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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.

Contents

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)

As noted by Mills et al. (2011) preparation of products that contain probiotic strains via the industrial pipeline can be stressful to the strain itself and can even induce stress-response genes. An important prerequisite of the selection of probiotic strains are the ability to withstand stresses such as temperature, osmotic, solvent stress and/or freeze-drying as they progress during their manufacture to final form (Bron et al., 2011). These processes may leave the final strain(s) in a variety of physiological states before they can reach the environment in which they have biological activity in their host. This review proposes that the definition of live, viable probiotic microbes should be extended to encompass all microbes in the population that are metabolically active and/or have intact membranes. These strains still possess the capability of exhibiting beneficial function(s) when re-acclimated to the host environment. Instead of only identifying those cells that are live as those that are capable of forming septae and accumulating biomass to form a colony when isolated on appropriate artificial media, the new operational definition encompasses the various states that an organism may need to go through before progeny are formed. Fig. 1 illustrates a concept map for the two separate possibilities for viable probiotic strains after preparation (center green ovals). The next layer of circles (both above and below the white line) identifies either injured or dormant cells which may be metabolically active but cannot replicate yet still possess the capability to do so. It also recognizes that cells may be irreparably damaged if severely injured (Fig. 1 — outer gray circles) and are therefore, nonviable/dead and not capable of enumeration. If only culture techniques had been applied to this mixed population, underestimation of the true number of strains capable of biological activity would occur. Some stressed or injured cells may not be capable of immediate replication for a vigorous culture response, but may reestablish that capacity through repair mechanisms. By contrast, dead bacteria or irreparably damaged bacteria (undergoing an irreversible decline in metabolic activity with or without damaged membranes), are not viable and should be excluded from the enumeration of live cells. Conventional and alternative methods to quantify the biomass of microorganisms present are assessed from this standpoint in the following review. Obtaining information about all individual bacteria and their physiological status is relevant since many probiotic effects may depend on their metabolic activity and subsequent byproducts than culturability/replicating status. An example would be the production of lactic acid. It has been noted that even dead cells and/or genomic DNA may have some probiotic effect(s) (Ghadimi et al., 2008; Bunthoff and Abee, 2002; Ouwenhand et al., 2000; Pessi et al., 1999; Ouwenhand and Salminen, 1998).

3. Evaluation of culture-dependent techniques for enumerating probiotic organisms

3.1. Availability and reliability of selective media for strains of probiotic interest

Probiotics were initially characterized by their phenotypic characteristics (such as colony morphology) microscopic details (such as Gram stain reaction and cell morphology), and physiologic characteristics (such as fermentation patterns and enzymatic activity) (Conway and Henriksson, 1994). The range of selective media available to identify and enumerate strains of probiotic interest is relatively limited and it should be noted that no one single medium and/or set of techniques for isolation of the strain is applicable to all probiotic strains Vinderola and Reinheimer (1999) (Table 2). Selective media for specific species of *Lactobacillus* are available; by contrast, members of the genus, *Bifidobacterium*, can be identified, but no standard selective media are available to differentiate among *Bifidobacterium* species (Hartemink and Rombouts, 1996; Roy, 2001). To overcome this, selective differential media have been developed, but the subjectivity of identification requires skilled personnel for reliable results.

The development of selective media for a broader range of strains of probiotic interest remains a challenge. Different organisms require distinct sets of nutrients in varying concentrations and forms. These must be identified and formulated into the medium at the appropriate concentrations to sustain growth yet avoid co-precipitation of the introduced chemicals. Moreover, depending on the nature of the samples, the cultivation efficiency of active cells by standard plating techniques is estimated between 0.001 and 1% (Amman et al., 1995). No single culture-based methodology is applicable to all probiotic organisms, as there is considerable variability between species and even strains in their response to plating procedures.

3.2. Quantification of culturable probiotic microbes by heterotrophic plate counts

Quantification of bacteria in a given sample is routinely achieved by counting the total number of colony-forming units (CFUs) grown on an agar plate from serial dilutions, expressed as CFU per gram or mL of the original sample. This yields an estimate of the number of cells present based on a skilled interpretation of the number of colonies on a plate. It is a skewed estimate, as only cells that can form colonies under the given experimental conditions (e.g. incubation media, temperature, time, and oxygen conditions) are counted. Colonies may arise from individual cells or from cell clusters that happened to be sufficiently separated after plating to be distinguished following growth. Thus, depending on original concentration estimates prior to dilution, a colony could arise from one cell or several thousand. Hence, they are referred to colonies, not cells.

Reliable quantitation requires an acceptable range of countable colonies on a plate. These are based on historical ranges and have been refined by various authoritative bodies. Commonly used ranges for countable numbers of colonies on a plate are 25–250 and 30–300 (Table 2). In their seminal work on milk analysis, Breed and Dotterrer (1916) proposed the original estimates for acceptable, countable plates. Factors such as colony size, and hence the number of colonies per plate, varied by species; nutrients could be limiting, and neighboring colonies inhibit or stimulate growth or merge altogether. Thus, almost a century ago, these scientists noted that "… certain plates in any series made from a given sample are more satisfactory for use in computing a total than are others. The matter of selecting plates to be used in computing a count becomes a matter of considerable judgment" (Breed and Dotterrer, 1916).

The upper limit of the enumeration is reached when bacteria compete for space and nutrients. This depends on bacterial swarming behavior as well as the plating surface area, a critical factor when using small membranes instead of standard plates. TNTC (too numerous to count) can be reported in several ways. ASTM (1998) recommends reporting this as greater than the upper limit (e.g. a 1:10 dilution with more than 200 CFU on a spread plate would be reported as >2000 CFU/mL). FDA's BAM recommends counting the colonies from the dilution giving plate counts closest to 250 and estimating the total number and then using that number as the estimated aerobic count. The lower limit of enumeration can be based on the limit of quantification (LOQ) (25 CFU, from a countable range of 25–250) or the limit of detection (LOD) (i.e. 1 CFU). ASTM recommendations rely on the LOD and to report that answer if no colonies are recovered (e.g., <10 CFU/mL for 1:10 dilution) (ASTM, 1998). Different regulatory bodies have suggested and/or identified acceptable ranges of colonies to count from spread plates over the years since the original 1916 proposal by Breed and Dotterer and appear in Table 3.

In summary, culture-based techniques provide estimates of those microbes that are capable of replicating under experimental conditions. Selective media exist only for a limited subset of potential strains of interest. Reliable plate count enumeration is based on a relatively narrow countable range (generally considered to be 25–250 CFU bacteria on a standard petri dish) and the lack of consensus on the use of a LOD (1 CFU) or LOQ for the lower limit of quantitation introduces a larger degree of variability than is necessary. It is also worth noting that although

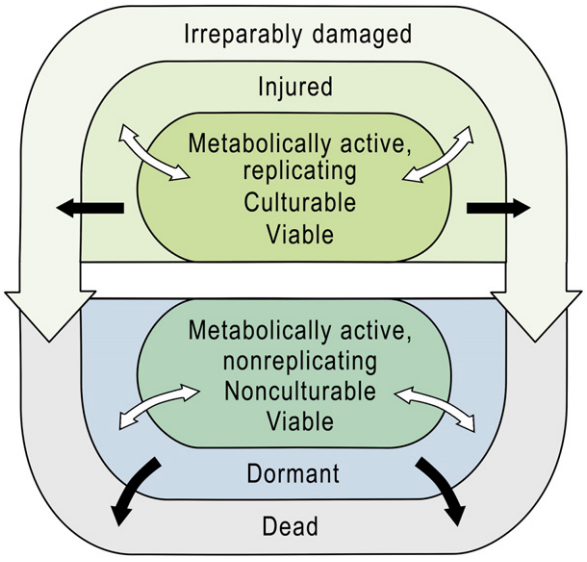

Fig. 1. A concept map for probiotic strains that describes metabolically active, replicating/culturable/viable states and the transitions that are possible. The arrow on the perimeter and the black one-way arrows indicate that once a cell is non-viable/dead it does not return to a viable state.

counts of CFU follow a Poisson distribution, mention is rarely made of the transformation used to approximate a normal distribution prior to the use of normal statistical analytical tools. Consequently, despite its common usage, the plate count method does not support precise, reproducible estimations of cell densities of probiotic strains, especially in mixed cultures. (Sohrabvandi et al., 2012) Moreover, it estimates only the subset of viable organisms that replicated under the conditions of culture. It should also be noted that beyond the art and skill of the technician to culture the sample under the correct environmental conditions, that rapid turn around is not possible as a minimum of 24–72 h of growth in an incubator is necessary before enumeration of colonies on agar plates is possible.

The International Scientific Association for Probiotics and Prebiotics recognized that culture based analysis of strains can underestimate the number of viable cells and fails to account for the impact of bacterial growth modes (Champagne et al., 2011). Numerous investigators have published research on the use of culture-independent methods to provide more insight into the enumeration of viable probiotic strains.

4. Alternative culture-independent methods for enumeration of viable microbes

In the recent years, alternative, culture-independent methods have been used to accurately enumerate probiotic strains based on viability and deliver results in a timely manner (Table 4). Enumeration techniques that lend themselves to quantifying viable cells either use dyes to differentiate live and dead cells by direct observation, measure the presence of an intact cell membrane (membrane integrity), or characterize some aspect of metabolic activity, such as the synthesis of nucleic acids, or respiration; these parameters indicate that the cells are alive even if they are unable to develop into colonies on culture media (Fig. 2). These newer techniques are described in more detail below and a tabular list of various published studies using the various techniques appears in Table 4.

4.1. Direct imaging and visual enumeration — Fluorescent in situ hybridization

Bacteria in a sample can be directly visualized microscopically, but enumeration of viable microbes requires differentiating live and dead bacteria.

Direct epifluorescent counting has been described as a suitable method for enumeration of total bacteria in environmental samples (Kepner and Pratt, 1994). The optical sectioning capability of Confocal

Table 2
Selected examples of culture-based methods for identification/enumeration of probiotic strains[a].

Methods and media for selective enumeration of probiotic strains based on viable replicating technique				
Medium	Base	Selectivity/supplement	Notes	Reference(s)
Bifidobacterium spp.				
Bifidobacterium selective medium (BSM)	MRS (de Man, Rogosa, Sharpe)	Cysteine HCl and Mupurocin	Incubated for 72 h @ 37 °C; Potential concerns regarding development of Mupurocin-resistant *Staphylococcus aureus*	Leuschner et al. (2003), Upton et al. (2003), Simpson et al. (2004)
NPNL (Neomycin sulfate, paromycin, nalidixic acid, and Lithium chloride)	MRS or BL (blood–liver–glucose)	Neomycin sulfate, paromycin sulfate, nalidixic acid and lithium chloride	When L-cysteine not present, *Bifidobacteria* do not grow or form pinpoint colonies; Time consuming to prepare	Teraguchi et al. (1978), Dave and Shah (1996)
Raffinose *Bifidobacterium* medium	LCL (liver–cysteine–lactose)	Proprionate, lithium chloride, and raffinose	Antibiotic free-medium Some *B. bifidum* strains do not grow well on this agar	Hartemink et al. (1996)
MRS-raffinose	MRS	Raffinose, lithium chloride (0.05%)	Incubation @ 45 °C specific for enumeration of *B. lactis BB12* LiCl inhibits lactobacilli	Tabasco et al. (2007)
Lactobacillus acidophilus group (*L. acidophilus*, *L. johnsonii*, *L. gasseri*, *L. crispatus*)				
MRS-clindamycin	MRS	Clindamycin	Anaerobic incubation @ 37 °C for 72 h Use of antibiotic for suppression	ISO (2002), Van de Casteele et al. (2006)
X-Glu	Rogosa agar	5-Bromo-4-3-indoyl-β-D-glucopyrananoside	Visualization of the β-D glucosidase activity. More selective than MRS and Rogosa for yogurt and related products	Kneifel and Pacher (1993)
Lactobacillus casei group (*L. casei*, *L. paracasei*, *L. rhamnosus*)				
MRS-salicin	MRS	Salicin	Conflicting reports; Cannot be used in products containing *L. acidophilus*	Dave and Shah (1996), Ravula and Shah (1998)

[a] Adapted from Kneifel W., "Probiotic Products: How can they meet requirements?" in Probiotics and Health Claims. (2011) Kneifel W and Salminen S. (eds.) Wiley Blackwell, England and Ashraf, R. and Shah, N.P., Selective and differential enumeration of *Lactobacillus debrueckii supsp. bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus caseii* and *Bifodobacterium* spp. In yoghurt — A review. (2011) *Intl J Food Micrbiol* 149:194–208.

Table 3
Acceptable plate counts recommended by authoritative organizations and others.

CFU range acceptable	CFU range unsatisfactory	Notes	Reference
50–200 CFU/mL	<400 > 30	The number of colonies needed to be within 20% of the average	Breed and Dotterrer (1916)
25–250 CFU/mL	–	–	Tomasiewicz (1980)
25–250 CFU/mL	–	–	USP 2011a,b
25–250 CFU/mL	–	–	Food and Drug Administration et al. (1998)
20–80 CFU/membrane, 20–200 CFU/spread plate, 30–300 CFU pour plate	–	–	ASTM (1998)

Scanning Laser Microscopy (CSLM) increases sensitivity and reduced out-of focus blur, enabling observation of subsurface structures of foods in situ (Brooker, 1995; Heertje et al., 1987). Digital acquisition of images by CSLM enables rapid enumeration of bacteria by digital image analysis (Caldwell et al., 1992). This technique may be of value for the rapid estimation of viable bacteria in some dairy products, which could take over three days (Auty et al., 2001). FISH consistently estimates higher yields than plate counts for dairy products but lower for cheese products and spray-dried cultures, highlighting the need for further work to establish the effect of the matrix. The use of this technique as well as the combination of species specific qPCR has allowed unequivocal methods for enumeration of probiotic strains into a variety of cheese products (Auty et al., 2001; Villlarreal et al., 2013).

4.2. Nucleic acid-based enumeration methods

4.2.1. Polymerase chain reaction (PCR)

Detection of nucleic acid sequences (DNA, mRNA and rRNA) is a molecular technique that can be applied to bacterial enumeration. Most molecular analyses target amplification of nucleic acid to maximize analytical sensitivity. DNA amplification by PCR was investigated for enumeration of live bacteria based on the assumption that DNA would be degraded more rapidly after cell death than other cellular components and that intact DNA sequences would indicate cell viability (Jamil et al., 1993). Although most DNA detection is undertaken by PCR (McKillip et al., 1999), hybridization-based detection methods also have been employed (Meijer et al., 2000). However the presence of DNA does not necessarily indicate viability, although the detection of longer intact DNA sequences correlates more closely with viability than shorter sequences (McCarty and Atlas, 1993).

4.2.2. Reverse transcriptase PCR (RT-PCR)

RT-PCR is one of the many variants of PCR and allows multiple copies of a particular sequence through amplification. It should be noted that ribonucleic acid (RNA) is first transcribed in reverse into its DNA complement that utilizes the reverse transcriptase. Attention has turned to the use of mRNA as a marker of viability. This marker is a highly labile molecule with a very short half-life (seconds) in bacteria. Hence, detection of bacterial mRNA transcription should provide a more reliable indication of viability than DNA-based methods. The most common amplification techniques for detecting mRNA are reverse transcriptase PCR (RT-PCR) and nucleic acid sequence based amplifications (NASBA) (Chan and Fox, 1999). Both have been applied to the determination of bacterial viability with variable success. More recently, reverse transcriptase-strand displacement amplification (RT-SDA) has been used as an indicator of bacterial viability (Hellyer and Nadeau, 2004).

Ribosomal RNA (rRNA) has also been investigated as an indicator of viability and can be positively correlated with viability under some bacterial regimes (McKillip et al., 1998).

4.2.3. Real time-quantitative polymerase chain reaction (RT-qPCT or qPCR)

RT-qPCR, is a DNA amplification technique that uses fluorescent reporter dyes to combine the amplification and detection steps of the PCR reaction in a single tube format. Whereas traditional PCR measures the accumulation of the PCR product at the end of all the PCR cycles, RT-qPCR quantifies PCR amplification as it occurs. RT-qPCR detection measures the increase in fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle. A quantification cycle (Cq) value is determined by plotting fluorescence against the cycle number. Cq corresponds to the number of cycles for which the fluorescence is higher than the background fluorescence. RT-qPCR is a quantitative technique because data are collected during the exponential growth (log) phase of PCR when the quantity of the PCR product is directly proportional to the amount of template nucleic acid. Using this technique allows microbial populations to be quantified by measuring the abundance of a target sequence in DNA samples extracted from food products (Postellec et al., 2011). Combined with reverse transcription (RT), this technique can also be used to estimate the amount of mRNA transcripts. An investigator could choose a particular transcript related to metabolic activity (such as production of lactic acid during fermentation) for a more direct indication of the activity of living cells.

Guidelines on Minimum Information for the Publication of Quantitative Real-time PCR (MIQE) provide criteria to help achieve high quality results with valid conclusions (Bustin et al., 2009). A simplified roadmap for obtaining solid data using MIQE guidelines has been published (Taylor, 2013).

Table 4
Published studies related to enumeration of probiotic strains by culture-independent techniques.

Culture-independent methods for enumeration of probiotic bacteria			
	Method	Structural target probed	Reference
Imaging	Fluorescent in situ hybridization (FISH)	Presence of nucleic acid	Bernardeau et al. (2001), Lahtinen et al. (2006)
	Live–dead staining and microscopic counting	Cellular integrity	Auty et al. (2001), Lahtinen et al. (2006), Maukonen et al. (2006), Zotta et al. (2009, 2012), Perndana et al. (2012)
Molecular Biology	EMA or PMA-qPCR (vPCR)	Cellular integrity/Nucleic Acid	Fujimoto and Watanabe (2013), Desfosses-Fouchault et al. (2012)
	Real-Time qPCR	Presence of nucleic acid	Furet et al. (2004), Lahtinen et al. (2005, 2006), Guieimonde et al. (2004), Garcia-Cayuela et al. (2009), Bogovic-Matijasic et al. (2010), Kramer et al. (2009), Sheu et al. (2010), Herbel et al. (2013), Sattler et al. (2014)
	Quantification of 16S rRNA	Presence of nucleic acid	Lahtinen et al. (2008)
	MALDI-TOF mass spectrometry	Presence of nucleic acid	Angelakis et al. (2011)
Cell Sorting	Flow cytometry/FACS	Cell integrity or metabolic activity	Ben Amor et al. (2002), Chen et al. (2012), Doherty et al. (2010), Martin-Dejardin, et al. (2013), Maukonen et al. (2006), Rault et al. (2007), Sunny-Roberts et al. (2007), Volkert et al. (2008), Kramer et al. (2009), Bunthoff and Abee (2002), Patchett et al. (1991)

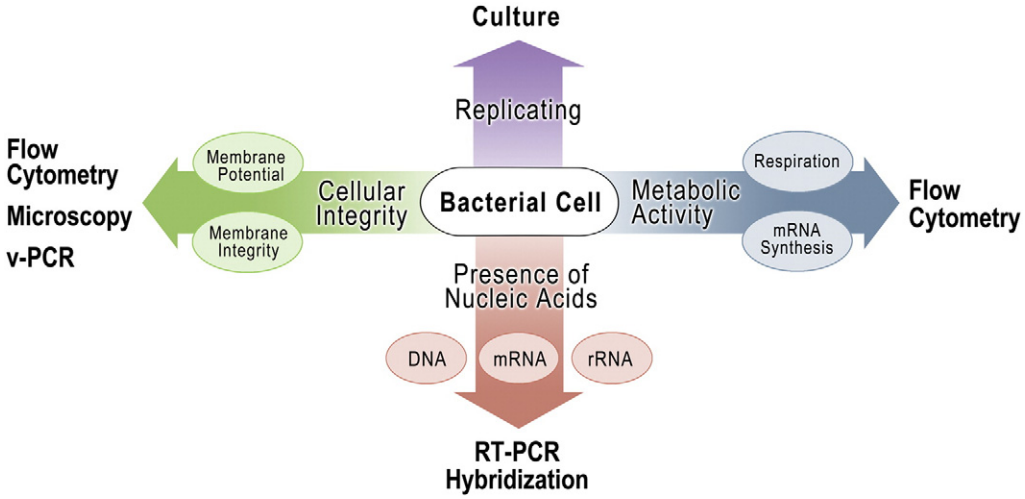

Fig. 2. Summary of the facets of a probiotic bacterial cell that may be probed via various culture and non-culture based techniques to assess viability of probiotic strains.

4.2.4. Ethidium monoazide-PCR and propidium monoazide-PCR

Ethidium monoazide-PCR (EMA-PCR) and propidium monoazide-PCR (PMA-PCR) are emerging techniques that limit enumeration to live cells (Nogva et al., 2003) and can also be referred to as viability-PCR (vPCR). (Fittipaldi, et al., 2012). Cells with intact membranes are assumed to be viable. Ethidium monoazide (EMA) is an azide-bearing, DNA-intercalating dye thought to enter only membrane-compromised cells. EMA covalently crosslinks DNA when the azide group converts to a highly reactive nitrene radical upon exposure to bright visible light. Water simultaneously inactivates unbound EMA and the reaction product remains free in solution. EMA treatment is followed by genomic DNA extraction and qPCR analysis. Crosslinking strongly inhibits PCR amplification of the modified DNA, such that only unmodified DNA (from presumptively intact cells) can be amplified. EMA treatment in conjunction with qPCR led to signal reduction of up to four $\log_{10}$ units in the case of membrane-compromised cells (Rudi et al., 2005).

It was later shown that, in some bacterial species, EMA does penetrate cells with intact membranes (Nocker et al., 2006). However, propidium monoazide (PMA), an analog of EMA that functions through similar chemistry, is efficiently excluded from cells with intact cell membranes, probably due to an increased positive charge. PMA-qPCR is applicable to a wide range of gram-negative and gram-positive bacteria. This approach has been used successfully to assess the killing efficacy of disinfectants (Nocker et al., 2007a) and to detect viable *Escherichia coli* and *Pseudomonas aeruginosa* for water quality assessments (Gensberger et al., 2013). PMA treatment also limits detection to intact microbial cells when used with end-point PCR in combination with denaturing gel electrophoresis (Nocker et al., 2007b). Challenges have been encountered when applying PMA-PCR to samples that have insufficient light transparency (Wagner et al., 2008). This limitation might be overcome by using a trigger other than light to induce DNA cross-linking or by manipulating pH or temperature to alter turbidity. Fittipaldi, et al. (2012) published the most recent technical review of vPCR.

4.3. Flow cytometry (FC)/fluorescent activated cell sorting (FACS)

Cell sorting methods, such as Coulter counters and flow cytometry (FC) were originally developed for counting red blood cells. Today, FC has been upgraded to analyze much smaller cells, such as bacteria, and to deliver high-throughput data. The technique allows simultaneous multiparametric analysis of physical and/or chemical characteristics of up to thousands of particles per second. The cell surface or its components must first be labeled with one or more fluorescent dyes. A monodisperse suspension (single, unclumped cells) is made so that single, labeled cells are aligned to pass individually through a laser beam. Laser-excitation of the fluorescent molecules causes them to emit light at various wavelengths and the amount and type fluorescence indicates the percentage of various cell types or cell components present in the sample.

FC allows the examination of a large number of cells at a time (200 to 2000 cells per second), recording, for each cell, several different parameters that can later be linked to a wide variety of cellular characteristics (Tracy et al., 2010). A variety of fluorescent probes can be applied to examine physiological characteristics of living cells, such cell membrane integrity, intracellular enzyme activity, cytoplasmic pH, and membrane potential, all of which provide a measure of viability (Chen et al., 2012). Fluorescent Activated Cell Sorting (FACS) is a specialized form of flow cytometry that sorts a heterogeneous mixture of biological cells into two or more containers based on the fluorescent characteristics as well as light scattering.

These powerful and rapid cell-sorting techniques could reduce the time needed to determine probiotic strain abundance, size, and metabolic activity. Fluorescent DNA stains, nucleic acid probes, and immunofluorescence probes directed at cell proteins, extend the capabilities of the technique, enabling cells to be discriminated based on amount and type of nucleic acids, amount of respiratory enzymes, or membrane integrity. The potential exists to measure cell size, cell granularity, and indicators of viability such as levels of newly synthesized DNA, specific gene expression from transcription of messenger RNA, and even transient signaling events in living cells. Such techniques offer significant promise for more robust enumeration of viable probiotic strains (whether replicating or VBNC).

5. Limitations of assays

It should be noted that all assays have some limitations and no perfect methods exists even when the procedure is optimized. Some of the limitations the techniques summarized in this review appear in Table 5.

6. Conclusion and future directions

Both traditional cell culture methods, as well as the newer, alternative techniques reviewed herein, offer advantages and limitations for enumerating probiotics. Use of the variety of techniques and the targets of the probiotic cell that they probe to assess viability are summarized in Fig. 2. Conventional culture methods are commonly used for the microbiological quality assurance for probiotic preparations and are identified as CFU/gram or capsule or mL of product. These methods are simple but

Table 5
Parameters for consideration in selection of approach to enumeration probiotic species.

Method	Material cost	Time to execute	Time to availability of results	Specificity	Automation	Challenges (examples)
Culture	Inexpensive	++	+++	++	No	Identifies replicating cells only if placed on appropriate synthetic media; Fermentation patterns may be similar between strains; Tedious to prepare some media; Some media incorporate antibiotics
EMA/PMA-PCR (v-PCR)	+	++	++	+++	Yes	Toxic materials; Sensitive to small variations in sample preparation
RT-PCR	+	+++	++	++	No	
Fluorescent microscopy	+	+	<2 h	++	No	Optimization of permeabilization of cell wall methods for penetration fluorescent probe
MALDI-TOF mass spectrometry	+	+	++	+	No	Variability in reproducibility reported
Flow cytometry/FACS	+++	++	++	+++	Yes	LOD 1×10^4 cells/mL, however, most probiotic preparations contain $\geq 1 \times 10^6$ cells per preparation

generally require more than 24 h to yield reliable results and some media that are necessary to selectively identify these strains are tedious to make, can contain antibiotics (Bujalence et al., 2006), and even the use of pour or spread plate techniques may affect results that were presented in Table 2. It should be noted that underestimation of bacterial numbers also may occur, because the cells that are viable but no longer culturable by culture methods are difficult to detect. Therefore, rapid and simple culture-independent methods are required. It is recommended that the operational definition of live probiotic bacteria be updated to address the fact that viable cells exist in metabolic states that may not be amenable to culturable. Newer methods that probe cellular integrity, presence of nucleic acids and/or the metabolic activity are summarized in Fig. 2 by the two horizontal and downward pointing arrows. Use of these techniques shows considerable promise for quantifying live microbes in different metabolic states. An important caveat, however, is the recognition that probiotic efficacy cannot be predicted solely on the basis of viable cell quantities, as components of non-viable cells also may have a probiotic effect. For example cell wall components, whether from dead or living cells, have been reported to contribute to efficacy (Salminen et al., 1999). Nevertheless, in the context of enumeration rather than efficacy, alternative techniques that measure viable cells appear promising and afford the opportunity to develop and validate standardized protocols to quantify specific probiotic strains in the future. This would set the probiotics field on par with other industries, such as water quality analysis, that have amended their views on strict use of culture analyses and adopted newer techniques (such FACS/FC with viability stains) to increase accuracy and precision in enumeration. The methods selected should take into account all the cells present in a sample/product and be able to distinguish and quantify the various states of cells based on the agreed parameter(s) of cell viability. Criteria such as detection limit and sensitivity of the method, time required to obtain results, and laboratory outlays in skill, labor and cost need to be taken into consideration for selection of the method. Precedence for use and validation of alternative methods for microbiological analysis of food, animal feeding stuffs and environmental and veterinary samples has been outlined in ISO 16140:2003. It defines the general principles and the technical protocol for the validation of alternative methods in the field of which could be used in the framework of the official control, and the international acceptance of the results obtained by the alternative method. The use of these guidelines provides a framework that there is the possibility of utilizing an alternative method for enumeration of probiotics for human use. The culture-independent alternative methods reported herewith should be considered as important tools in the armamentarium of the quality assurance of probiotic organisms.

Even with the limitations noted in Table 5, FC appears as a promising tool for use in the manufacturing of products containing probiotic strains (Diaz et al., 2010; Tracy et al., 2010; Davey, 2011). Beyond the simple enumeration of cells, FC provides insight regarding microbial fitness and metabolic activities during bioprocessing of product formulations. This could improve processes optimization involving strains for commercial use, prediction of microbial performances along the whole process and the presence/absence of activity during storage could benefit the quality control of probiotic products during their shelf life (Sohier et al., 2014; Diaz et al., 2010). Inclusion of FC as a validated technology for this purpose is supported by those in both the academic and industrial arenas (Sohier et al., 2014; Burguiere, 2013; and Lahtinen et al., 2006) and it has been discussed by project groups within organizations such as the International Dairy Federation and ISO (Agenda-IDF/ISO Analytical Week 2012, Standing Committee on Analytical Methods for Dairy Organisms — D06; 3–7 June, Rotterdam, The Netherlands).

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References

Al-Otaibi, M.M., 2009. Evaluation of some probiotic fermented milk products from Al-Ahsa markets, Saudi Arabia. Am. J. Food Technol. 4, 1–8.
Amman, R.I., Ludwig, W., Schleifer, K.-H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microb. Rev. 59, 143–169.
Angelakis, E., Million, M., Henry, M., Raoult, D., 2011. Rapid and accurate bacterial identification in probiotics and yoghurts by MALDI-TOF mass spectrometry. J. Food Sci. 76, M568–M572.
ASTM, D5465-93, 1998. Standard Practice for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods.
Auty, M.A.E., Gardinere, G.C., Mc Breaty, S.J., O'Sullivan, E.O., Muerill, D.M., Collins, J.K., Fitzgerald, G.J., Stanton, C., Ross, R.P., 2001. Direct in situ viability assessment of bacteria in probiotic dairy products using viability staining in conjunction with confocal scanning laser microscopy. Appl. Environ. Microbiol. 67, 420–425.
Ben Amor, K., Breeuwer, P., Veraarschot, P., Rombouts, F.M., Akkermans, A.D.L., De Vos, W. M., Abee, T., 2002. Multiparimetric flow cytometry and cell sorting for the assessment of viable, injured and dead *Bifidobacterium* cells during bile stress salts. Appl. Environ. Microbiol. 58, 5209–5216.
Bernardeau, M., Vernoux, J.P., Gueguen, M., 2001. Usefulness of epifluorescence for quantitative analysis of lactobacilli in probiotic feed. J. Appl. Microbiol. 91, 1103–1109.

Bogovic-Matijasic, B., Obermaier, T., Rogelj, I., 2010. Quantification of *Lactobacillus gasseri*, *Enterococcus faecium and Bifidobacterium infantis* in a probiotic OTC drug by real-time PCR. Food Control 21, 419–425.
Breed, R., Dotterrer, W.D., 1916. The number of colonies allowable on satisfactory agar plates. J. Bacteriol. 1, 321–331.
Breeuwer, P., Abee, T., 2004. Assessment of the membrane potential, intracellular pH and respiration of bacteria employing fluorescence techniques. Molecular Microbial Ecology Manual 2nd edition. Kluwer Academic Publishers, (The Netherlands), pp. 1563–1580.
Bron, P.A., van Bokhorst-van deVeen, Weiss, M., Kleerebezeem, M., 2011. Engineering robust lactic acid bacteria. In: Tasakalidou, E., Papadimitriou, K. (Eds.), Stress Response of Lactic Acid Bacteria. Springer, New York City, New York, USA, pp. 369–370.
Brooker, B.E., 1995. Imaging food systems by confocal scanning laser microscopy. In: Dickinson, E. (Ed.), New Physico-chemical Techniques for the Characterization of Complex Food Systems. Blackie Academic and Profession, London, United Kingdom, pp. 43–68.
Bujalence, C., Jimenez-Valera, M., Moreno, E., Reuiz-Bravo, A., 2006. A selective medium for *Lactobacillus plantatarum*. J. Microbiol. Methods 66, 572–575.
Bunthoff, C.J., Abee, T., 2002. Development of a flow cytometric method to analyze subpopulations of bacteria in probiotic products and dairy starters. Appl. Environ. Microbiol. 68 (6), 2934–2942.
Burguiere, P., 2013. Enumeration and identification methods for probiotics. 127th AOAC Annual Meeting and Exposition. International Symposium on the Challenges with the Analysis of Probiotics (Chicago, IL).
Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.
Caldwell, D.E., Korber, D.R., Lawrence, J.R., 1992. Confocal laser microscopy and digital image analysis in microbial ecology. Adv. Microb. Ecol. 12, 1–67.
Carr, J., Ibrahim, S.A., 2005. Viability of Bifidobacterium in commercial yogurt products in North Carolina. Milchwissenschaft 60, 414–416.
Carter, K.C., 1987. Essays of Robert Koch, Greenwood, Portsmouth, NH.
Champagne, C.P., Ross, R.P., Saarela, M., Hansen, K.F., Charalampopulos, D., 2011. Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices. Int. J. Food Microbiol. 149, 185–193.
Chan, A.B., Fox, J.D., 1999. NASBA and other transcription-based amplification methods for research and diagnostic microbiology. Rev. Med. Microbiol. 10, 185–196.
Chen, S., Cao, Y., Ferguson, L.R., Shu, Q., Garg, S., 2012. Flow cytometric assessment of the protectants for enhanced in vitro survival of probiotic lactic acid bacteria through simulated human gastro-intestinal stresses. Appl. Microbiol. Biotechnol. 95, 345–356.
Conway, P.L., Henriksson, A., 1994. Strategies for the isolation and characterization of functional probiotics. In: Gibson, S.A.W. (Ed.), Human Health: The Contribution of Microorganisms, vol. 1. Springer-Verlag, New York.
Dave, R.I., Shah, N.P., 1996. Evaluation of media for selective enumeration of *Streptococcus thermophiles*, *Lactobacillus delbrueckii* ssp. *bulgaris*, *Lactobacillus acidophilus*, and bifidobacteria. J. Dairy Sci. 79, 1529–1536.
Davey, H.M., 2011. Life, death, and in-between; meanings and methods in microbiology. Appl. Environ. Microbiol. 77, 5571–5576.
Desfosses-Fouchault, E., Dussault-Lepage, V., Le Boucher, C., Savarad, P., LaPointe, G., Roy, D., 2012. Assessment of probiotic viability during cheddar cheese manufacture and ripening using propidium monazide-PCR quantification. Front. Microbiol. 3, 1–11.
Diaz, M., Herrero, M., Garcia, L.A., Quiros, C., 2010. Application of flow cytometry to industrial bioprocesses. Biochem. Eng. J. 48, 385–407.
Doherty, S.B., Wang, L., Ross, R.P., Stanton, C., Fitzgerald, G.F., Brodkorb, A., 2010. Use of viability staining in combination with flow cytometry for rapid viability assessment of *Lactobacillus GG* in complex protein matrices. J. Microbiol. Methods 82, 301–310.
Fittipaldi, M., Nocker, A., Codony, F., 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J. Microbiol. Methods. 91 (2), 276–289.
FAO/WHO, 2001. Guidelines for the evaluation of probiotics in food. Food and Agriculture Organization of the United Nations and World Health Organization Working Groups Report (http://www.fao.org/es/ESN/food/foodanadfood_probio_en.stm).
Food and Drug Administration, Maturin, L.J., Peeler, Jt, 1998. Chapter e: Aerobic Plate Count Bacteriological Analysis Manual. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/Bacteriological-AnalyticalManualVAM/ucm063346.htm.
Fujimoto, J., Watanabe, K., 2013. Quantitative detection of viable *Bifidobacterium bifidum* BF-1 cells in human feces by using propidium monoazide and strain-specific primers. Appl. Environ. Microbiol. 79, 2182–2186.
Furet, J.P., Quenee, P., Tailliez, P., 2004. Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. Int. J. Food Microbiol. 7, 197–204.
Garcia-Cayuela, T., Tabasco, R., Pelaez, C., Requena, T., 2009. Simultaneous detection of viable lactic acid bacteria and bifidobacteria in fermented milk by using propidium monoazide and real-time PCR. Int. Dairy J. 19, 405–409.
Gensberger, E.T., Sessistsch, A., Kostic, T., 2013. Propidium monoazide-quantitative polymerase chain reaction for viable *Escherichia coli* and *Pseudomonas aeruginosa* detection from abundant background microflora. Ann. Biochem. 441, 69–72.
Ghadimi, D., Folstr-Holst, R., de Vrese, M., Winkler, P., Heller, K.J., Schrezenmeirer, J., 2008. Effects of probiotic bacteria and their genomic DNA on TH1/TH2 cytokine production by peripheral blood mononuclear cells (PBMCs) of healthy and allergic subjects. Immunobiology 213 (8), 677–692.
Guieimonde, M., Tolkk, S., Korpimaki, T., Salminen, S., 2004. New real time−(q) quantitative PCR procedure for quantification of bifidobacteria in human faecal samples. Applied and Environmental Microbiology 70, 4165–4169.
Hartemink, R., Rombouts, F.M., 1996. Comparison of media for the detection of bifidobacteria, lactobacilli and total anaerobes from faecal samples. (1999). J. Microbiol. Methods 36 (3), 181–192 (Jun).
Hartemink, P., Kok, B.J., Weenk, G.H., Rombouts, F.M., 1996. Raffinose-Bifidobacterium (RB) agar a new selective agar for bifidobacteria. J. Microbiol. Methods 27, 33–43.
Heertje, I., van der Vlist, P., Blonk, J.C.G., Hendrickx, H.A.C., Brackenhof, G.J., 1987. Confocal scanning laser microscopy in food research: some observations. Food Microstruct. 6, 115–120.
Hellyer, T.J., Nadeau, J.G., 2004. Strand displacement amplification: a versatile tool for molecular diagnostics. Expert. Rev. Mol. Diagn. 4, 251–261.
Herbel, S.R., Lauzat, B., von Nickisch-Rosenegk, Kuhn M., Murugaiyan, J., Wieler, L., Guenter, S., 2013. Species-specific quantification of probiotic lactobacilli in yoghurt by quantitative real-time PCR. J. Appl. Microbiol. 115, 1402–1410.
ISO, 2002. Milk products — enumeration of *Lactobacillus acidophilus* — colony count technique at 43 °C. International Standard ISO/DIS 20128 (IDF 220).
ISO, 2003. Microbiology of food and animal feeding stuffs. Protocol for the validation of alternative methods. International Standard ISO 16140:2003.
Jackson, M.S., Bird, A.R., McOrist, A.L., 2002. Comparison of tow selective media for the detections and enumeration of lactobacilli in human feces. J. Microbiol. Methods 51 (3), 313–321.
Jamil, S., Keer, J.T., Dockrell, H.M., Stoker, N.G., Lucas, S.B., Chiang, T.J., Hussain, R., 1993. Use of polymerase chain reaction to assess efficacy of leprosy chemotherapy. The Lancet 342, 264–268.
Kepner, R.J., Pratt, J.R., 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol. Rev. 58, 603–615.
Kneifel, W., 2011. Probiotic products: how can they meet requirements? In: Kneifel, W., Salminen, S. (Eds.), Probiotics Health Claims. Wiley Blackwell, West Sussex England.
Kneifel, W., Pacher, B., 1993. An X-Glu based agar medium for the selective enumeration of *Lactobacillus acidophilus* in yogurt-related milk products. Int. Dairy J. 3, 277–291.
Kramer, M., Obermajer, N., Matijasic, B., Rogerlj, I., Kmetee, I.R., 2009. Quantification of live and dead probiotic bacteria in lyophilized product by real time PCR and by flow cytometry. Appl. Genet. Mol. Biotechnol. 84, 1137–1147.
Lahtinen, S.J., Gueiumonde, M., Ouwehand, A.C., Reinikainen, J.P., Salminen, S.J., 2005. Probiotic bacteria may become dormant during storage. Appl. Environ. Microbiol. 71, 1662–1663.
Lahtinen, S.J., Gueiumonde, M., Ouwehand, A.C., Reinikainen, J.P., Salminen, S.J., 2006. Comparison of four methods to enumerate probiotic bifidobacteria in a fermented food product. Food Microbiol. 23, 571–577.
Lahtinen, S.J., Ahokoski, H., Reinikainen, J.P., Gueimonde, M., Nurmi, J., Ouwenhand, A.C., Salminen, S.J., 2008. Degradation of 16S rRNA and attributes of viability of viable but nonculturable probiotic bacteria. Lett. Appl. Microbiol. 46, 693–698.
Le, T.T., Ruyssen, T., Uyttendaele, M., Van Assche, P., Dewettinck, K., 2008. Assessment with a compact flow cytometer of live, injured and dead probiotic lactobacilli and bifidobacteria after subjecting to osmotic, freeze and heat stress. Conference on Food Science and Technology — Mekong Delta, 20th–22nd March 2008, Can Tho University, Can Tho, Vietnam.
Leuschner, R.G.K., Bew, J., Simpson, P., Ross, P.R., Stanton, C., 2003. A collaborative study of a method for the enumeration of probiotic bacteria in animal feed. Int. J. Food Microbiol. 83, 161–170.
Lin, W.H., Hwang, C.F., Chen, L.W., Tsen, H., 2006. Viable counts, characteristic evaluation for commercial lactic acid bacteria products. Food Microbiol. 23, 74–81.
Mahdi, A., Esteban, M.A., Hmila, Z., Bekir, K., Kamoun, F., Bakhrouf, A., Krifi, B., 2012. Survival and retention of the probiotic properties of *Bacillus* sp. Strains under marine stress starvation conditions and their potential use as a probiotic in Artemia culture. Res. Vet. Sci. 93 (3), 1151–1159.
Martin−Dejardin, F., Ebel, B., Lemetais, G., Nguyen Thi Minh, H., Gervais, P., Cachon, R., Chambin, O., 2013. A way to follow the viability of encapsulated Bifidobacterium bifidum subjected to a free−drying process in order to target the colon: Interest in flow cytometry. European Journal of Pharmaceutical Sciences 49, 166–174.
Maukonen, J., Alakomi, H.-L., Nohynek, L., Hallamaa, K., Leppamaki, S., Matto, J., Saarela, M., 2006. Suitability of the fluorescent techniques for the enumeration of probiotic bacteria in commercial non-dairy drinks and in pharmaceutical products. Food Res. Int. 39, 22–32.
McCarty, S.C., Atlas, R.M., 1993. Effect of amplicon size on PCR detection of bacteria exposed to chlorine. PCR Methods Appl. 3, 181–185.
McKillip, J.L., Jaykus, L., Drake, M.A., 1998. rRNA stability in heat-killed and UV-irradiated enterotoxigenic *Staphylococcus aureus* and *Escherichia coli* 0157:H7. Appl. Environ. Microbiol. 64, 4264–4268.
McKillip, J.L., Jaykus, L., Drake, M.A., 1999. Nucleic acid persistence of *Escherichia coli* O157:H7 in artificially contaminated skim milk. J. Food Prot. 62 (904), 908.
Meijer, A., roholl, P.J., Gielis-Proper, S.K., Meulenberg, Y.F., Osewaarde, J.M., 2000. Chlamydia pneumoniae in vitro and in vivo: a critical evaluation of in situ detection methods. Journal of Clinical Pathology. 53, 904–910.
Mills, S., Stanton, C., Fitzgerald, G., Ross, R.P., 2011. Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. Microbial Cell Factories S19 (http://www.microbialcellfactories.com/content/10/S1/S19).
Nocker, A., Cheung, C.Y., Camper, A.K., 2006. Comparison of propidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Methods 70, 252–260.
Nocker, A., Sossa, K., Campber, A.K., 2007a. Molecular monitoring of disinfection efficacy. J. Microbiol. Methods 70, 252–260.
Nocker, A., Sossa, P., Burr, M., Camper, A.K., 2007b. Use of propidium monoazide for live-dead distinction in microbial ecology. Appl. Environ. Microbiol. 73, 5111–5117.
Nogva, H.K., Dromtorp, S.M., Nissen, H., Rudi, K., 2003. Ethidium monazide for DNA-based differentiation of viable and dead bacteria by 5′ nuclease. PCR BioTech. 810, 804–813.

Ouwenhand, A.C., Salminen, S.J., 1998. The health effects of cultured milk products with viable and non-viable bacteria. Int. Dairy J. 8, 749–756.
Ouwenhand, A.C., Tolkkos, S., Kulmala, J., Salminen, S., Salminen, E., 2000. Adhesion of inactivated probiotic strains to intestinal mucus. Lett. Appl. Med. 31, 82–86.
Patchett, R.A., Back, J.P., Pinder, A.C., Kroll, R.G., 1991. Enumeration of bacteria in pure cultures and in foods using a commercial flow cytometer. Food Microbiol. 8, 119–126.
Perndana, J., Bereschenko, L., Roghair, M., Fox, M.B., Boom, R.M., Kleerebezem, M., Schutyser, M.A., 2012. Novel method for enumeration of viable *Lactobacillus planatarum* WCFS1 cells after single droplet drying. Appl. Environ. Microbiol. 78, 8082–8088.
Pessi, T., Sutas, Y., Saxelin, M., Kallioinen, H., Isolauri, E., 1999. Antiproliferative effects of homogenates derived from five strains of candidate probiotic bacteria. Applied and Environmental Microbiology. 65, 4725–4728.
Postellec, F., Falentin, H., Pavan, S., Combrisson, J., Sohier, D., 2011. Recent advances in quantitative PCR (qPCR) applications in fold microbiology. Food Microbiol. 28, 848–861.
Rault, A., Beal, C., Ghorbal, S., Ogier, J.—C., Bouix, M., 2007. Multiparametric flow cytometry allows rapid assessment and comparison of lactic acid bacteria viability after freezing and during frozen storage. Cryobiology. 55, 35–43.
Ravula, R.R., Shah, N.P., 1998. Selective enumeration of *Lactobacillus caseii* from yogurts and fermented milk drinks. Biotechnol. Tech. 12, 819–822.
Roy, D., 2001. Media for the isolation and enumeration of bifidobacteria in dairy products. Int. J. Food Microbiol. 69, 167–182.
Rudi, K., Moen, B., Dromtrop, S.M., Holck, A.L., 2005. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl. Environ. Microbiol. 71, 1018–1024.
Salminen, S., Ouwenhand, A., Benno, Y., Lee, Y.K., 1999. Probiotics: how should they be defined? Trends Food Sci. Technol. 10, 107–110.
Sattler, V.A., Mohni, M., Klose, V., 2014. Development of a strain-specific real-time PCR assay for enumeration of a probiotic *Lactobacillus reuteri* in chicken feed and intestine. PLoS One 9 (2), e9028. http://dx.doi.org/10.137/journal.pone.0090208.
Shah, N.P., 2000. Symposium: Probiotic bacteria. Probiotic bacteria: selective enumeration and survival in dairy foods. J. Dairy Sci. 83 (4), 894–907.
Sheu, S.-J., Hwang, W.Z., Chiang, Y.-C., Lin, W.-H., Chen, H.-C., Tsen, H.-Y., 2010. Use of TUF gene based primers for the PCR detection of probiotic *Bifidobacterium* species and enumeration of Bifidobacteria in fermented milk by cultural and quantitative real-time PCR methods. J. Food Sci. 75, M521–M527.
Simpson, P.J., Fitzgerald, G.F., Stanton, C., Ross, R.P., 2004. The evaluation of a mupirocin-based selective medium for the enumeration of bifidobacteria from probiotic animal fee. J. Microbiol. Methods 57, 9–16.
Sohier, D., Pavan, S., Rious, A., Comvrisson, J., Postollec, F., 2014. Evolution of microbiological analytical methods for dairy industry needs. Front. Microbiol. 5, 16. http://dx.doi.org/10.3389/fmicb.2014.0016.
Sohrabvandi, S., Mortazavian, A.—M., Dolatkhnejad, M.—R., Monfared, A.B., 2012. Suitability of MRS—bile agar for the selective enumeration of mixed probiotic bacteria in presence of mesophilic lactic acid cultures and yoghurt bacteria. Iranian Journal of Biotechnology. 10, 16–21.
Staley, J.T., Konopka, A., 1985. Measurements of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. 39, 321–346.
Sunny-Roberts, E.O., Ananta, E., Knorr, D., 2007. Flow cytometry assessment of *Lactobacillus rhamnosus* GG (ATCC 53103) response to non-electrolytes stress. Nutr. Food Sci. 37, 184–200.
Tabasco, R., Paarup, T., Janer, C., Pelaez, C., Requenca, T., 2007. Selective enumeration and identification of mixed cultures of *Streptococcus thermophiles*, *Lactobacillus delbrueckii* subsp *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei and Bifidobacterium lactis* in fermented milk. Int. Dairy J. 17, 1107–1114.
Taylor, S., 2013. A map to more reliable RT-qPCR results using MIQE guidelines. Am. Lab. 45 (10), 18 (20-21).
Teraguchi, S., Uehara, M., Ogasa, K., Mitsuoka, T., 1978. Enumeration of bifidobacteria in dairy products. Jpn. J. Bacteriol. 33, 753–761.
Tomasiewicz, D.M., 1980. The most suitable number of colonies on plates for counting. J. Food Prot. 43, 282–286.
Tracy, B.P., Gaida, S.M., Papoutsakis, E.T., 2010. Flow cytometry for bacteria: enabling metabolic engineering, synthetic biology and the elucidation of complex phenotypes. Curr. Opin. Biotechnol. 21, 85–99.
Upton, A., Lang, S., Heffernan, H., 2003. Mupirocin and *Staphylococcus aureus*: a recent paradigm of emerging antibiotic resistance. J. Antimicrob. Chemother. 51, 613–617.
<1227>USP, 2011. Validation of microbial recovery from pharmacopeial articles. United States Pharmacopeia, 34, pp. 783–786.
<61>USP, 2011a. Microbial examination of nonsterile products: microbial enumeration tests. USP 34, United States Pharmacopeia pp. 52–56.
Van de Casteele, S., Vanheuverzwijn, T., Ruyssen, T., van Assche, P., Swings, J., Huys, G., 2006. Evaluation of culture media for selective enumeration of probiotic strains of lactobacilli and bifidobacteria in combination with yoghurt or cheese starters. Int. Dairy J. 16, 1470–1476.
Vankerckhoven, V., Huys, G., Vancanneyt, M., Vael, C., Klare, I., Romond, M.-B., Entenza, J., Moreillon, P., Wind, R., Know, J., Wiertz, E., Pot, B., Vaughn, E., Kahlmeter, G., Goossens, H., 2008. Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. Trends Food Sci. Technol. 19, 102–114.
Villlarreal, M.L.M., Padiha, M., Vieira, A.D.S., de Melo Franco, B.D.G., Ruiz Martinez, R.C., Isay Saad, S.M., 2013. Advantageous direct quantification of viable closely related probiotics in *Petit-Suisse* cheeses under in vitro gastrointestinal conditions by propidium monoazide-qPCR. PLoS One. http://dx.doi.org/10.137/journal.pone.009212 (December 17).
Vinderola, C.G., Reinheimer, J.A., 1999. Culture media for the enumeration of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in the presence of yoghurt bacteria. Int. Dairy J. 9, 497–505.
Volkert, M., Ananata, E., Luscher, C., Knorr, D., 2008. Effect of air freezing, spray freezing, and pressure shift freezing on membrane integrity and viability of *Labctobacillus rhamonosus* GG. J. Food Eng. 87, 532–540.
Wagner, A.O., Malin, C., Knapp, B.A., Illmer, P., 2008. Removal of free extracellular DNA from environmental samples by ethidium monoazide and propidium monoazide. Appl. Environ. Microbiol. 74, 2537–2539.
Xu, H.-S., Roberts, N., Singleton, F.L., Attwell, R.W., Grimes, D.J., Colwell, R.R., 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholera* in the estuarine and marine environment. Microb. Ecol. 8, 313–323.
Zotta, T., Parente, E., Ricciardi, A., 2009. Viability staining and detection of metabolic activity of sourdough lactic bacteria under stress conditions. World J. Microbiol. Biotechnol. 25, 1119–1124.
Zotta, T., Guidone, A., Tremonte, P., Parente, E., Ricciardi, A., 2012. A comparison of fluorescent stains for the assessment of viability and metabolic activity of lactic acid bacteria. World Journal of Microbiology and Biotechnology. 28, 919–927.