Titolo del progetto di ricerca proposto:

FLOW CYTOMETRY BACTERIAL ENUMERATION METHOD VALIDATION, LONG TERM AND ACCELERATED STABILITY STUDY ACCORDING TO ICH-Q1A CONDITIONS AND MODELIZATION WITH MECHANISTIC APPROACH, EVALUATION OF INTRINSIC PROPERTIS OF PROBIOTIC BACTERIA DETERMINED BY FLOW CYTOMETRIC VIABILITY ASSAY AND RESUSCITATION TEST

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State of the Art

A World Health Organization working group has recommended that probiotics be defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host"[1]. This working group has recommended that product labels should include information on "minimum viable numbers of each probiotic strain at the end of the shelf-life". Notably they did not recommend a specific method for probiotic enumeration nor the adoption of Colony Forming Unit (CFUs) as the standard for presenting this information. Indeed, another of this group's recommendation was "further development of methods (*in vitro* and *in vivo*) to evaluate the functionality and safety of probiotics". Consequently, with this definition (*adequate amounts*), probiotic bacteria enumeration is the main evaluation tool for product feasibility, formulation, stability, quality control and the first parameter used for commercial agreements. Moreover, viability (*live microorganisms*) at the point of consumption by the consumer is essential for product efficacy; therefore, the accurate determination of viability is of fundamental importance for probiotics. At the present time, there is still a lack of knowledge and agreement about the definition of "viability", and in the probiotic industry the term, "viability," is still limited to, "enumeration through cultivability," of the selected microorganisms based on traditional methods that involve viable cell count (VCC) on selective media such as M17 or MRS agars. Results generating from these methods are expressed as Colony Forming Unit (CFU). However, plate counts methods (established by Koch more than a century ago) show some limitations, mainly in two specific contexts:

- Technical aspects:

Plate counts methods that express results in CFU, considered the "gold standard" for probiotic enumeration, are far from accurate and universal. Officially, there is availability of only few ISO methods (i.e. ISO 20128:2006 – IDS 192:2006 for *Lactobacillus acidophilus* group and ISO 29981:2010 – IDF 220:2010 for *Bifidobacterium* genus) and the field is populated by numerous Internal Methods, belonging to different producers, which generates confusion and frustration among customers. In general, even different Internal Methods are used for the same bacterial species!

Moreover, plate count methods are laborious (in terms of laboratory work-load and sample throughput). Conventional microbiological techniques are not suitable for the analysis of large numbers of samples (limiting the possibility to run the analysis in triplicate for example), they are time consuming (48-72 hour for a result), and expensive. The lack of international probiotic standards makes it impossible to harmonize results among different laboratories and control authorities, causing loss of technological, clinical, and economic significance. Furthermore, operator generated bias and sample preparation complexity, can lower the precision of data in terms of repeatability (r) and, consequently, reproducibility (R), leading to a generally low accuracy.

The Italian Health Authority in its Probiotic and Prebiotic Guidelines (May, 2013) defines: "*The amount of cells present must be listed on the label, and moreover, this amount has to be guarantee until the end of the product shelf-life, at the specified storage conditions, with uncertainty of 0,5 log. It is emphasized that the analytical method of quantification of living bacterial cells may differ from specie to specie.*"[2] An uncertainty of 0,5 log means that the multiplication ratio between two different results count can be up to approximately *10 times*. Such variability is at the very core of practically all disputes between producers and customers, generating much confusion and frustration in the probiotic industry. The accuracy of the viable plate count method may also be

influenced by the degree of cell clumping, inhibition by nearby cells, composition of the growth media, and final formulation. An unfortunate trend in recent years is to establish expectations for data generated by the plate count method that the accuracy of the method cannot support[3].

- Biological and functional aspects:

Plate count methodology requires that microorganisms are both viable and cultivable in order to be enumerated, which limits detection to a specific physiological state. If the microorganism is not capable of growing under defined conditions, these methods lead to false-negative results, underestimating the population within a given sample.

Therefore, microorganisms that do not form colonies because they are dead, sub lethally injured, or viable but non-culturable (VBNC), are generally undetected by standard plate count methods[4]. However, these subpopulations are highly important for their potential effect on the host[5-6].

For example all these subgroups can contribute on the activation and modulation of the immune system (i.e. peptidoglycan from cell walls) or contribute, as a DNA reservoir, to the genetic horizontal sharing with other members of the intestinal microbiota or with the leakage of intracellular enzymes. VBNC also often “resuscitate” once in their native ecosystem -the gastrointestinal tract- while being undetectable with classic plate count methods[4]. Enumeration with Plate Count cannot give insight on the total potentially beneficial bacteria, which includes the VBNC, inactivated and dead cells.

VBNC status can be frequently observed in finished probiotic products due to numerous unavoidable stress factors that probiotic cultures must undergo during industrial production.

Fermentation, biomass concentration and washing, freezing and drying during lyophilization, powder grinding of pure probiotic cultures, and storage represent critical points that can, even when fully optimized, drive cells to enter in a VBNC state.

Over the last 20 years, multi-parametric flow cytometry (FC) has become a powerful tool in microbiology, particularly in biotechnological processing, food preservation, chemical disinfection processes and pathogen enumeration in clinical and industrial practice. Many attempts have been made to develop rapid and unbiased methods, usually based on the exclusion, uptake or metabolism of colored, fluorescent, or fluorogenic stains, designed to provide information that correlates with viability. Flow cytometry can provide data on different structural and functional properties of cells, which then enable quantification of viability beyond the traditional reproductive capacity on agar and other microbiological media, providing greater insight into the functional strain-related responses to various applications[7-9].

In fact, from the point of view of a microbiologist, a key advantage of FC is the ability to simultaneously collect multiple data outputs for an individual cell regarding viability, vitality, structural integrity, physiological status, or stage of growth cycle. Flow cytometers offer the advantage of being able to analyze thousands of cells/events per second, depending on sample type and cell concentration in the sample. Bacterial enumeration can be obtained in less than one hour in triplicate and with high accuracy. For these reasons, FC is emerging as an alternative rapid method for microbial detection, enumeration, and population profiling.

The use of FC not only allows the determination of viable cell counts but also allows for enumeration of damaged and dead cell subpopulations. Absolute counts of each of the subpopulations can be achieved through the inclusion of defined numbers of fluorescent beads as an internal standard or, with more modern equipment, through the use of particular software capabilities. This is achieved when the volume of the sample going through the cytometry system

can be accurately measured and, hence, this information is subsequently used to calculate the absolute numbers of events/cells per ml.
By the optimal selection of compatible (spectrally distinct) cocktails of fluorescent probes, multi-parametric measurements can be used to quantify uptake of fluorescent dyes that discriminate subpopulations of cells according to characteristics of interest. In the case of viability measurements, this might include measurement of metabolic activity, membrane permeability and potential, RNA and/or DNA content, etc.
For example, one of the most important criteria for distinguishing between viable and irreversibly damaged cells is membrane integrity. In fact, a structurally intact cytoplasmic membrane is recognized as being essential for metabolic activity of the cell to reproduce and grow.
Therefore, live cells with intact membranes are able to exclude DNA-binding dyes that easily penetrate dead or membrane-compromised cells. With fluorescent stains, it is possible to have a dual approach involving the staining of intact cells with one stain followed by the counterstaining of membrane-compromised cells with another stain, thus detecting concentrations of bacteria in a cell suspension and dead/live/compromised subpopulations.
The increased development of cell-based clinical diagnostic and therapeutic/food supplement products begs for more comprehensive regulatory requirements and attention to enumeration, standardization, and better insight of cell population heterogeneity. The recent approval of FC as a tool for cell based processes and products development and quality control by the U.S. Pharmacopeia shows promise for more widespread acceptance of cytometric profiling as a measure of cell number and viability (FLOW CYTOMETRY USP <1027>). Moreover, in December 2015, the International Standard ISO 19344 – IDF 232 "*Milk and milk products - Starter cultures, probiotics and fermented products - Quantification of lactic acid bacteria by flow cytometry*" was published. This particular ISO, as the name suggests, can be applied universally and independently of the species of interest (one method for all the species).
Results are expressed as TFU (Total Fluorescent Units) and AFU (Active Fluorescent Units) and define viability according to three different parameters based on staining protocols described in the method. Therefore, any revision to the labeling of probiotic preparation should allow for flexibility in the approach used to determine the "dose" of probiotics provided so as to permit continued innovation in the industry, while providing consumers with useful information. Presenting the data in relation to the end of the shelf-life could be desirable (in the food supplement category it is not mandatory) with testing under consumer-relevant conditions.

Project description

The project has three different scope, below summarized:

1) To Validate Flow Cytometry Method for probiotic enumeration according to the pharma ICH guidelines USP39<1225>/ICH Q2R1 in terms of:
    a. Accuracy
    b. Precision: Repeatability
       Intermediate precision (ruggedness)
    c. Specificity
    d. Limit of quantification
    e. Linearity
    f. Range

g. Robustness

2) To run long term and accelerated stability studies according to ICH-Q1A guidelines using both Plate Count and Flow Cytometry Method. Tested condition will be stability up to 30 months tested in climate chambers at:
   a. Zone II condition (25°C – 65% RH)
   b. Zone IVb condition (30°C – 75% RH)
   c. Accelerated condition (40°C – 75% RH)

   Results interpretation will be based on the calculation of Half-life times and with a novel mechanistic approach using an appropriate kinetic Arrhenius model that could shorten the time required for a stability study[10].
3) To evaluate any possible correlation between Plate Count and Flow Cytometry Results and to investigate the potential resuscitation of null product by Plate Count that are still "active" when enumerated in Flow Cytometry, when in contact with eukaryotic cell lines. The scope of this test is to evaluate if a loss of cultivability and a potential Viable but nonculturable status (that is not detectable by Plate Count methods) can be reverted to cultivability when in contact with a proper stimulus.

Expected Results

During the Validation process according to ICH guidelines we expect to obtain robust statistically significant data on the evaluated parameter. This step is key in order to be confident in the application of Flow Cytometry for probiotic enumeration and subpopulation analysis according to the staining protocol. Once validated the same method together with Plate count method will be used for long term and accelerated stability studies. Expected results are the implementation of the mechanistic Arrhenius equation for accelerated studies on probiotic stability and to evaluate any potential correlation between Plate Count and Flow cytometry results. Moreover, it is expected that during stability studies there will be a shift in the bacterial population mainly based on the progression of VBNC/cultivable ratio. During the third part of the project when the probiotic product will not generate anymore positive results by Plate Count but still effective if evaluated by Flow Cytometry, it will be tested for resuscitation. If successful this experiment will demonstrate that limiting probiotic activity to cultivability give only a partial view on the probiotic function.

Timing of the project

First year
- Bibliography study;
- Definition of the Validation Protocol
- Validation of the Flow Cytometry Method on three industrial batches
- Start of the stability studies
- Definition of the exel spreadsheet

Second year
- Stability study
- Resuscitation tests

Third year
- Conclusion stability study
- Analysis of the results

Proposed criteria for results evaluation

- Written report and poster publication
- Key-point update with University

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