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Review

The enumeration of probiotic issues: From unavailable standardised culture media to a recommended procedure?

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ABSTRACT

Probiotic products are becoming increasingly popular worldwide. Regulators and quality control personnel need a clear path to count viable cells within each product. We have reviewed progress on the enumeration of probiotics in foods and supplements. Today, no single culture medium or combination of culture media can accurately enumerate all probiotics available. Culture-independent techniques can speed the counting process compared with traditional agar plating, but their sophistication makes them unsuitable for routine quality control in many laboratories. The enumeration of probiotics in a given product needs to be tailor-made: the response of each probiotic and starter culture used in the food or supplement to a narrow set of culture media should be assessed first to identify the proper conditions for an accurate enumeration. A standardised procedure should be developed in the near future with the consensus of the scientific, industrial and regulator communities.

Contents

1. Introduction

The middle 1990's are pointed to as the beginning of the massive incorporation of probiotics into fermented milk products and the development of probiotic supplements. Since then there has been a global increase in popularity and diversity of probiotic products and the market is expected to grow for at least the next ten years. However, as the range of complexity of probiotic formulations has increased, so too has the importance of quality and regulatory control. A key factor to support market growth is therefore the ability to have an accurate count of viable cells in any product, but as yet there is no single culture medium or combination of culture media that can be used to accurately enumerate all probiotics. In this review, we first provide context for the issue, and we take a brief look at the various techniques and culture media used to date. A simple procedure is then proposed for regulatory authorities and quality control laboratories to use for enumeration of probiotic

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bacteria in foods and supplements, its limitations are discussed, and the need for a global consensus action is highlighted.

2. Definition and proper use of the term probiotics

Reunited in Argentina, the expert panel commissioned in 2001 by the Food and Agriculture Organisation of the United Nations and the World Health Organisation issued an evaluation of the health and nutritional properties of probiotics in food, including powdered milk with live lactic acid bacteria. As part of the resulting report, this expert panel defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). From that moment on, this definition grew in acceptance and use by the scientific and industrial communities, and many regulatory organisations worldwide incorporated it into their local legal framework for probiotics.

More recently, the FAO/WHO definition was revisited and further supported by the International Scientific Association for Probiotics and Prebiotics (ISAPP). ISAPP organised a meeting of clinical and scientific experts on probiotics in 2013 to re-examine the concept of probiotics; this meeting included participants in the original FAO/WHO expert panel and members of the FAO/WHO working group. As a result of that meeting, the FAO/WHO definition for probiotics was endorsed, with a minor grammatical correction. Probiotics are now defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". This document also proposed an overall framework for the proper use of the term probiotic, which did not limit the regulatory category of probiotics, that excludes fermented foods with no evidence of health benefits and with undefined microbial content and undefined consortia such as faecal microbiota transplants (Hill et al., 2014).

In this context, the proper use of the term probiotic implies the need to know the identity of the microbe(s) and to assess the level of viable cells in food and supplements in an accurate way so that a sufficient number of live probiotics is delivered in a product. Both regulatory and quality control authorities should have well-defined means to assess the cell viability of each probiotic contained in foods, supplements or any substances properly regarded as probiotic to ensure that consumers get the required dose of viable cells of the probiotic strain with each intake.

3. Diversity of carriers and microorganisms used as probiotics

The term probiotics comprises all microbial strains administered alive with health benefits to the host demonstrated with at least one clinical study (human intervention in the case of probiotics meant for human health). However, these microbes may belong to many different microbial groups, which possess very different nutritional growth requirements. The many different probiotics display very different responses to the ingredients used for formulating culture media, including drugs added to make culture media selective or differential. The main genera used as probiotics for humans are *Lactobacillus* and *Bifidobacterium* and, to a lesser extent, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Saccharomyces* and *Bacillus*. These genera represent diverse microbial groups such as lactic acid bacteria, bifidobacteria, yeasts and spore-forming bacilli. In dairy products, the most researched probiotic lactic acid bacterium is *Lactobacillus rhamnosus* GG (Westerik Kort, Sybesma, & Reid, 2018), whereas the most researched bifidobacteria is *Bifidobacterium animalis* subsp. *lactis* BB12 (Jungersen et al., 2014). In non-dairy products, *Lactobacillus plantarum* 299v is one of the probiotic strains with the most intensive use in commercial products (mainly oat-based fruit juices). *Saccharomyces boulardii* and *Bacillus*, however, are less frequently used in food, but they have been largely used in dietary supplements. *Bacillus coagulans* BC30 has been used in a variety of food such as pasta, tea bags, non-fermented milk, muffins, ice cream and protein bars. In short, spore-forming bacilli are used in food products that represent a challenge for survival for traditional vegetative *Lactobacillus* and *Bifidobacterium* cells. Although fermented milks are the main foods in which probiotics have been included, other food, such as cheese, fruit juices, cereal bars, tea bags, pasta and chocolate bars, sometimes includes probiotics as well. Among supplements, different formats, such as liquid, drops, capsules, powders, tablets or gums, can be found in the market.

Table 1 shows some examples of commercial foods and supplements containing probiotics from different species. The microbiological composition of commercial probiotic foods and supplements may be as simple as containing a single strain or very complex and containing more than 10 species or strains. The complexity involved in an accurate enumeration of each strain increases with the number of different strains used. Differential agar media may differentiate at the genus level and in some cases at the species level, but it is virtually impossible to distinguish among different strains in multi-strain products using agar media.

4. Techniques for the enumeration of probiotics

The enumeration of viable cells using solid media (agar based) dates back to the 19th century when bacteriology was founded. Culture methods enabled the detection, enumeration and isolation of viable bacteria. The scientific community typically considers a cell "viable" if it reproduces to form a colony on an agar plate that contains the necessary nutrients. However, this definition is limited and at the same time too broad. Microbes exist in a variety of metabolic states depending on environmental conditions and the presence of stressors. From being viable to being dead, cells can undergo different physiological states such as non-replicating (in stationary phase; inhospitable conditions for replication or injured), starving, dormant or irreparably damaged. Therefore, a consensus on the operational definition of live, as stipulated in the definition of probiotic, needs to be established (Davis, 2014).

Today, the most commonly used forms of bacteriological analysis in food microbiology are detection and enumeration using liquid or agar media (Gill, 2017). Plate count methods, whereby cells can replicate on a given agar medium until forming a visible colony, are currently accepted for determining probiotic viability and are intensively used in routine quality control laboratories. Since probiotic bacteria are expected to be present in a concentration higher than 10^6–10^7 cfu g^{-1} or mL^{-1}, the sensitivity of cultural methods for bacterial detection is not an issue. Such an approach fails to count vegetative cells that are dormant or in a viable but non-culturable state. Methods are available that allow for the resuscitation of bacteria in such a state, but still the key question is the physiological fitness of those cultures, i.e., their ability to produce a health benefit once consumed. As the definition of probiotics implies that cells must be viable, cells entering this physiological state may be challenging to count, but they can resume replication following exposure to an appropriate resuscitation stimulus (Pinto, Santos, & Chambel, 2015).

Identifying the correct method for cell resuscitation should be the path to the proper enumeration of viable cells. For instance, the simple addition of 0.05% (w/v) cysteine to the agar media, coupled with anaerobic incubation even for facultative anaerobes like most lactobacilli, may work in many cases. Di Lena et al. (2015) developed a selective medium and applied it to reliably detect members of the *Lactobacillus casei* group regardless of their physiological condition. The use or non-use of resuscitation stimuli for cell counts should be discussed and agreed upon to determine whether strictly viable cells are of interest (no use of resuscitation stimuli) or if cells

Table 1
Foods and supplements containing single and multiple strains of probiotic microorganisms.[a]

Strains/species	Type of product	Brand name	Reference
Lb. reuteri DSM 17938	Drops	BioGaia Protectis	Kołodziej and Szajewska (2017)
E. coli Nissle 1917	Capsules	Mutaflor	Kruis et al. (2004)
Bif. infantis 35624	Capsule	Allign	Fredua-Agyeman and Gaisford (2015)
Lb. rhamnosus GG	Capsules	Amerifit Brands	Cabana et al. (2017)
Sac. boulardii	Capsules	Nexabiotic	Guslandi, Giollo, and Testoni (2003)
Lb. acidophilus, *Lb. rhamnosus*	Capsules	Bacilac	Temmerman, Scheirlinck, Huys, & Swings (2003)
Lb. rhamnosus GR-1 and *Lb. reuteri* RC-14	Gelatin capsules	Fem dophilus	Anukam et al. (2006)
Lb. acidophilus, *Lb. rhamnosus*, *Bif. bifidum*	Capsules	Bififlor	Temmerman et al., 2003
Lb. paracasei ŁOCK 0919, *Lb. casei* ŁOCK 0900, *Lb. casei* ŁOCK 0908	Powder	Latopic	Zawistowska-Rojek, Zareba, Mrówka, and Tyski (2016)
S. thermophilus DSM 24731, *Bif. breve* DSM 24732, *Bif. longum* DSM 24736, *Bif. infantis* DSM 24737, *Lb. acidophilus* DSM 24735, *Lb. plantarum* DSM 24730, *Lb. paracasei* DSM24733, *Lb. delbrueckii* subsp. *bulgaricus* DSM 24734	Capsules	VSL#3	Connell et al. (2018)
B. subtilis PXN21, *Bif. bifidum* PXN23, *Bif. breve* PXN25, *Bif. infantis* PXN27), *Lb. acidophilus* PXN3, *Lb. delbrueckii* subsp. *bulgaricus* PXN39, *Lb. casei* PXN37, *Lb. plantarum* PXN47, *Lb. rhamnosus* PXN54, *Lb. helveticus* PXN45, *Lb. salivarius* PXN57, *Lc. lactis* PXN63, *S. thermophilus* PXN66	Capsule	Bio-kult	Fredua-Agyeman and Gaisford (2015)
B. clausii	Liquid ampoules	Enterogermina	Patrone, Molinari, and Morelli (2016)
Lb. rhamnosus, *Lb. planatarum*, *Lb. acidophilus*, *Enterococcus faecium*	Liquid	Symprove	Fredua-Agyeman and Gaisford (2015)
Lb. reuteri prodentis	Chewing gum	GUM PerioBalance	Vivekananda, Vandana, and Bhat (2010)
Lb. acidophilus, *Lb. casei*	Chocolate bar	Attune	Gadhiya, Patel, and Prajapati (2015)
Lb. helveticus, *Bif. longum*	Chocolate bar	Ohso	Gadhiya et al. (2015)
Lb. paracasei 8700:2, *Lb. plantarum* HEAL 9	Fruit juices	Golden circle	Ranadheera, Vidanarachchi, Silva Rocha, Gomez Cruz, and Ajlouni (2017)
Lb. casei Lc431	Fruit-flavoured water	Perkii	Ranadheera et al. (2017)
Lb. plantarum 299v	Fruit juice and fermented oats	ProViva	Ranadheera et al. (2017)
Lb. rhamnosus GG	Fruit juice	Biola	Ranadheera et al. (2017)
Lb. acidophilus LA-5, *Bif. animalis* subsp. *lactis* BB-12	Fermented milk	Cultura	Laake et al. (2005)
Lb. casei GG, *Bif. bifidum*, *Lb. acidophilus*	Fermented milk	Vitamel	Temmerman et al., 2003
Lb. casei DN 114-001, *S. thermophilus*, *Lb. bulgaricus*	Fermented milk	Actimel	Fredua-Agyeman and Gaisford (2015)
Bif. animalis subsp. *lactis* DN 173-010, *S. thermophilus*, *Lb. bulgaricus*, *Lc. lactis*.	Fermented milk	Activia	Temmerman et al., 2003
Lb. casei Shirota	Fermented milk	Yakult	Fredua-Agyeman and Gaisford (2015)
B. coagulans BC30	Tea	Lavender Chamomile	https://www.bigelowtea.com
B. coagulans BC30	Coffee	Cooper Moon Cappuccino	https://www.foodnavigator-usa.com
B. coagulans BC30	Microwavable muffins	FlapJacked	http://www.nutritionaloutlook.com
Lb. acidophilus, *Lb. bulgaricus*, *Lb. casei*, *Lb. fermentum*, *Lb. plantarum*, *Lc. lactis*, *B. subtilis*, *Bif. bifidum*, *Bif. infantis*, *Bif. longum*; *S. thermophilus*, *Sac. cerevisiae*	Fruit juices	Bio-Live Gold & Dark	Ranadheera et al. (2017)
Lb. acidophilus LA-5	Cereal	Yogactive	https://www.yogactive.com/

[a] Microorganism abbreviations are: *Lb.*, *Lactobacillus*; *E.*, *Escherichia*; *Bif.*, *Bifidobacterium*; *Sac.*, *Saccharomyces*; *S.*, *Streptococcus*; *B.*, *Bacillus*; *Lc.*, *Lactococcus*.

close to vitality will also be recovered and counted, thereby contributing to the count of total viable cells. It is worth mentioning that some non-viable cells still have health benefits (Rodríguez-Nogales et al., 2015; Sakar, 2018).

The use of general, selective or differential agar-based culture media has been the gold standard for the enumeration of probiotic bacteria in food and supplements. In many cases it is the only option available. Most lactobacilli and bifidobacteria grow satisfactorily in many different commercial versions of the well-known de Man–Rogosa–Sharpe (MRS) agar. However, it lacks differential capacity: many different species of *Lactobacillus* render exactly the same type of colony when surface-plated on this medium. If the food or supplement contains only one strain (see examples in Table 1), then a proper enumeration should be achieved by spreading suitable dilutions of the products on the surface of MRS agar, desirably supplemented with cysteine to promote the recovery of injured cells. Surface plating is always encouraged compared with pour plating, even in single (pure) culture, as any contamination is more readily detected by the naked eye when grown on the surface rather than inside the agar.

Some drawbacks of culture-based techniques for probiotic enumeration include 1) the labour-intensive and time-consuming preparation times of culture media, 2) the lack of differentiation of closely related species, 3) the lack of selectivity when antibiotics are used as selective agents, 4) the prolonged incubation times (up to 96 h in certain cases to achieve proper differentiation among colonies of different species), 5) the lack of selectivity for cases in which relative proportions of different strains in a product are not equal and 6) counting one chain of cells as a single cfu, resulting in a high probability of an underestimated count. The simultaneous presence of several species (starter cultures and probiotic bacteria in fermented milks, for instance) sharing similar cultural characteristics (they may all be lactic acid bacteria in the end) can make the differential or selective colony count of each species a challenging task (Ashraf & Shah, 2011). It is at this point that molecular techniques become of great value.

Genomic technologies are attractive for culture-independent detection and include molecular techniques comprised of PCR-based methods, non-PCR-based methods or their combination, which have been extensively reviewed (Bagheripoor-Fallah,

Mortazavian, Hosseini, Khoshgozaran-Abras, & Rad, 2015). Recently, flow cytometry methodologies were revised and proposed as a potential method of measuring bacterial viability in probiotic products (Wilkinson, 2018). Culture-independent methods can provide reliability through the simultaneous detection of several genes or their transcripted products; they have higher accuracy, sensitivity and simplicity when compared to culture-dependent techniques. However, at the same time, these methods can be expensive and too sophisticated for widespread routine use, and they require very well-trained personnel (Bagheripoor-Fallah et al., 2015), limiting their availability to the research and academic domains. Additionally, genomic technologies evolve very fast, and sometimes specific reagents or devices and accessories for existing equipment are no longer available after some years on the market. However, the main limitation of genomic technologies is that they are not suitable for addressing viability (Gill, 2017).

It is interesting to take a look at Sorbiflore, a product approved in the European Union by the European Food Safety Authority as a piglet feed additive. The product is a preparation of *Lb. rhamnosus* and *Lactobacillus farciminis* (EFSA, 2008). The strains were enumerated using epifluorescent microscopy; 4′,6-diamino-2-phenylindole-2HCl (DAPI) was used to label the bacterial cells irrespective of their viability, while viable staining (VFU counts) was performed using Chemchrom B dye. The dye requires a functional membrane and active non-specific cytoplasmic esterases to create fluorescence. Chemchrom B stains both cultivable and non-cultivable cells. Although it has not been demonstrated that non-cultivable Chemchrom B stained cells are truly viable in the physiological sense, the EFSA expert panel considered the possibility that the measured VFU could be related to the active agents in the additive and therefore offers a means of monitoring the viability of the strains in the product (EFSA, 2008).

When levels of viable cells are compared using culture-dependent and culture-independent techniques, differences in counts may arise. Four methods of enumeration (plate counts, quantitative real-time PCR, fluorescent in situ hybridisation (FISH) and a commercial LIVE/DEAD BacLight bacterial viability kit) were compared by monitoring the levels of probiotic bifidobacteria in foods. Plate counts were lower than the PCR and FISH counts, whereas the LIVE/DEAD counts were comparable with those obtained by PCR and FISH, suggesting that the cells unable to grow on plates may have become dormant. Lahtinen, Gueimonde, Ouwehand, Reinikainen, and Salminen (2006a) concluded that the choice of enumeration method for probiotic bacteria may have a significant effect on the results of the analysis.

Cells may enter a viable but non-culturable state, in which they are dormant but metabolically active, in response to the stresses encountered during processing, formulation and addition to food. For example, it was reported that three probiotic bacteria showed a reduction in plate counts but were able to maintain esterase activity, an intact cytoplasmic membrane and pH gradient (Lahtinen et al., 2006b). These apparently uncultivable probiotic cells were still active and stress resistant. These microbes are, or may be, capable of replicating on agar plates once they are exposed and acclimated to a more favourable environment.

Whether or not the definition of live probiotic bacteria will include this range of metabolic states for the reliable enumeration of total live bacteria should still be discussed and agreed upon. Alternative methods (fluorescent in situ hybridisation, real-time quantitative PCR, reverse transcriptase PCR, propidium monoazide PCR and cell-sorting techniques) offer the potential to enumerate both culturable and viable but non-culturable bacteria (Davis, 2014), but are expensive and challenging to set up for routine analysis. Because standard culture-dependent methods enumerate only replicating cells, culture techniques may underestimate the number of viable organisms that contribute to the functional capacity of the probiotic (Zielińska, Ołdak, Rzepkowska, & Zieliński, 2018). In terms of the industry, regulatory authorities and day-to-day use in most routine laboratories, the availability of culture-dependent methodologies is still of value, and it will be so in the near future.

5. Culture media proposals over history

The International Dairy Federation (IDF) characterises itself as a representative of the global dairy sector to ensure that the best scientific expertise is used to achieve and maintain high-quality milk and nutritious, safe and sustainable dairy products. At the beginning of the 1990s, the IDF was the first institution that addressed the issue of the enumeration of probiotics. IDF bulletin 252/1990 reported the assessment of 14 culture media for the detection and enumeration of bifidobacteria in fermented milk products. The authors of the bulletin concluded that the choice of a culture medium depended first on the type of products under study, emphasising the use of BL (blood liver) and NPLN (neomycin paromomycin lithium chloride and nalidixi acid) agar. They warned that 90% may be the maximum recovered, suggesting that these media may be partially inhibitory to the cultures aimed at being enumerated.

In 1995, IDF bulletin 306 (on the detection and enumeration of *Lb. acidophilus*) was published as a joint effort of the IDF/ISO/AOAC E104 group of experts on lactic acid bacteria and starters. This review proposed the use of six culture media when *Lb. acidophilus* was the only fermenting microorganism, five culture media when *Lb. acidophilus* occurs in combination with mesophilic LAB or bifidobacteria, and 10 culture media when *Lb. acidophilus* occurs in combination with *Streptococcus thermophilus* (or yoghurt bacteria) or *S. thermophilus* (or yoghurt bacteria) and bifidobacteria. However, no single culture media was effective for all of the possible cases presented. It is worth mentioning that the Guidelines for the Evaluation of Probiotics in Food released in 2002 by the FAO/WHO did not address the issue of the enumeration of probiotics in foods or feeds.

IDF bulletin 411/2007 reported a major effort in the development of a standard method for the selective enumeration of bifidobacteria in dairy products. For this collaborative trial, seven samples of yoghurt or infant formula containing different bifidobacteria species, alone or in different combinations with *S. thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. acidophilus*, *Lb. casei* or *Lactobacillus gasseri*, were sent to 15 research institutes or dairy companies in Europe and six in Japan and New Zealand. All participants were instructed to follow a given procedure and to report their results. This trial was published as ISO 29981:2010 (IDF 220:2010) standard entitled "Milk products – Enumeration of presumptive bifidobacteria – Colony count technique at 37 °C". This standard specifies a method for the selective enumeration of presumptive bifidobacteria in milk products (fermented and non-fermented milks, milk powders, infant formula) using the antibiotic Li-Mupirocin, which inhibits the growth of most lactic acid bacteria commonly used in dairy products.

In 2006, the standard ISO 20128:2006 (IDF 192:2006) entitled "Milk products – Enumeration of presumptive *Lactobacillus acidophilus* on a selective medium – Colony-count technique at 37 °C" was released. The method is applicable to fermented and non-fermented milks, milk powders and infant formulae in which presumptive *Lb. acidophilus* is present and in combination with other lactic acid bacteria and bifidobacteria. The method is not applicable when the number of presumptive *Lb. acidophilus* is less than 10^4 cfu g^{-1} and the numbers of *L. rhamnosus*, *Lactobacillus reuteri* and *Lactobacillus paracasei* subsp. *paracasei* are greater than 10^6 cfu g^{-1}. It is worth noting that in terms of both IDF/ISO standards the results obtained are presumptive, and further analysis

must be performed to confirm that the colonies counted belong to the genus *Bifidobacterium* or to the species *Lb. acidophilus*. It is also worth noting that no standard was developed to enumerate the commercially important species of *Lb. casei* or *Lb. plantarum*, the latter species being most often used in non-dairy probiotic products. In terms of culture-independent procedures, ISO/IDF also released a methodology for the quantification of lactic acid bacteria and probiotics through flow cytometry (ISO 19344:2015, IDF 232).

Researchers have been searching for culture media able to accurately enumerate probiotics in foods and supplements. Dave and Shah (1996) evaluated 15 culture media for the enumeration of yoghurt bacteria, *Lb. acidophilus* and bifidobacteria. They determined that for the selective enumeration of *Lb. acidophilus*, MRS-salicin agar or MRS-sorbitol agar could be used. For the selective enumeration of bifidobacteria, MRS–NNLP (nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate) agar was found to be a suitable culture medium. However, the determination of the bifidobacteria count as the difference between the count of *Lb. acidophilus* (on MRS-salicin agar or MRS-sorbitol agar) and the total counts of *Lb. acidophilus* and bifidobacteria (obtained in MRS-maltose agar) resulted in a higher recovery of some strains of bifidobacteria.

Roy (2001) reviewed culture media for the enumeration of bifidobacteria in dairy products. Culture media were classified as basal, elective, differential or selective. Non-selective media are useful for the routine enumeration of bifidobacteria when present in non-fermented milks. It was concluded that reinforced clostridial agar and MRS were the preferred media for industrial quality control laboratories. Eighteen media for the selective enumeration of bifidobacteria from other lactic acid bacteria were proposed, but it was concluded that there is no standard medium for the detection of bifidobacteria. However, if a recommendation should be made, then Columbia agar supplemented with LiCl and Na propionate and MRS supplemented with LiCl, neomycin, paromomycin and nalidixic acid could work.

Ashraf and Shah (2011) reviewed results on the use of more than 40 culture media for the selective and differential enumeration of starter and probiotic bacteria (*Lb. acidophilus*, *Lb. casei* and *Bifidobacterium*) in yoghurt. The authors concluded that the differential enumeration of yoghurt bacteria could be performed on seven different agar media. Regarding probiotics, the total count of *Lb. acidophilus* and bifidobacteria could be achieved in MRSM (MRS + maltose) agar. The selective enumeration of *Lb. acidophilus* could be performed in BAM (basal agar maltose), MRS-clindamycin or X-Glu agar in products containing mixed lactobacilli, streptococci and bifidobacteria. *Lb. casei* could be selectively enumerated on LC agar, whereas *Bifidobacterium* could be enumerated on MRS–NPNL agar.

Tharmaraj and Shah (2003) used 19 bacteriological media to assess their suitability for the selective enumeration of starter

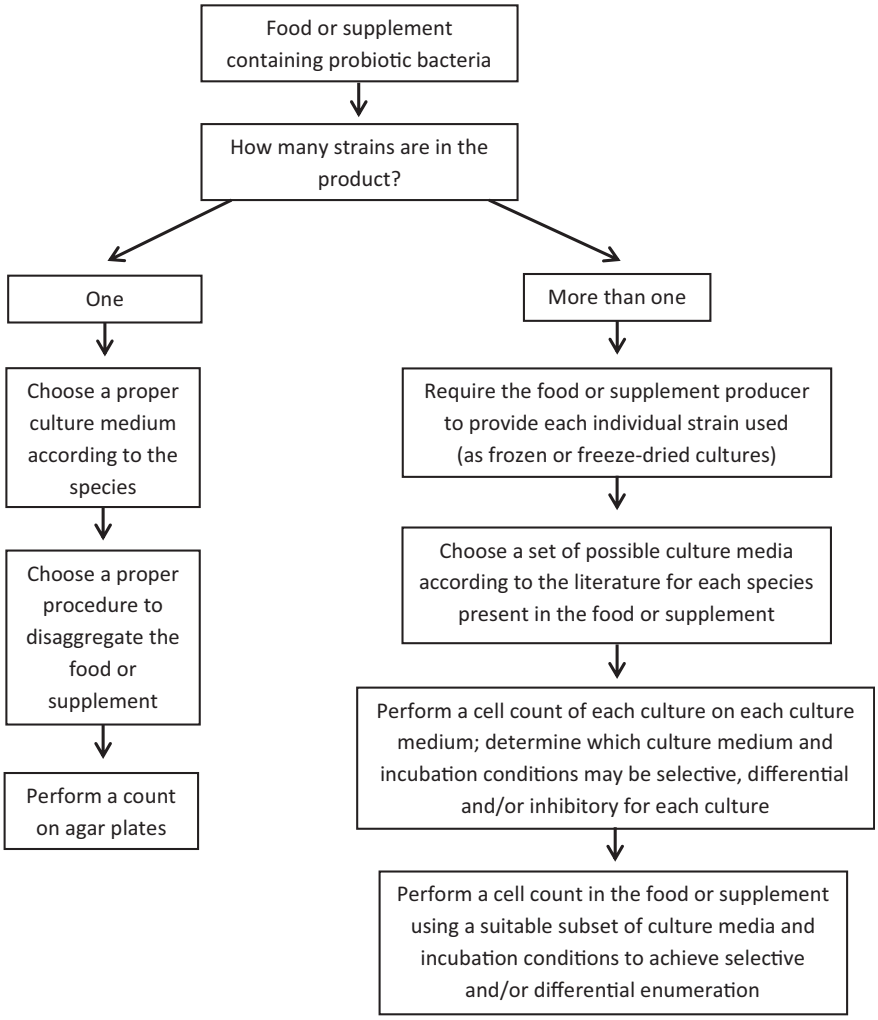

Fig. 1. Procedure for the choice of a set of culture media to perform the selective and/or differential enumeration of probiotics in foods or supplements. The procedure is not applicable when different strains of the same species are present.

yoghurt bacteria and probiotic strains of *Lb. casei*, *Lb. rhamnosus*, *Lb. acidophilus*, bifidobacteria and propionibacteria. MRS-vancomycin agar at 43 °C and 37 °C was suitable to enumerate *Lb. rhamnosus* and *Lb. casei*, respectively. *Lb. acidophilus* could be enumerated using MRS, basal agar-maltose or BA-sorbitol agar. MRS–NNLP could be used for bifidobacterial, whereas propionibacteria could be enumerated on sodium lactate agar. However, most proposals in this work relied on subtracting specific counts from total counts, which may lead to inaccurate estimations.

The American Public Health Association updated in 2015 their compendium of methods for the microbiological examination of foods, including a chapter devoted to probiotics (Schoeni, 2015). Recently, Majhenič, Lorbeg, and Treven (2018) revised the enumeration and identification procedures for mixed probiotic and lactic acid bacteria starter cultures, concluding that plate counting remains the most frequently used method for this purpose and that no single medium or combination of defined media is applicable to all dairy products, making the selection of suitable selective media a remaining challenge.

6. A proposed procedure for regulatory authorities and quality control laboratories for the enumeration of probiotic bacteria in foods and supplements

The enumeration of probiotic bacteria is a challenge as probiotics represent a heterogeneous group of microbes in an array of product formats that may also include food fermentation microbes (starter cultures in fermented milks, for example). Although many efforts have been made by public institutions and researchers, we still lack a standardised method or culture media that is useful irrespective of the product and the species present. In many cases, public institutions devoted to food control are challenged to assay the level of viable bacteria in probiotic products, but the choice of methods is not clear. One path would be for institutions in charge of registering this kind of product to require that companies include the precise methodology used to assess the level of viable cells of each strain on the product label, as the total count is not sufficient. In the case that this information is not available, the procedure described in Fig. 1 may be useful in laboratories requested to perform quality control for a given product. However, as colonies of strains of the same species are generally not possible to differentiate on the surface of agar plates, the procedure may not be valid when mixed strains are present.

The flow diagram proposed in Fig. 1 suggests assessing the performance of all cultures used to formulate the food or supplement under analysis (in their frozen or freeze-dried form) in a set of culture media chosen from those that have been proposed so far for the selective and/or differential enumeration of probiotics. Some of the culture media were discussed above and recently reviewed (Zielińska et al., 2018). When performing this task, it is very important to take into consideration the many operative factors in cell enumeration that may affect the results, such as sample preparation (rehydration, thawing, temperature, time, ratio of sample to diluent) and dilutions (homogenisation, serial dilution, media), and that have been reviewed in detail in the work of Champagne, Ross, Saarela, Hansen, and Charalampopulos (2011).

This approach was used in our laboratory in the late 1990s to identify suitable culture conditions to enumerate *Lb. acidophilus*, *Lb. casei* and bifidobacteria in Argentinian fresh cheese manufactured with *S. thermophilus* and *Lactococcus lactis* as starter cultures (Vinderola, Prosello, Ghiberto, & Reinheimer, 2000). Fifteen culture media were assessed based on their capacity to allow for the selective or differential growth of two strains of *Lb. acidophilus*, two strains of bifidobacteria and four yoghurt starter cultures using three incubation conditions (aerobiosis, anaerobiosis and microaerobiosis; Vinderola & Reinheimer, 2000). Two culture media were shown to be effective with commercial MRS with added bovine bile salts (MRS-B) or added lithium hydrochloride and sodium propionate (MRS-LP). Then, the recovery capacity of these media using MRS as a control for four strains of *Lb. casei* and more strains of *Lb. acidophilus* and bifidobacteria was assessed (Vinderola & Reinheimer, 2000), observing that these two culture media were useful for the selective and differential count of three probiotic bacteria in cheese, while inhibiting the growth of lactic acid starters used for cheese production (Fig. 2).

7. Is consensus action needed?

INFOGEST (http://www.cost-INFOGEST.eu/) is a network action that aims to harmonise in vitro protocols to simulate the human gastrointestinal digestion. A harmonised static in vitro digestion method using skim milk powder as a model food was developed. To validate this protocol, inter-laboratory trials were conducted (Egger et al., 2016).

This protocol with consensus may also be applied to study the in vitro survival of probiotics in the intestine (Minekus et al., 2014), a test for which no standardised procedure has yet been agreed (Burns, Lafferriere, Vinderola, & Reinheimer, 2014). In 2014 (Hill et al., 2014) and 2017 (Gibson et al., 2017), ISAPP led a consensus action about probiotics and prebiotics, respectively. It would be convenient for leading actors such as ISAPP, IPA (International Probiotics Association) and the IDF to come together to produce a consensus document with a reference for viable cells (Culture-dependent or culture-independent technique? If agar plating is used, with the addition of resuscitation ingredients or not?) and a standardised procedure for probiotic enumeration in simple and

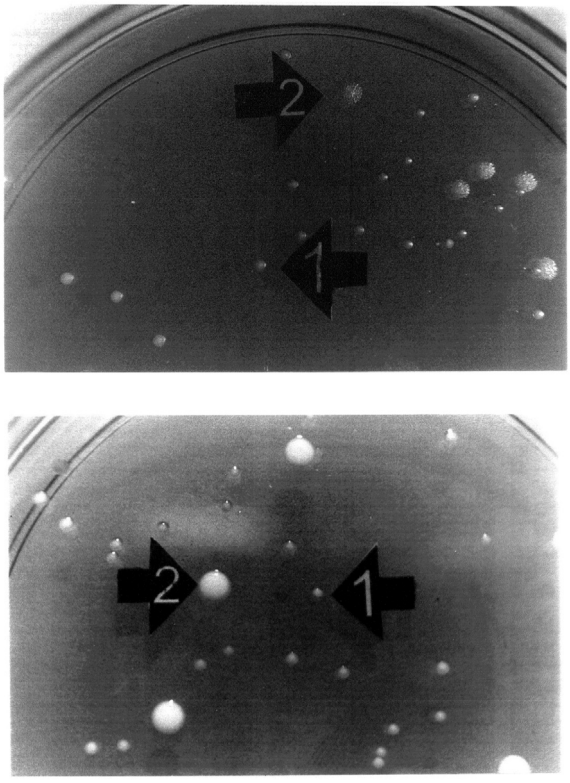

Fig. 2. Colonies of *Lb. casei* (1) and *Lb. acidophilus* (2) (top panel) on MRS-bile (72 h, aerobiosis, 37 °C) and colonies of *Lb. casei* (1) and *Bifidobacterium* (2) (bottom panel) on MRS-LP (96 h, anaerobiosis, 37 °C) from probiotic fresh cheese. *Streptococcus thermophilus* and *Lactococcus lactis* (cheese starters) were inhibited in both culture media.

complex foods and supplements. This document would be invaluable for regulators and quality control laboratories in many places where legal frameworks and guidelines are still scarce. Additionally, such a consensus procedure could finally be incorporated into the standard being constructed following the proposal made by IPA during the 39th session of the Codex Alimentarius (CA) Committee on Nutrition and Foods for Special Dietary Use (CCNFSDU), held in Berlin (Germany) in November 2018. IPA presented a proposal for new work on harmonised probiotic guidelines for use in foods and dietary supplements (IPA, 2018).

8. Conclusions

More than 30 years have passed since the intensive arrival of probiotic products on the market in foods and dietary supplements, and the future seems bright in terms of the development and launch of new products. Ranging from simple formulations containing one strain to those containing more than 10 cultures, the enumeration of viable cells on agar media is of increasing complexity and may reach a point at which the differentiation of species and strains is no longer possible. The most accurate approach may be the use of molecular techniques, but affordable methods that can identify only live microbes are essential.

Many culture media have been proposed as effective for the selective and/or differential enumeration of probiotics. However, today no single culture medium is able to carry out this task in an accurate manner for all probiotic products. Culture-independent techniques can speed up the counting process compared with traditional plating and can give insight into cell populations that are underestimated on agar plates. However, some characteristics (cost of the equipment and reagents, need for a specialist to manage the technique) still makes them unsuitable for day-to-day routine quality control in many laboratories around the world, especially in developing countries.

Leaving behind the idea of a culture media or a set of culture media universally suitable for enumerating probiotics, the solution needs to be tailor-made by considering the specific strains used in the product and its characteristics. The response of each probiotic and starter culture used in a food or supplement to a narrow set of culture media should be assessed first to find those suitable to recover each culture, while inhibiting the others or making them differential on agar plates. A consensus on what viable cells mean, how to measure them and a standardised procedure will help regulators and quality control experts and will support and promote this growing market.

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References

Anukam, K., Osazuwa, E., Ahonkhai, I., Ngwu, M., Osemene, G., Bruce, A. W., et al. (2006). Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: Randomized, double-blind, placebo controlled trial. *Microbes and Infection, 8*, 1450–1454.

Ashraf, R., & Shah, N. P. (2011). Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt-a review. *International Journal of Food Microbiology, 149*, 194–208.

Bagheripoor-Fallah, N., Mortazavian, A., Hosseini, H., Khoshgozaran-Abras, S., & Rad, A. H. (2015). Comparison of molecular techniques with other methods for identification and enumeration of probiotics in fermented milk products. *Critical Reviews in Food Science and Nutrition, 55*, 396–413.

Burns, P., Lafferriere, L., Vinderola, G., & Reinheimer, J. (2014). Dairy practices influence the capacity of probiotic bacteria to overcome simulated gastric digestion. *International Journal of Dairy Technology, 67*, 448–457.

Cabana, M. D., McKean, M., Caughey, A. B., Fong, L., Lynch, S., Wong, A., et al. (2017). Early probiotic supplementation for eczema and asthma prevention: A randomized controlled trial. *Pediatrics, 140*. Article 20163000.

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. *International Journal of Food Microbiology, 149*, 185–193.

Connell, M., Shin, A., James-Stevenson, T., Xu, H., Imperiale, T. F., & Herron, J. (2018). Systematic review and meta-analysis: Efficacy of patented probiotic, VSL#3, in irritable bowel syndrome. *Neurogastroenterology and Motility, 30*. Article 13427.

Dave, R. I., & Shah, N. P. (1996). Evaluation of media for selective enumeration of *Streptococcus thermophilus, Lactobacillus delbrueckii* ssp. *bulgaricus, Lactobacillus acidophilus*, and bifidobacteria. *Journal of Dairy Science, 79*, 1529–1536.

Davis, C. (2014). Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. *Journal of Microbiological Methods, 103*, 9–17.

Di Lena, M., Quero, G., Santovito, E., Verran, J., De Angelis, M., & Fusco, V. (2015). A selective medium for isolation and accurate enumeration of *Lactobacillus casei*-group members in probiotic milks and dairy products. *International Dairy Journal, 47*, 27–36.

EFSA. (2008). *Safety and efficacy of the product Sorbiflore, a preparation of* Lactobacillus rhamnosus *and* Lactobacillus farciminis*, as feed additive for piglets - scientific opinion of the panel on additives and products or substances used in animal feed*. https://www.efsa.europa.eu/en/efsajournal/pub/771. (Accessed 11 September 2018).

Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunção, R., Balance, S., et al. (2016). The harmonized INFOGEST in vitro digestion method: From knowledge to action. *Food Research International, 88*, 217–225.

FAO/WHO. (2001). *Health and nutrition properties of probiotics in food including powder milk with live lactic acid bacteria*. Rome, Italy: FAO/WHO. http://isappscience.org/wp-content/uploads/2015/12/FAO-WHO-2001-Probiotics-Report.pdf last. (Accessed 11 September 2018).

Fredua-Agyeman, M., & Gaisford, S. (2015). Comparative survival of commercial probiotic formulations: Tests in biorelevant gastric fluids and real-time measurements using microcalorimetry. *Beneficial Microbes, 6*, 141–151.

Gadhiya, D., Patel, A., & Prajapati, J. B. (2015). Current trend and future prospective of functional probiotic milk chocolates and related products – a review. *Czech Journal of Food Science, 33*, 295–301.

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., et al. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology & Hepatology, 14*, 491–502.

Gill, A. (2017). The importance of bacterial culture to food microbiology in the age of genomics. *Frontiers in Microbiology, 8*. Article 777.

Guslandi, M., Giollo, P., & Testoni, P. A. (2003). A pilot trial of *Saccharomyces boulardii* in ulcerative colitis. *European Journal of Gastroenterology and Hepatology, 15*, 697–698.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology, 11*, 506–514.

IPA. (2018). *Proposal for new work on harmonized probiotic guidelines for use in foods and dietary supplements*. Rome, Italy: FAO/WHO. http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FMeetings%252FCX-720-39%252FCRDs%252FCRD03.pdf.

Jungersen, M., Wind, A., Johansen, E., Christensen, J. E., Stuer-Lauridsen, B., & Eskesen, D. (2014). The science behind the probiotic strain *Bifidobacterium animalis* subsp. *lactis* BB-12®. *Microorganisms, 2*, 92–110.

Kołodziej, M., & Szajewska, H. (2017). *Lactobacillus reuteri* DSM 17938 in the prevention of antibiotic-associated diarrhoea in children: Protocol of a randomised controlled trial. *BMJ Open, 7*. Article 013928.

Kruis, W., Fric, P., Pokrotnieks, J., Lukás, M., Fixa, B., Kascák, M., et al. (2004). Maintaining remission of ulcerative colitis with the probiotic *Escherichia coli* Nissle 1917 is as effective as with standard mesalazine. *Gut, 53*, 1617–1623.

Laake, K. O., Bjørneklett, A., Aamodt, G., Aabakken, L., Jacobsen, M., Bakka, A., et al. (2005). Outcome of four weeks intervention with probiotics on symptoms and endoscopic appearance after surgical reconstruction with a J-configurated ileal-pouch-anal-anastomosis in ulcerative colitis. *Scandinavian Journal of Gastroenterology, 40*, 43–51.

Lahtinen, S. J., Gueimonde, M., Ouwehand, A. C., Reinikainen, J. P., & Salminen, S. J. (2006a). Comparison of four methods to enumerate probiotic bifidobacteria in a fermented food product. *Food Microbiology, 23*, 571–577.

Lahtinen, S. J., Ouwehand, A. C., Reinikainen, J. P., Korpela, J. M., Sandholm, J., & Salminen, S. J. (2006b). Intrinsic properties of so-called dormant probiotic

bacteria, determined by flow cytometric viability assays. *Applied and Environmental Microbiology, 72*, 5132–5134.
Majhenic, A. C., Lorbeg, P. M., & Treven, P. (2018). Enumeration and identification of mixed probiotic and lactic acid bacteria starter cultures. In A. Y. Tamime, & L. V. Thomas (Eds.), *Probiotic dairy products* (2nd ed., pp. 207–252). Chichester, West Sussex, UK: Wiley-Blackwell.
Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., et al. (2014). A standardised static in vitro digestion method suitable for food – an international consensus. *Food and Function, 5*, 1113–1124.
Patrone, V., Molinari, P., & Morelli, L. (2016). Microbiological and molecular characterization of commercially available probiotics containing *Bacillus clausii* from India and Pakistan. *International Journal of Food Microbiology, 237*, 92–97.
Pinto, D., Santos, M. A., & Chambel, L. (2015). Thirty years of viable but non-culturable state research: Unsolved molecular mechanisms. *Critical Reviews in Microbiology, 41*, 61–76.
Ranadheera, C. S., Vidanarachchi, J. K., Silva Rocha, R., Gomez Cruz, A., & Ajlouni, S. (2017). Probiotic delivery through fermentation: Dairy vs. non-dairy beverages. *Fermentation, 3*. Article 67.
Rodríguez-Nogales, A., Algieri, F., Vezza, T., Garrido-Mesa, N., Olivares, M., Comalada, M., et al. (2015). The viability of *Lactobacillus fermentum* CECT5716 is not essential to exert intestinal anti-inflammatory properties. *Food and Function, 4*, 1176–1184.
Roy, D. (2001). Media for the isolation and enumeration of bifidobacteria in dairy products. *International Journal of Food Microbiology, 69*, 167–182.
Sarkar, S. (2018). Whether viable and dead probiotic are equally efficacious? *Nutrition & Food Science, 48*, 285–300.
Schoeni, J. L. (2015). Probiotics. In Y. Salfinger, & M. L. Tortorello (Eds.), *Compendium of methods for the microbiological examination of foods*. Washington, DC, USA: American Public Health Association (Chapt. 20).
Temmerman, R., Scheirlinck, I., Huys, G., & Swings, J. (2003). Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology, 69*, 220–226.
Tharmaraj, N., & Shah, N. P. (2003). Selective enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus*, bifidobacteria, *Lactobacillus casei, Lactobacillus rhamnosus*, and *Propionibacteria*. *Journal of Dairy Science, 86*, 2288–2296.
Vinderola, G., Prosello, W., Ghiberto, T. D., & Reinheimer, J. A. (2000). Viability of probiotic (*Bifidobacterium, Lactobacillus acidophilus* and *Lactobacillus casei*) and non-probiotic microflora in Argentinian Fresco cheese. *Journal of Dairy Science, 83*, 1905–1911.
Vinderola, G., & Reinheimer, J. A. (2000). Enumeration of *Lactobacillus casei* in the presence of *L. acidophilus*, bifidobacteria and lactic starter bacteria in fermented dairy products. *International Dairy Journal, 10*, 271–275.
Vivekananda, M. R., Vandana, K. L., & Bhat, K. G. (2010). Effect of the probiotic *Lactobacillus reuteri* (Prodentis) in the management of periodontal disease: A preliminary randomized clinical trial. *Journal of Oral Microbiology, 2*. Article 5344.
Westerik, N., Kort, R., Sybesma, W., & Reid, G. (2018). *Lactobacillus rhamnosus* probiotic food as a tool for empowerment across the value chain in Africa. *Frontiers in Microbiology, 9*. Article 1501.
Wilkinson, M. (2018). Flow cytometry as a potential method of measuring bacterial viability in probiotic products: A review. *Trends in Food Science & Technology, 78*, 1–10.
Zawistowska-Rojek, A., Zareba, T., Mrówka, A., & Tyski, S. (2016). Assessment of the microbiological status of probiotic products. *Polish Journal of Microbiology, 65*, 97–104.
Zielińska, D., Ołdak, A., Rzepkowska, A., & Zieliński, K. (2018). Enumeration and identification of probiotic bacteria in food matrices. In A. M. Holban, & A. M. Grumezescu (Eds.), *Advances in biotechnology for food industry* (pp. 167–196). New York, NY, USA: Academic Press (Chapt. 6).