Viability assessment of probiotics as concentrated cultures and in food matrices

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Abbreviations

a_w – water activity
CFU = colony-forming units
FD = freeze-drying
FFFC = free-flowing powder of free-cell cultures
FFME = free-flowing powder of microencapsulated cultures
GIT = gastro-intestinal tract
Lb. = *Lactobacillus*

Abstract

Due to the fact that probiotic cells need to be alive when they are consumed culture-based analysis (plate count) is critical in ascertaining the quality (numbers of viable cells) of probiotic products. Since probiotic cells are typically stressed, due to various factors related to their production, processing and formulation, the standard methodology for total plate counts tends to underestimate the cell numbers of these products. Furthermore, products such as microencapsulated cultures require modifications in the release and sampling procedure in order to correctly estimate viable counts. This review examines the enumeration of probiotic bacteria in the following commercial products: powders, microencapsulated cultures, frozen concentrates, capsules, foods and beverages. The parameters which are specifically examined include: sample preparation (rehydration, thawing), dilutions (homogenization, media) and plating (media, incubation) procedures. Recommendations are provided for each of theses analytical steps to improve the accuracy of the analysis. Although the recommendations specifically target the analysis of probiotics, many will apply to the analysis of commercial lactic starter cultures used in food fermentations as well.

1. Introduction

Products containing probiotic bacteria are generally found in two forms: supplements and foods. It is estimated that the global probiotic supplement market (pills, caplets) in 2008 alone was worth approximately $1.5 billion (39). With respect to foods, probiotic bacteria were initially incorporated into yogurt products. Today, however, they are found in numerous other foods and

beverages ranging from fruit juices to breakfast cereals. It is estimated that the probiotic industry holds about a 10 % share of the global functional food market (82), which represented 10 billion Euros in 2008. Recent data demonstrate that products containing probiotic bacteria show a growth rate of 25 % in North America and Eastern Europe, while the larger and more mature markets of Asia and Western Europe are still showing increases of between 5 and 8 % per annum (39). Therefore, products which contain probiotic bacteria are of considerable and growing economic importance.

A widely accepted definition of probiotics states that they are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (6). In this respect, a product containing probiotic organisms should therefore contain a number of viable cells which has shown to be efficacious (which is generally > 10^6-10^8 CFU/g, or > 10^8-10^{10} CFU/day). Although there is no cell count level recognized to guarantee a health effect (69), some agencies are providing recommendations. Although somewhat an arbitrary number, the Canadian Food Inspection Agency (7) recommends a level of 10^9 CFU per serving to be able to present generic health claims. Studies on probiotic bacteria with higher (35) or lower (89) doses have been published. In any case, determining the population of viable cells in a probiotic-carrying product is critical in the evaluation of its quality. Concerns about the quality of probiotic products have been widely expressed (52). Many studies report that commercial products did not contain the stated cell numbers (50, 88) or that some were at unacceptably low levels (2, 8, 38, 51, 55, 57, 75). These data suggest that the viability levels of probiotic bacteria in commercial products may be a problem and point to the need to be able to correctly assess the various populations. Unfortunately, enumeration of specific bacteria often requires specialized and standardized

93 methodologies, which have not been established for many probiotic strains. The overall aim of
94 this review is to provide recommendations as to the appropriate procedures for the enumeration
95 of probiotics in foods and supplements. Not only would this be useful to industry and regulatory
96 agencies, it will also serve all scientists working with lactic and bifidobacteria cultures. Many
97 studies still currently published omit to incorporate or describe important parameters for the plate
98 count analyses.
99
100 There are instances where non-viable cells have shown biological effects (62, 90). Fermentation-
101 derived bioactive compounds which are responsible for the health benefits (for example peptides,
102 enzymes, polysaccharides) are increasingly being identified. If the definition of probiotic is to
103 remain linked to "live cultures" then the concept of "probioactives" (a bioactive compound
104 influencing health which is synthesized by a probiotic culture or which specifically results from
105 the bioconversion of a food matrix by a probiotic microorganism (31)) might need to be used.
106 Since, by definition, probiotics must be alive, and until the science on probiotic-linked health
107 effects expands into this "probioactives" field, the viability of probiotic cultures will be a
108 paramount to the "estimation" of their functionality, hence the need for this review.
109
110
111 ## 2. Scope
112
113 A certain number of parameters need to be established to limit the scope of this review.
114

115 The “gold standard” for viability counts is still colony counts on plating media. Therefore, this
116 review will focus on the standard plate count methodology. Obviously, newer technologies are
117 being explored (flow cytometry, PCR). These more modern tools have shown that viability has
118 multiple facets and they have identified “stressed” and “viable but non-culturable” states (46).
119 Such cell “states” will obviously influence viable counts by traditional plating. The approach
120 adopted for this review will be to suggest techniques aimed at obtaining the highest plate counts
121 possible and enable the recovery of damaged cells when possible. Such an approach is adequate
122 when the intention is to recognise as much as possible the biomass produced and marketed
123 (suppliers), or the “population” added into the product, which is still viable (food processors).
124
125 Numerous microorganisms are recognized as candidates for probiotic status. This review will
126 exclusively focus on *Lactobacillus (Lb.)* and *Bifidobacterium* species, since they constitute the
127 majority of probiotic cultures on the market. It must be kept in mind, however, that there are
128 many other probiotic candidates in the *Enterococcus, Pediococcus, Bacillus, Streptococcus,*
129 *Lactococcus, Propionibacterium* and *Saccharomyces* genera, which could also warrant specific
130 examinations as to enumeration procedures.
131
132 Probiotic cultures are usually analysed in three basic forms: dried, frozen and liquid/moist
133 products. The goal of this review is to examine the plating procedures for all these formats. Thus,
134 this review will examine two steps of the enumeration procedure: sample preparation, which is
135 matrix-dependent and dilution and plating, which is generally independent of the matrix.
136 Variations in plate count results can also be linked to the analyst and apparatus and this has been
137 reviewed by Corry et al. (20).

3. Sample preparation for dilution and plating

3.1 Frozen cell cultures and foods

When one compares viability assessments in a liquid culture versus its frozen counterpart, four operations can result in differences in viability: the freezing step, the storage conditions, the thawing process and the homogenization of the thawed product. Recommendations can be made for each step (Table 1).

As a rule, the colder the storage temperature, the higher viability is maintained. Storage at -80°C is significantly better than at -30°C to maintain viability (33). But even at -80°C, significant viability losses can occur over a few months of storage (33).

Less conclusive data are available on the thawing process. When analysing frozen foods for total plate counts, it has been suggested to thaw samples at refrigeration temperatures (less than 5°C) between 10 and 18 h (5, 54). It is not clear if the thawing practice recommended for microbiological analysis of foods, can apply to concentrated probiotic cultures. Indeed, with mesophilic starter cultures, rapid warming in a water bath at 20-45°C was better than slow thawing at 4°C (78), while with *Lb. Leichmanii*, heating to 30-48°C gave best survival levels (43). Therefore, different thawing temperatures seem advisable as a function of the product or the culture (Table 1). With respect to thawing rate, a few studies showed that rapid thawing ensured the highest survival levels (59, 65, 81). Manufacturers of frozen starter cultures

recommend to add them directly to processing milk, which varies between 4°C (unfermented milk or ice cream blend) and 42°C (yogurt). These data suggest that rapid thawing is advisable for concentrated cultures (Table 1). Rapid thawing should obviously not be carried out at a temperature which can kill the cells (generally above 50°C). Suppliers of lactic cultures warn the users that a culture must be immediately used once thawed, especially if it was thawed at room temperature or higher. Presumably, the high cell levels would result in very rapid acidification and this would damage the culture. It therefore seems wise to analyse the culture for CFU levels as soon as it is thawed (Table 1). It is also desirable to carry out preliminary assays aimed at ascertaining the time required for this thawing step (Table 1).

Interestingly, CFU counts are sometimes higher in freshly frozen samples (33) than in the original liquid cell suspension. Since growth below -20°C is doubtful, increased CFU following the freeze-thaw steps must be related to physical occurrences on the cell chains (58). Therefore, increases in CFU after freezing could reflect chain breakup. Homogenization methods are available to break chains and improve the precision of the analysis, and this will be further addressed. Although data would suggest that the freeze-thaw steps break cell chains, it still seems recommendable to carry out a homogenization step on thawed cultures (Table 1).

One question sometimes raised by laboratory analysts is “can a culture which was inadvertently thawed be re-frozen?” Processors often use rapid or ‘snap’ freezing with liquid nitrogen (temperature drops can be up to 230°C per minute) (34). Even if survival is as good in cabinet freezers (for ex. 2°C per minute) survival will vary as a function of strain or medium (33, 42, 64,

81). Whatever the methodology used, re-freezing will almost certainly negatively affect viable counts.

Please place Table 1 here

3.2 Dried cell cultures

Dried cells may have undergone six stressful processes during their production: growth to high cell densities, pumping (shear and oxygen stresses), centrifugation, freezing, desiccation and powder grinding (potentially a heat stress). Not surprisingly, data show that freeze-dried bacteria suffer cell damage at all these process steps (4, 9, 70). As a result, conditions of rehydration of these damaged cells will have a major influence on the CFU readings obtained.

Various forms of dried cultures are available: 1) free-flowing powders, 2) powders in capsules, 3) powders compressed to obtain tablets (pills). In these three cases the powder used can be the original product obtained after FD or it may be a microencapsulated product, which is typically obtained by spray-coating the freeze-dried powder with a lipid (12). Additionally, some capsules can have an additional enteric coating. Such treatments will undoubtedly complicate viability assessment and need to be addressed in each specific case when it comes to drawing up standard procedures for sample preparation prior to plating.

3.2.1 Free-flowing powders of free-cell cultures

206 This product consists of a powder with a particle size ranging from 50 to 250 µm (17), as
207 opposed to a compressed powder in a pill form. The cells in the powder are free, e.g. not
208 microentrapped in gels, such as alginate (10, 48). This form is mainly sold to the to the food
209 industry, as adjunct or starter cultures. It will be referred to "as free-flowing powders of free-cell
210 cultures" (FFFC).
211
212 Many parameters will influence viability assessment in the preparation of samples of FFFC:
213 storage conditions prior to analysis, hydration matrix (pH, composition, solids level), hydration
214 temperature and hydration time before carrying out dilutions. Arguably, the analysis of dried
215 probiotics is subject to more variables than that of frozen cultures, and many recommendations
216 can be made (Table 2).
217
218 Freeze-dried cultures can be stored at higher temperatures than frozen ones, and refrigeration at
219 4°C is considered adequate (15). At 22°C, mortality rates are 10 times higher than at 4°C (10).
220 Lately, thanks to improvements in processing and powder formulation, great advances in room-
221 temperature stability have been achieved (36), but it is still considered prudent to store the
222 unopened product between 0 and 4°C prior to analysis (Table 1). It is important to emphasize
223 here that storage of viable probiotics in food products at room temperature represents a major
224 goal of many food industries.
225
226 It is often the case under industrial conditions that only a fraction of a culture package is needed
227 for the incorporation of a given CFU level (15). Therefore, situations occur where a commercial
228 product is opened, a sample taken and the rest is kept for later use. In general, moisture

229 absorption by the powder will increase the water activity (a_w) in dried products. Indeed, it has
230 been shown that an increase from 0.1 to 0.3 in a_w of a milk-based product will result in only a
231 2% increase in moisture, but that the stability during storage will be 10 times lower. Therefore,
232 when a portion of a package containing a freeze-dried powder is taken, the sachet must be closed
233 as rapidly as possible and re-stored at 4°C. Since the relative humidity can be very high in some
234 environments, prudence suggests that products which are opened should be analysed within 48 h
235 even if they are kept at 4°C (Table 2).
236
237 Industry generally standardizes the product to obtain a given CFU/g level and thus a "filling"
238 powder is often added to the freeze-dried culture. Also, powders from different culture
239 production batches can be blended. Therefore a given commercial product may contain two
240 powders, and stratification can occur. In order to prevent variations in CFU due to uneven
241 powder mixing, it seems useful to sample a rather large amount (10 to 50 g).
242
243 With FFFC, the rehydration medium is critical for obtaining the highest possible CFU levels.
244 Three factors need to be considered here: solids level, composition and pH. Some data suggest
245 that rehydrating in too dilute solutions generates lower CFU readings (24, 79). Presumably, an
246 osmotic shock occurs in these situations. A frequent practice is to rehydrate the powder to the
247 solids level prior to FD. Since media used to suspend cells destined for FD (skim milk or others)
248 typically contain between 10 and 20 % solids, this practice therefore results in a rehydration at
249 relatively high solids levels. Rehydrating at 20 to 50% solids can give good results (24) but may
250 also lead to increased variability (17). Presumably, at high solids contents some particles are not

251 completely hydrated before the sample is taken for subsequent dilutions. These data explain our
252 recommendation to rehydrate the cells in solutions having between 10 and 20 % solids (Table 2).
253
254 The second aspect of the rehydration medium is composition and pH. Water is not recommended
255 if the powder is to be rehydrated in dilute solutions (79). However, water works well with
256 cultures dried in milk solids, especially if the amount of water added brings the suspension to the
257 original solids level of the freeze-drying medium (typically between 10 to 20%). In addition to
258 the presence of sugar, vitamins, minerals and nitrogenous compounds, milk provides a very
259 suitable buffered environment. This is an important consideration in light of data showing very
260 high viability losses when cells are rehydrated in an acid environment such as fruit juices (70).
261 Sugars alone can be used with some success as protective ingredient to FD (25, 79), but they lack
262 buffering capacity. Not surprisingly, De Valdez et al. (25) showed that rehydration media
263 containing peptones tended to improve CFU readings. Sugar-only formulations are often seen in
264 dried products in order to reduce the presence of potential allergens. These however can lead to
265 pH problems. With such products, it would seem advisable not to solely use water for
266 rehydration but, rather, a solution containing buffering compounds. Logically, the pH should be
267 close to that which is optimum for growth.
268
269 An aspect of rehydration conditions which warrants further study is the redox level. Many
270 plating media for probiotic bacteria contain cysteine to lower the redox level. Homogenization
271 will obviously aerate the medium which, arguably, can be detrimental to the viability of strains,
272 which are sensitive to oxygen (84). McCann et al (53) added cysteine to a rehydration medium
273 for the enumeration of 6 cultures but its protective effect during homogenization was not studied.

In summary, these considerations suggest that solids levels, composition, pH and potentially redox level must be managed during rehydration (Table 2).

Rehydration temperature and time must also be standardised. One would assume that rehydrating at 4°C would be ideal but this is not the case. Temperatures between 30 and 37°C are best for post hydration viabilities (56, 79). Care must be taken, however, not to go above 46°C, which can be detrimental to cell function (13). A minimum hydration period allows the particles to dissolve and generate a homogenous cell suspension. Therefore, it is advisable to incubate the cell suspension between 15 and 30 min at the selected hydration temperature before carrying out the subsequent dilutions and plating (24, 60). However, extended times of hydration prior to carrying out the dilutions would not be advisable as they could result in growth initiation or, at the opposite, detrimental acidification of the medium.

One must also keep in mind that there are wide differences between species and strains of probiotic bacteria with respect to behaviour during rehydration (24, 25). Muller et al., (60) concluded that the reconstitution conditions should be optimised for the strain used in order to achieve accurate viable probiotic numbers from dried probiotic cultures. All these data point to the need to standardize the FFFC rehydration procedure (Table 2).

Please place Table 2 here

3.2.2 Free-flowing powders of microencapsulated cell cultures (FFME)

297

298 A number of methods have been proposed to microencapsulate lactic cultures and probiotic
299 bacteria (12). Spray-coated products are the most widely used by industry. Although spray-
300 coating increases the size of the freeze-dried particles, they are still in the 90 to 250 µm range
301 (17) and remain free-flowing in nature. The most widely studied microencapsulation system for
302 probiotics is microentrapment in alginate beads, and at least one product is currently marketed in
303 this form (14). These products will be referred to as free-flowing powders of microencapsulated
304 cell (FFME).

305

306 In general, the hydration of FFME cultures followed by immediate plate counting will
307 significantly underestimate the CFU. This has been noted for the alginate (16) and fat-based
308 spray-coated (17) products. Therefore, the methodology with FFFC must be adapted to allow
309 adequate release of the bacteria from the encapsulant. In all cases, a high-shear homogenization
310 step is recommended. The blender and ultraturax methods seem to give comparable results, at
311 least with the spray-coated products (17). It is unknown if homogenization with the Stomacher
312 system also works for these products. As a function of the technology used to microencapsulate
313 the cells, additional measures might be required (19, 47) (Table 3). Considerable documentation
314 is found on the dissolution of alginate beads. Typically, the rehydration medium would contain 1
315 to 2 % citrate or phosphate (19). Little has been done, however, with respect to the amount of
316 emulsifier required when cultures are coated with fats. A level of 0.1 % Tween 80 has been
317 proposed (17). If it is unknown if the product is microencapsulated, the addition of citrate as well
318 as of Tween to the rehydration medium seems advisable.

319

Please place Table 3 here

3.2.3 Individual supplements

Individual dietary supplements containing one or more different probiotic strains are the most frequently encountered dried format sold directly to consumers. There are mainly three forms:

1) Powders which are incorporated into a soft gel capsule; they will be referred to as a "capsules".
2) Powders compressed in to a solid mass; they will be referred to as a "tablets" but could also be termed caplets.
3) Powders inserted into small laminated envelopes, which are in a single dose form (about 0.5 g); they will be referred to as "sachets".

The analytical procedure for the sachets and capsules is basically the same. The capsule or the envelope is opened manually, under aseptic conditions, and the powder is recovered. Since the manufacturers have probably mixed different powders to carry out the standardization of CFU to the level specified on the label, it seems wise to use the complete content of the capsule/sachet for the analysis. The rehydration procedure is then basically the same as that for the FFFC or FFME products (Table 2). If it is unknown that a microencapsulated culture was used, it is best to use the FFME procedure.

Compressed cultures are typically found as tablets, some being chewable. The compressed products need to be homogenized to resuspend the cells. Some studies suggest that the

343 Stomacher is satisfactory for that purpose (18). It has been stated that FFME cultures (typically
344 lipid spray-coating) show higher survival to the compression step (77), which generates heat.
345 Therefore, it is to be expected that tablets increasingly contain the cultures microencapsulated by
346 spray-coating as in their formulation. As a result, the addition of the emulsifier in the hydration
347 medium would be recommendable (Table 3) and all the recommendations for FFME (Table 2)
348 would apply.
349
350 3.2.4 Probiotics in dried foods
351
352 There are only a few food products with dried probiotics e.g. breakfast cereals, infant formulas
353 and dry milk formulations. However it is likely that the market for these will increase. To our
354 knowledge, no standardised methodology has been recommended for these particular products. It
355 can be hypothesized that the methodologies recommended for the FFME would be best for these
356 products. This means applying media/temperature/time hydration parameters (Table 2)
357 accompanied by high-shear homogenization. In foods, the cultures may be in a lipid matrix, for
358 example chocolate. Therefore, when in doubt, the rehydration medium should contain an
359 emulsifier as recommended for the spray-coated FFME products.
360
361
362 ***3.3 Beverages or foods***
363
364 Dairy products are currently the most common platform for delivery of probiotics. They include
365 products such as yogurt and cultured milk drinks (73), pasteurized unfermented milk, cheese, as

366 well as frozen yogurt and ice cream. Probiotics are increasingly found in fruit juices and inn
367 cereals.
368
369 A number of factors in the food matrix influence probiotic viability including acidity, hydrogen
370 peroxide production, oxygen content, concentration of sugars (osmotic stress), water activity (a_w)
371 and metabolites, as well as storage temperature (28, 41, 51, 70, 84). In yoghurt and juices, the pH
372 at the end of fermentation has been reported to be the most important factor influencing the
373 growth and viability, particularly for species of bifidobacteria (76). The damage caused by acid
374 exposure includes an intracellular accumulation of protons, as well as structural damages to the
375 cell membrane, DNA and proteins (21).
376
377 Generally, probiotics in liquid food environments are stored at low temperatures, ranging from 1
378 to 6^{o}C. It has been reported that bifidobacteria are less tolerant to the lower temperatures than
379 lactobacilli (40). From a physiological point of view, cold stress can reduce membrane fluidity,
380 affect the level of DNA supercoiling, increase the rate of DNA strand breakage, stabilize
381 secondary structures of DNA and RNA and thus alter replication, transcription and translation
382 (21). Enzyme activity, protein and ribosome functions are also affected at low temperatures (37)
383 and the bacteria have generally been shown to become more sensitive to bile salts and NaCl (32).
384 Nebra *et al*. (61) found that pre-incubation of injured *Bifidobacterium* cells for 4 hours at 37^{o}C
385 (the necessary time to recover injured cells avoiding cell multiplication) in the presence of a
386 reducing agent increased the recovery of injured cells damaged through oxidative stress.
387

All these data point to potential variability in physiological cell states. Highly stressed cells might still retain metabolic activities but may not be culturable. In general, there appears to be no defined procedure for the recovery of stressed probiotic cells from food environments. It remains to be ascertained if recommendations made for dried cultures with respect to homogenization in a recovery medium (Table 2) would improve viable counts and stabilize the variance.

4. Dilutions

4.1 Homogenization methods to break lumps or chains

Once a fermented dairy drink is added to the diluent, the preparation of samples most often involves either simply manual shaking, vortexing, or macerating in a Stomacher or a blender (63, 71, 83, 87). Data suggest that high shear homogenization would be desirable even with beverages. When the strain grows in chains, the CFU readings significantly increase when a. Waring®-type blender or a Stomacher™ homogenizer is used (26).

Treating a sample with high-shear generator probes (Ultra Turax®, Omni) is another method applied to lactic and probiotic cultures (47). Thus, when a fresh M17-grown *Streptococcus thermophilus* culture was treated with generator probes for 1 minute at 20000 rpm, the CFU counts gradually increased by a factor of 2 (Gardner and Champagne, unpublished). However, the CFU readings started to drop as the homogenization period was extended over 90 seconds.

411 Thus, the benefit of high-shear homogenization can be time-linked and might even be strain-
412 dependant. It is therefore advisable to test the procedure on the samples to find the optimum time.
413 This is done by carrying out CFU as well microscopic examination of the samples before and
414 after homogenization.
415
416 High shear homogenization reduces the variability of the CFU analysis in addition to increasing
417 the CFU counts by a factor of up to 3 (17). It can be hypothesized that the high-shear
418 homogenization increases the precision and readings of CFU by two means: break down of small
419 clumps of bacteria, as well as by breaking the cell chains.
420
421 Other methods of homogenizing the samples to reduce variability are 1) to include glass beads in
422 the dilution bottles (80), and 2) to sonicate the samples for 5 seconds (27). These procedures are
423 certainly better that the simple hand shaking of bottles or vortex mixing, but they have not been
424 compared to the traditional homogenizing apparatus.
425
426 In summary, a few recommendations can be made with respect to sample homogenization in a
427 diluent or a beverage (Table 4).
428
429 ***Please place Table 4 here***
430
431 ***4.2 Dilution media***
432

433 Dilutions leading to plating on agar media are typically carried out in solutions containing
434 peptone (43), NaCl (1), Ringer and/or in phosphate salts (1, 53). In a study with *Lb. rhamnosus*
435 R0011 and *B. longum* ATCC 15708, cultures diluted in a peptone or a phosphate-based dilution
436 medium gave the same results (17), but Abe et al (1) found peptone to be better than phosphate
437 salts for bifidobacteria.
438
439 Many probiotic bacteria are sensitive to oxygen (84). As a result, the addition of antioxidants
440 (ascorbic acid, cysteine) to the growth medium, and/or incubation in an anaerobic environment is
441 often required for the successful growth of probiotic bacteria. Unfortunately, little data are found
442 on the necessity of these strategies for the dilution of the samples prior to plating. McCann et al.
443 (53) used cysteine in the dilution medium for lactobacilli, enterococci, and bifidobacteria but did
444 not demonstrate its need for improving the CFU readings. Recent data indicated that a 30
445 minutes delay in plating of *L. rhamnosus* R0011 and *B. longum* ATCC 15708 did not cause cell
446 viability losses (17). However, in another study on the effects of suspension-dilution buffers on
447 teh viable counts of bifidobacteria, Mitsuoka's buffer (containing phosphates, Tween, cysteine
448 and agar) proved to be the best for the enumeration of most *Bifidobacterium* strains in dried
449 cultures and in foods, although the benefit over simple peptone and PBS was only marginal (1).
450 It is unknown if the slight advantage of Mitsuoka's buffer was specifically linked to cysteine or
451 if Tween and agar were also involved. It must also be kept in mind that there are large variations
452 between bifidobacteria strains (1) with respect to the benefits of these protective ingredients on
453 the culturable levels.
454

It must be kept in mind that the dilution steps are critical components of variability in CFU counts. This is linked to the experimental errors of pipeting volumes which are added to those of variable levels of diluent volume (in test tubes or bottles) following sterilization of the dilution buffers. In summary, with probiotics, the dilution medium does affect viable counts and some recommendations can be made (Table 4).

5. Plating media

5.1 Pure cultures

The literature abounds with studies on the effectiveness of media for the enumeration of probiotic bacteria (74). The frustrating conclusion of these studies is that the results obtained may be very strain specific (1, 22, 29, 72, 83).

MRS agar (MRSA) is probably the most widely used base plating medium for pure cultures of bifidobacteria and lactobacilli (17, 50, 66). For increased effectiveness, it is often supplemented with 0.5% of cysteine or carbohydrates. However, recent data by Abe et al. (1) might challenge this practice with respect to bifidobacteria. Reinforced Clostridial Medium agar (RCMA) might well be a preferable medium, and a few studies point to the value of this formulation (3, 23, 72, 86). RCMA especially seems to work better (give higher CFU numbers) than MRSA for stressed cells, which warrants its recommendation (Table 4).

In light of the wide variations between strains, finding an optimum plating medium for each strain to be studied is recommendable before long-term studies with probiotics are initiated. To estimate the recovery level, direct microscopic counts (DMC) of a fresh culture can serve as a reference for the estimation of the total population. Ideally, fluorescent dies of commercial viability indicator kits (for example LIVE/DEAD® *Bac*Light™) could be used to differentiate and enumerate live and dead cells (3, 45).

5.2 Mixed Cultures

Numerous studies have evaluated the effectiveness of selective and differential plating media for the detection and enumeration of specific probiotic species within mixed cultures 23, 74, 83, 85, 86). Media for the specific growth of bifidobacteria include such substances as cysteine, ascorbic acid, sodium sulphite, which lower the redox potential, and antibiotics such as kanamycin and mupirocin, against which bifidobacteria are generally resistant, whereas lactobacilli tend to be more susceptible (68).

The choice of culture medium and methodology for the selective enumeration can also strongly depend on the product matrix, the target microbe and the diversity of the background microbiota in the product (86). For example, LC medium (67), a basic medium containing bromocresol green and 1% ribose, pH 5.1, has been recommended for the selective enumeration of *Lb. rhamnosus* and *Lb. paracasei* from yogurt products, while MRS-AC medium (MRS with acetic acid, pH 5.2) has been recommended for cheese products (86). Generally, selective media used to enumerate *Lb. acidophilus* are incubated aerobically to prevent growth of bifidobacteria (30).

An evaluation of various selective and differential media for reliable counts of *Lb. acidophilus*, *Lb. casei* and *Bifidobacterium* species from a range of commercial products indicated that no selective or differential medium provided reliable counts, with the possible exception of LC agar (83). Furthermore, differential evaluation of colony size and were found too subjective.

In general, analogous to selecting the plating media for pure cultures, the choice of selective and/or differential media for probiotic detection and enumeration from mixed cultures should be investigated prior to commencing a long-term study where the various parameters are taken into account, from product matrix, to target group, etc (Table 4). Moreover, it is advisable to use a set of different selective media for each targeted species for more conclusive identification and enumeration (86).

The development of suitable standard plating methods is an area which still requires further research. Better selective and differential media can be developed as we gain a deeper understanding of the metabolic capabilities of strains, which is fast becoming a feasible pursuit with the upsurge in the total genome sequencing of strains. However, it is worth emphasizing that selective media generally impose greater stress on the target organism and consequently the count may be lower than on non-selective media due to the increased injury/stress imposed.

6. Incubation

6.1 Temperatures

Since probiotic strains typically originate from the gastrointestinal tract of humans incubation of their growth media is usually carried out at 37°C (Table 4).

There are instances where probiotic bacteria are not grown at 37°C. For example, incubating the Petri plates at 15°C is a method of selectively counting *Lb. casei* probiotics in yogurt (11). Presumably, this would be applicable to *Lb. rhamnosus* and *Lb. plantarum* cultures as well. Incubation temperature is also a parameter used to modulate the expression of certain biological activities such as for the production of exopolysaccharides by *Lb. rhamnosus* (13). Therefore, there are instances where probiotic cultures are not grown at 37°C, but these are rare occurrences and usually require a specific reason.

6.2 Anaerobiosis

Some cultures can successfully be grown in an aerobic environment, but this might require the presence of cysteine in the plating medium. Many researchers simply incubate the plates in an anaerobic environment. Two systems are successfully used for this purpose. The first is anaerobic jars, such as the GasPack (Becton Dickinson Microbiology Systems) or AnaeroGen (Oxoid) systems. The second approach is to inject oxygen-less gasses in specifically designed cabinet incubators. Typically, the gas mixture is composed of N_2, CO_2 and H_2.

The need for anaerobic incubation will vary between strains. If low CFU readings are obtained under aerobic incubation conditions, a test can be carried out in conjunction with microscopic

546 analyses (DMC w/o fluorescent viability dyes) to ascertain the effectiveness of the incubation
547 condition to deliver high plate counts, as was described earlier for the selection of the plating
548 medium. When in doubt, it seems recommendable to incubate under anaerobic conditions.
549

550 ## 7. Recommendations

551

552 In this review, we showed that a multitude of factors affect the determination of viable counts of
553 probiotic cultures, and sub-optimal procedures can have a major impact on the end-result.
554 Therefore recommendations were made on storing (Tables 1 and 2), thawing (Table 1) and
555 rehydrating (Table 2) frozen and dried cell cultures, resuspending encapsulated cultures (Table
556 3), as well as diluting and plating (Table 4). Unfortunately, no single universal methodology is
557 applicable to all probiotics since there is considerable variability between species and even
558 strains on their responses to plating procedures. Therefore, scientists working with probiotics
559 will find in these recommendations the parameters of importance for optimizing the plating
560 methodology for their own cultures. Hopefully, these will benefit all research groups working
561 with functional foods, frozen or dried lactic cultures, as well as commercial and governmental
562 (regulatory) laboratories.
563

564 Although this review specifically targets thee analysis on probiotics, be believe many of the
565 recommendations also apply to estimation of viable counts of commercial starter cultures used in
566 foods (cheese, yoghurt, dry sausages, sauerkraut, wine malolactic, specialty breads), and in
567 particular the following: storage practices, thawing and rehydration parameters, homogenization
568 procedures as well as the data that should be provided in the description of methodologies.

569

570 Authorities and research laboratories often carry out quality assessments of commercial probiotic
571 products on the market. In these cases, the characteristics of the cultures are often unknown and
572 there is uncertainty of the methodologies to be used. When in doubt, we recommend the analyst
573 should assume the following: the cells are stressed, damaged and sensitive to oxygen, the product
574 is encapsulated, and cells are in aggregates or form chains.

575

576 Since methodological differences will inevitably occur, it is critical that they are adequately
577 described in scientific publications. Recommendations as to what should be included in the
578 methodology section of publications, at least with respect to the preparation of the sample for
579 CFU analysis, are presented in Table 5.

580

581 ***Please place Table 5 here***

582

583 ## 8. Conclusions

584

585 The culture based analysis for CFU determinations is an old trusted and established technique
586 although it does underestimate the number of viable cells in some instances. It is also a reference
587 method for the determination of various food pathogens.

588

589 An increasing problem is the enumeration of viable probiotic cells in complex microbiota. In the
590 future, advances in quantitative PCR and flow cytometry may well enable the enumeration of
591 specific strains in these environments. Research in these fields is certainly warranted.

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935
936 Table 1: Recommendations for the storage and preparation of frozen cultures for subsequent
937 plating
938

Step	Recommendation
Storage	Prior to analysis, store frozen cultures/foods as cold as possible. Temperature should be lower than -20°C.
Thawing	1) Foods. Thaw at 4°C for no more than 18 h. 2) Concentrated cultures. They can be thawed rapidly but should not be exposed to high temperatures: keep thawing temperature at or below that for optimum growth. Ascertain the minimum time required for thawing of the sample. Analyse as soon as the liquid state is obtained.
Homogenization	When CFU in frozen samples are higher than prior to freezing, this suggests insufficient homogenization. A high-shear homogenization step aimed at breaking down lumps or cell chains should always be used in the analytical procedure. Note: ascertain appropriate homogenization time since too much can kill cells
Re-freezing	If thawed, a culture/food cannot be re-frozen prior to analysis. Sample is lost.

939
940
941
942 Table 2. Some recommendations for the storage and rehydration of dry cultures for subsequent
943 plating
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Product	Recommendation
Dried cultures of FFFC	1) Store at 4°C prior to analysis even if the label states that they are considered stable at room temperature. 2) Analyse an opened product within 48 h. 3) Make sure powder is well blended before sampling. Take 10 to 50 g if particles of different sizes are seen. 4) Rehydrate in solutions having between 10 and 20 % solids (including solids brought by the culture itself). 5) Rehydrate in a buffered medium (containing antioxidants if the strains is sensitive to oxygen), at a desirable pH (presumably close to optimum pH for growth). 6) Rehydrate between 30 and 37°C for 15 to 30 min prior to diluting.
Dried cultures of FFME	1) Prepare a specific rehydration medium by adding ingredients specifically designed to dissolve the encapsulation matrix (citrate/phosphate for alginate gels or emulsifier for fat coatings). 2) Carry out a high-shear homogenization step. 3) All 6 parameters of FFFC above also apply.
Tablets	1) Carry out homogenization step (Stomacher acceptable). 2) Add ingredients specifically designed to dissolve the encapsulation matrix (citrate/phosphate or emulsifier). 3) All 6 parameters of FFFC above also apply.
Capsules/single dose	1) Open capsule or sachet and recover as much powder as possible. 3) Apply appropriate parameters of FFFC or FFME above (when in doubt use FFME).

945

946
947 Table 3. Methods used to resuspend microencapsulated or microentrapped probiotic cultures for
948 subsequent plating
949

Technology	Encapsulation matrix	Method
Microentrapment in a gel particle. Extrusion or emulsion technologies.	Alginate or pectin w/o : - starch - chitosan	Dissolve in a medium which binds calcium (0.5% phosphate or 1 % citrate). Also use high shear homogenization.
	Carrageenan	High shear homogenization
Spray coating	Typically lipids	High shear homogenization. Medium contains an emulsifier; 0.1% Tween.

950
951
952
953
954 Table 4. Some recommendations with respect to dilution and plating steps in the CFU analysis
955 when strains are not well characterized with respect to their optimum plating conditions.
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Steps	Recommendation
Dilution of sample	1) Carry out a high-shear homogenization step on all samples. 2) Ascertain optimum agitation time if UltraTurax® type probes are used.
Dilution medium	Use peptone-based medium or Mitsuoka's medium (the latter contains an antioxidant).
Plating medium for pure cultures	Use MRS or RCMA based media depending on the species/strain. Verify effectiveness of the medium with DMC counts, preferably with viability stains.
Plating media for mixed cultures	Verify recovery level, selectivity or differentiation effectiveness with pure cultures before applying to the mixed cultures.
Incubation temperature of the plates	Incubate at 37°C unless there is a reason to do otherwise (e.g. a specific selective method).
Atmosphere during incubation	Incubate in anaerobic conditions

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Table 5. Data which should be included in the description of the methodology (in reports or in scientific publications) describing sample preparation of concentrated probiotic cultures for subsequent plating

Product	**Data that should be given**
Frozen foods or frozen concentrated cultures	1) Storage temperature of the samples as well as the time of storage prior to analysis. 2) Conditions of thawing: temperature, time, method (water bath, air incubator or other) 3) Homogenization method used (blender, Stomacher or UltraTurax) and duration of treatment
Dried cultures (FFFC, FFME, tablets, capsules)	1) Composition of the freeze-drying medium and carriers. 2) Storage temperature of the samples. 3) Composition of the rehydration medium 4) Ratio of powder to rehydration medium used. 5) Homogenization method used (blender, Stomacher or UltraTurax) and duration of treatment 6) Rehydration temperature and time prior to carrying out dilutions and plating.