On the other hand, PC analyses present several limits which a statement of 1916 by Breed and Dotterrer clearly summarizes: "The matter of selecting plates to be used in computing a count becomes a matter of considerable judgement".

First, no single methodology is applicable to all probiotic organisms because of considerable variability between species and strains in their response to plating procedures (Davis 2014). There are only a few ISO methods available and the probiotic market is populated by numerous internal methods developed by manufacturers, making it difficult to compare or confirm strains quantities in products.

Second, PC methods are laborious in terms of laboratory workload and sample throughput, and with a long time-to-result (often 72 hours) due to lengthy periods of incubation.

Third, there can be considerable technical difficulties in determining suitable growth conditions for each strain, especially with regard to oxygen-sensitive species which are highly adapted to the gastrointestinal environment and consequently challenging to cultivate *in vitro*.

Fourth, precision is quite low. Reproducibility (the degree of agreement between the results of experiments conducted by different individuals, at different locations, with different instruments) between the different PC ISO methods ranges from 0,5 to 1 log, meaning the methods' results have a multiplication range of 10 and more. The variability is such that the Italian Ministry of Health guidelines on probiotics and prebiotics advised the following: "The amount of cells present must be listed on the label /.../ with uncertainty of 0,5 log. It is emphasized that the analytical method of quantification of living bacterial cells may differ from species to species". The European Scientific League for Probiotics (ESLP) proposed a quality seal based on scientific evaluation and control of the CFU content with an acceptable variability of a 1 log factor (Warzee 2016). It means that the product maintains the claim within a reduction of ten times of the declared amount.

Finally, bacterial quantification in CFU may be a significant underestimation of the actual viable cells count in the sample, as Viable But Not Culturable (VBNC) cells by definition don't give rise to colonies, and because aggregates or chains of microbial cells can give rise to only one colony.

Flow Cytometry (FCM), a Modern Method to Measure Different Viability Parameters

The principle of FCM is that cells are sent in a nozzle and analyzed one by one by a laser. The forward light scatter provides information on the cell's dimension and the side scatter instructs on its granularity and morphology. In addition, three different staining protocols are described in the ISO 19344 IDF 232 method, published in 2015, to identify enzymatic activity, membrane integrity or membrane potential (which connect to the definition of bacterial viability). The membrane integrity protocol, for example, is based on a colorant that penetrates all bacteria (identifying Total Fluorescent Units, TFU: bacteria that are live, damaged and dead altogether) and another colorant which penetrates only bacteria with a damaged membrane (damaged and dead). The difference between the two groups is expressed as Active Fluorescent Units (AFU) and represents the viable (intact cells) based on this protocol (See Figure 1).

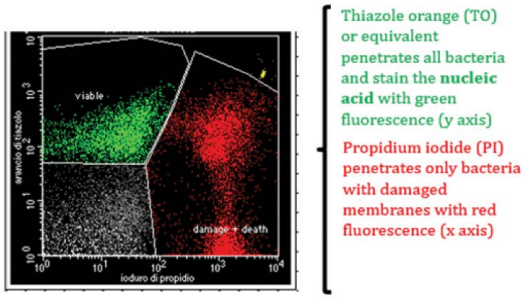


Figure 1: A result of a FCM analysis

The advantages of FCM are several:

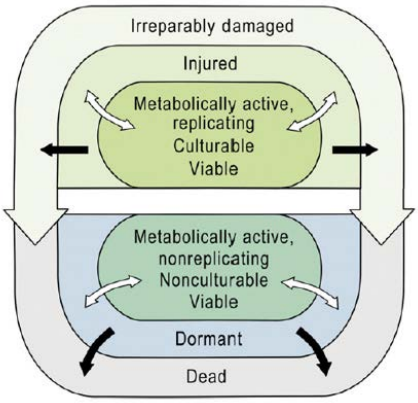
First, it can be applied universally and independently of the species. The same methodology allows the enumeration of live *Bifidobacteria, Lactobacilli, Streptococci,* yeasts, contaminants, etc.

Second, it is quick: in 30-45 minutes an operator can obtain a triplicate analysis.

Third, it does not depend on growth conditions and does not require the study of the best medium and parameters for multiplication as a prerequisite, though it requires control of sample preparation to avoid background noise.

Fourth, accuracy is much higher than PC's. The ISO method 19344-IDF 232, based on 1800 analyses done by 15 laboratories in 5 countries using 9 different flow cytometers indicated a high degree of equivalence (no statistical differences) for AFU results obtained from the 3 different staining protocols (enzymatic activity, membrane integrity and membrane potential). AFU/g results' repeatability (r) was determined as 0,06 log and reproducibility (R) as 0,45 log. This reproducibility can be further improved with proper training, as shown by Pane *et al.* (2018) who obtained a repeatability of 0,07 and reproducibility of 0,09 log with FCM between two different labs.

Finally, FCM provides greater insight into the functional strain-related responses to various applications (Wilkinson 2018) and can inform on the heterogeneity in a bacterial sample. Besides and crucially, FCM does not require cells to grow and can therefore recover dormant cells or Viable But Not Culturable (VBNC) cells: bacterial populations displaying metabolic activity but loss of culturability (Lennon & Jones 2011). They can be biologically active and their capacity to replicate can also be revived by favorable changes in environmental conditions (Rittershaus *et al.* 2013) (See Figure 2).



Microbes exist 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

Figure 2: 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.

Of VBNC Cells Biological Importance

Metabolically active VBNC cells may recover from sublethal injuries and thus become viable under optimal conditions, e.g. in contact with the enteric system, but these cells may also be too injured to proliferate even under optimal conditions (Barer 1997). VBNC cells can frequently be observed in probiotic products due to numerous unavoidable stressful processes that probiotic cultures must undergo during industrial production. Indeed, fermentation, biomass concentration, cryopreservation, drying by lyophilization, powder grinding, and storage represent critical processes that can, even when fully optimized, drive cells to enter in a VBNC state (Lahtinen *et al.* 2006; El Arbi *et al.* 2011).

Most food-borne pathogens (*Campylobacter jejuni, Campylobacter coli, Enterococcus faecalis, Escherichia coli, Helicobacter pylori, Salmonella, Shigella, Vibrio cholerae* etc.,) enter VBNC states (Ramamurthy *et al.* 2014). For this reason FCM enumeration is now officially recommended for all freshwater analysis in Switzerland for the detection of pathogens (Egli and Kotzsch 2015; Van Nevel *et al.* 2017). If VBNC pathogens are dangerous and need to be kept under control by FCM because they are able to bloom once arrived in a favorable ecosystem, can't we imagine that probiotics, which are also enteric bacteria in origin, would do the same?

When Staley and Konopka (1985) studied soil bacteria, they found that approximately only 0.1-1.0% of the total bacteria can be enumerated by the plating procedure. Indeed, as a general rule the maximum recovery of heterotrophic soil bacteria is 1% of the total direct count using plating procedures. From a microbiological perspective, only a few percent of the bacterial cells enumerated by direct microscopic count can be cultured and identified. Should we take into account that using plate count as a proxy for viability could exclude 99% of the bacteria? In our gut, about 20% of the bacteria present are in a dormant state (Lennon and Jones 2011), which contributes to the maintenance of microbial diversity, allowing strains to bloom in the presence of a rare or new nutrient source. VBNC bacteria can resuscitate when entering a favorable environment, such as in co-culture with eukaryotic cells (Takeda *et al.* 2012; Davey 2011). If this is a strategy employed by our gut microbiota, should we believe that probiotic bacteria are able to do the same, enter a VBNC state when going under stress, and revitalize when deployed in the gut?

Even without taking into consideration that VBNC cells can resuscitate, there is good reason to believe that dead strains play a biological role too. 90 years ago, Frederick Griffith made an experiment: he injected mice with a virulent pathogen and the mice died. He injected other mice with a non-virulent pathogen and the mice lived. He heat-killed the virulent pathogen and injected it in other mice and they lived. Then, he tested whether a mix of heat-killed virulent bacteria and the live non-virulent pathogen would be harmful and the mice died. Could it be that the virulent, dead cells transferred their virulence genes to the non-virulent neighbors? Could it be that probiotics could also share their genes in the surrounding microbial community? Isn't it a clue that the EFSA safety guidelines require the absence of antimicrobial resistance genes on probiotics? Can we imagine that other positive features could be horizontally transmitted during probiotic supplementations? One example is *Lactobacillus plantarum* (Siezen and Van Hylckama Vlieg 2011) described as a "natural metabolic engineer" due to functional gene cassettes, in particular for complex carbohydrates utilization, which can be acquired, shuffled, substituted or deleted in response to niche requirements. Could probiotic efficacy also be directly linked to the fact that during supplementation are provided not only "cells" but as well their DNA content which can be functionally transferred to other members of the microbiota?

Many other studies since Griffith have showed that in certain cases, bacteria killed by heat or pressure can prove as or even more effective than the live same strains, thanks to the transfer of information (DNA) in some cases, and thanks to the interaction between elements expressed on the bacteria's membranes or cell walls and our immune system (Cani *et al.* 2017; Mogna *et al.* 2018; Sugahara *et al.* 2017).

These hypotheses need to be further substantiated, however they could open to a re-imagination of probiotics applications and possibilities. Using PC as a proxy of viability is somewhat equivalent to considering that infertile individuals are not alive. It is excluding whole bacteria groups (up to 99% of all bacteria) and whole bacteria states (about 20% of VBNC bacteria in our gut) which however play a role in our health. Complementary methods such as FCM allow to retrieve more of these bacteria and to recover more information on their viability and heterogeneity.

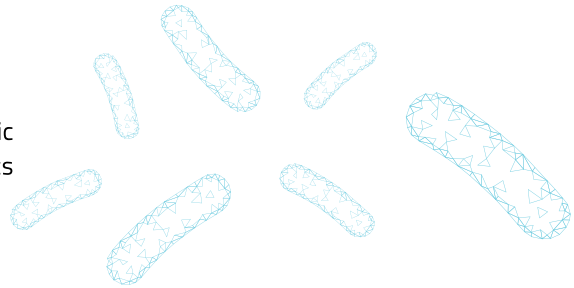
Next-generation Probiotics will Need Next-generation Analytics

More and more, the literature reports that non-culturable, heat-killed, tyndalized or micronized microorganisms (Cani *et al.* 2017; Mogna et al. 2018; Sugahara *et al.* 2017) and "probiotic derived factors" (Howarth and Wang 2013) may also confer a health benefit, it is likely that the definition of probiotics will continue to evolve as more research leads to greater understanding.

Moreover, in recent years, there is an increasing interest in "novel" probiotics that belong to intestinal microorganisms. Most of the promising new strains are strictly anaerobic (for example *Akkermansia municiphila, Faecalibacterium prausnitzii, Eubacterium hallii,* etc.) and adding to the technological difficulties to industrially produce them, the industry is faced with the objective difficulty to enumerate them. Classical PC methodology is arduous to implement on these new species due to their often unknown growth requirements, lack of enumeration methodology and necessity to operate in oxygen-free laboratories (Bircher *et al.* 2018). In some extreme situation, certain microbial strains strictly require the presence of other strains to grow, and thus will intrinsically be impossible to isolate and cultivate *in vitro.*

For the quality standards of all these next-generation probiotics and non-culturable states of strains (inactivated by tyndalization, sonication or micronization for example), in addition to all the new information that it can already provide on commercial probiotics, flow cytometry is

bound to become a major opportunity in probiotics quality standards. In addition, flow cytometry methods can be further developed to identify selectively strains within a mix thanks to the generation of strain-specific antibodies (Chiron *et al.* 2017 and Buckman *et al.* 2017). For the probiotics industry to be ever more accurate, reproducible and reliable including with regard to next generation strains, the adoption of complementary methodologies such as flow cytometry will be essential.



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