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Review

Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria ☆

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ABSTRACT

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. Standard culture techniques are commonly used to quantify probiotic strains, but cell culture only measures replicating cells. In response to the stresses of processing and formulation, some fraction of the live probiotic microbes may enter a viable but non-culturable state (VBNC) in which they are dormant but metabolically active. These microbes are capable of replicating once acclimated to a more hospitable host environment. An operating definition of live probiotic bacteria that includes this range of metabolic states is needed for reliable enumeration. Alternative methods, such as fluorescent in situ hybridization (FISH), nucleic acid amplification techniques such as real-time quantitative PCR (RT-qPCR or qPCR), reverse transcriptase (RT-PCR), propidium monoazide-PCR, and cell sorting techniques such as flow cytometry (FC)/fluorescent activated cell sorting (FACS) offer the potential to enumerate both culturable and VBNC bacteria. Modern cell sorting techniques have the power to determine probiotic strain abundance and metabolic activity with rapid throughput. Techniques such as visual imaging, cell culture, and cell sorting, could be used in combination to quantify the proportion of viable microbes in various metabolic states. Consensus on an operational definition of viability and systematic efforts to validate these alternative techniques ultimately will strengthen the accuracy and reliability of probiotic strain enumeration.

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Abbreviations: ASTM, American Society for Testing Materials; CFU, colony forming unit; CSLM, confocal scanning laser microscopy; Cq, quantitative cycle; EMA, ethidium monoazide; EPS, extracellular polysaccharide; FACS, fluorescent activated cell sorting; FC, flow cytometry; FISH, fluorescent in situ hybridization; IDF, International Dairy Federation; ISO, International Organization for Standardization; LOD, limit of detection; LOQ, limit of quantification; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NASBA, nucleic acid sequence based amplification; PMA, propidium monoazide; RT-qPCR, real time-quantitative polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; RT-SDA, reverse transcriptase-strand displacement amplification; TNTC, too numerous to count; VBNC, viable but non-culturable; vPCR, viability-polymerase chain reaction.

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1. Introduction

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). Probiotics have a long history of safe consumption in fermented foods such as yogurts and pickled edibles and considerable interest exists in their use as food additives and supplements. *Lactobacillus* and *Bifidobacterium* constitute the bacterial genera most frequently employed in probiotic preparations for human use. Probiotic preparations must meet strict criteria related to quality, safety and functionality (Vankerckhoven et al., 2008). A key quality criterion is that they contain accurately defined numbers of viable cells as expressed on the product label. Some investigators, however, have found that commercial products did not contain the stated cell numbers (Lin et al., 2006), but had significantly lower levels than reported (Carr and Ibrahim, 2005; Al-Otaibi, 2009).

As probiotics are live organisms, it is critical to enumerate accurately the population of viable microbes in the preparation and express this information to the consumer on the product label. Several significant challenges exist. First, culture-based enumeration of specific organisms requires specialized and standardized methodologies, which will only detect bacteria that are able to replicate on synthetic media and under specific conditions. As noted almost thirty years ago, there may be orders of magnitude differences between the numbers of cells isolated from natural environments which are countable by microscopic examination versus those that can form colonies on agar media which was coined "the great plate anomaly" (Staley and Konopka, 1985). Further, cells that divide and form chains or "clumps" of cells or become encased in the thick extracellular polysaccharide (EPS) during growth have a high probability of being missed if enumerated via traditional culture dependent analyses. Selective culture techniques do no always provide an accurate representation of all species within as sample as highlighted in a 2002 study of lactic acid bacteria enumeration using culture vs. DNA techniques (Jackson et al., 2002). Use of culture-independent techniques, with a more holistic definition of viable probiotic bacteria, have the potential to provide direct, rapid enumeration methods for both researchers and industry-based scientists faced with the challenge of providing the dose available for the final product.

Standardized methods are available for a limited number of species in certain dairy products, such as publications from the International Organization of Standardization (ISO) regarding enumeration standards for *Lactobacillus acidophilus* (ISO 20128/IDF 192:2006) and *Bifidobacterium* (ISO 29981/IDF 220:2010). Secondly, a consensus on the operational definition of live, viable cells needs to be established. Most probiotic strains are well adapted to living in or on the mammalian host, but may be poorly adapted to other environments (Mills et al., 2011). When subjected to environmental stress during formulation and storage, constituent microbes may transition to a viable but nonculturable state (VBNC), a protective response in which they are dormant yet metabolically active (Xu et al., 1982; Lahtinen et al., 2008). Microbes in this state can reestablish broad functioning and replicate when they encounter a more hospitable environment (Lahtinen et al., 2008). Because standard culture-dependent methods enumerate replicating cells only, culture techniques may underestimate the numbers of viable organisms that contribute to the functional capacity of the probiotic preparation once constituent microbes reach the anatomical niche in the host to which they are well-adapted.

The purpose of this review is two-fold: (1) to examine the metabolic states of probiotic microbes pertinent to a working definition of viability, and (2) to review the advantages and limitations of both culture-based methods and the newer visual imaging, molecular biology techniques that include cell sorting techniques. These techniques can be optimized so that enumerating microbes in various metabolic states can be achieved as well as ultimately developing validating more robust methods for enumerating live probiotic strains.

2. Microbial metabolic states and an operating definition of viability

To accurately enumerate live microbes in probiotic preparations, scientific consensus on the definition of a viable microbial cell is paramount. By a convention that dates back to the time of Koch, who in the 19th century first described the growth of bacteria into a colony (Carter, 1987), the scientific community typically considers a cell "viable" if it reproduces to form a colony on an agar plate that supplies key nutrients for the strain. Recent advances, however, reveal this to be a limited definition. Microbes exist in a variety of growth phases and metabolic states depending on environmental conditions and stressors (Volkert et al., 2008; Garcia-Cayuela et al., 2009), and only a subset of these states involve active replication. Descriptions of these various states have been identified in probiotic strains (Table 1). 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 (the VBNC state). Hence, the fundamental questions become: "Is an organism that does not replicate but continues to metabolize, viable? Or must the organism meet classical culture specifications for enumeration even though a heterotroph is stressed when removed from its natural environment and forced to grow on synthetic media?"

Table 1
Major physiological states of probiotic strains.

Physiological state	Phenotype
Viable (live)	Intact cytoplasmic membrane, functional synthesis of protein and other cell components (nucleic acids, polysaccharides, etc.) and energy production necessary to maintain cellular metabolism, and, eventually, growth and multiplication. (Breeuwer and Abee, 2004).
Culturable (replicating)	Capable of division; will form a colony on agar plate or proliferate observably in liquid medium (see authors listed in Table 2)
Non-replicating (in stationary phase; inhospitable conditions for replication; or injured)	Will not form a colony on an agar plate nor proliferate observably in liquid medium; but may have active physiologic activity and intact cytoplasmic membrane. Cells may be inhibited by the medium or injured but capable of repair (Le et al., 2008).
Starving	Cells undergo dramatic decreases in metabolism, but remain fully culturable (Mahdi et al., 2012).
Dormant (viable but not culturable)	In a state of low metabolic activity and unable to divide or to form a colony on an agar plate without a preceding resuscitation phase. A protective response. Also seen in "post-acidification" (Lahtinen et al., 2008; Shah, 2000)
Irreparably damaged cells	Will not grow with vigor under any conditions due to progressive metabolic decline. These cells may be irreparably injured (Le et al., 2008).
Non-viable (dead)	No metabolic activity. (Lahtinen et al., 2008; Le et al., 2008)