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The determination of viable counts in probiotic cultures microencapsulated by spray-coating

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ABSTRACT

An assessment of various methods to determine viable counts (CFU) in freeze-dried and dried microencapsulated (ME) probiotic cultures was carried out. Microencapsulation was done by spray-coating of dried *Lactobacillus rhamnosus* R0011 or *Bifidobacterium longum* ATCC 15708 cultures with fat. Rehydration of the ME powders was incomplete when they were added to water and gently agitated. As a result analytical methods based on vortexing of rehydrated ME cultures and which did not incorporate a high-shear homogenization (HSH) step underestimated the viable counts. The CFU of ME cultures were identical when methods using either blender or generator probes high-shear homogenization (HSH) were carried out. Furthermore HSH reduced the variability of the CFU results of both free-cell and ME cultures by a factor of three. The addition of an emulsifier (Tween 80) in the rehydrating medium to dissolve fat did not improve CFU counts when generator probes were used for HSH. The presence of fat in the ME product, or when added to the rehydration medium, improved CFU counts of *B. longum* but not of *L. rhamnosus*.

1. Introduction

A recognised definition of probiotics is “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Araya et al., 2002), and national organizations are beginning to introduce it in their guidelines for probiotics in foods (Health Canada, 2009). Therefore, at this point in our knowledge, viability is an essential component of probiotics functionality. It is therefore critical to assess the viable population in foods and supplements.

The enumeration of probiotic and lactic acid bacteria hold many challenges (Corry et al., 2007). Many cultures are fastidious and require numerous growth factors (Morishita et al., 1981; Carvalho et al., 2004). As a result, growth varies considerably as a function of medium composition, which is complicated even more by the fact that companies often add multiple species in their products (Dave and Shah, 1996; Talwalkar and Kailasapathy, 2004a). Some probiotic bacteria are sensitive to oxygen and may require media ingredients or modified gas environments to enable their growth (Dave and Shah, 1997; Talwalkar and Kailasapathy, 2004b). Furthermore, stressed lactobacilli and streptococci, in particular, may develop chains of cells which require homogenization to disrupt (Deibel and Banwart, 1982). Due to these numerous requirements and strain variations, there are no “universal” standard medium or growth conditions for the assessment of viable counts in probiotic-containing products.

Dried products present additional challenges (McCann et al., 1996). Viable counts of dried products are affected by rehydration temperature (Mille et al., 2004; De Valdez et al., 1985a), time (De Valdez et al., 1985b) and media (Sinha et al., 1982). Again, there is no standard procedure for the rehydration of lactic and probiotic cultures.

Abbreviations: CF-Lr, commercial free-cell *Lactobacillus rhamnosus* culture; CFU, colony-forming units; CM-Lr, commercial microencapsulated *Lactobacillus rhamnosus* culture; LF-Bl, laboratory-grown free-cell cultures of *Bifidobacterium longum*; LF-Lr, laboratory-grown free-cell cultures of *Lactobacillus rhamnosus*; LM-Bl, laboratory-grown microencapsuated cultures of *Bifidobacterium longum*; LM-Lr, laboratory-grown microencapsuated cultures of *Lactobacillus rhamnosus*; ME, microencapsulated; PTM, peptone, tryptone, meat extract medium; SPTP, NaCl, phosphate, Tween, peptone medium.

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A newer challenge is the analysis of encapsulated probiotic cultures. Such products offer great advantages with respect to protection of the cells against processing technologies and gastrointestinal environments (Champagne and Kailasapathy, 2008), as well as during storage (Siuta-Cruce and Goulet, 2001). There are various technologies based on alginate gels (Chandramouli et al., 2004), carrageenan gels (Lamboley et al., 1997), oil emulsions (Shima et al., 2006) and spray-coating (Durand and Panes, 2003). The latter is of interest because it is probably the most widely used microencapsulation technology applied commercially to probiotics. Analysis of microencapsulated (ME) products require adapted techniques to resuspend or recover the cells prior to dilutions and plating. With alginate beads, the gel particles must dissolve in a calcium-binding solution (phosphates or citrates), while the carrageenan and fat-coated alginate particles, homogenization is required. Most techniques are carried out on fresh, moist products, and little is known of dried ME products. Rehydration and analysis of probiotic bacteria in alginate-based systems has been described (Selmer-Olsen et al., 1999; Champagne et al., 2000) but no data are available on the parameters which affect CFU counts in dried, fat-based spray-coated probiotic bacteria.

The aim of the study was to evaluate various homogenization, rehydration and plating practices on rehydration rates as well as CFU counts obtained from powders of free-cell and spray-coated ME *Lactobacillus rhamnosus* and *Bifidobacterium longum* probiotic bacteria.

2. Materials and methods

2.1. Cultures

Two commercial dried products were provided by Institut Rosell-Lallemand (Montréal, QC, Canada). The first was classical freeze-dried culture of *L. rhamnosus* R0011, which contained free cells; it will be refereed to as the commercial free-cell *L. rhamnosus* (CF-Lr) culture. The second was a ME product of the same *L. rhamnosus* R0011 strain and will be referred to as the commercial ME *L. rhamnosus* (CM-Lr) culture. The ME product was prepared by spray-coating technology in a process based on the Durand and Panes (2003) patent.

Some probiotic cultures were prepared in laboratory conditions. Stock cultures of *L. rhamnosus* R0011 (Institut Rosell-Lallemand) and *B. longum* ATCC 15708 were obtained by mixing MRS-grown (Difco, Detroit MI, USA) cell suspensions with sterile BHI medium (Difco) containing 15% glycerol (Sigma, St-Louis, MO, USA) in a 1:5 ratio, placing 1 mL in Cryovials (Nalgene, Rochester, NY, USA) and freezing at −80 °C. Fresh liquid inocula were prepared by adding 1 mL of a thawed stock culture to 100 mL of MRS medium (supplemented with 0.1% ascorbic acid for bifidobacteria) and incubating at 37 °C until a pH of 4.5 was reached.

2.2. Media

Two rehydration media were tested because the literature reports variability in recovery of viable cells as a function of strain and medium (De Valdez et al., 1985b; Sinha et al., 1982). The PTM medium consisted of 15 g/L peptone (Difco), 10 g/L tryptone (Difco), and 5 g/L Lab Lemco meat extract (Oxoid, Nepean, Ontario, Canada), in mili-Q water (Millipore, Billerica, MA, USA). The PTM medium was selected because it had proved to be one of the best for many lactic cultures (De Valdez et al., 1985b). The SPTP medium contained 8.5 g/L NaCl (Sigma), 2.5 g/L K_2HPO_4 (LabMat, Beauport, Québec Canada), 2.5 g/L KH_2PO_4 (LabMat), 1 g/L Tween 80 (Fisher, Ottawa, Ontario, Canada) and 1 g/L peptone (Difco) in Milli Q water. This medium was considered because it contained salts. Phosphates and NaCl are often ingredients of dilution media (Abe et al., 2009; Thantsha et al., 2009) and warranted consideration. Dilutions for CFU analyses were carried out in either a 1 g/L peptone solution or in the SPTP medium. All enumerations were done on MRS broth with 15 g/L agar (Difco) using the pour plate method. Laboratory fermentations for biomass production (3 L volumes) were carried out on MRS broth supplemented with 20 g/L glucose (LabMat) (MRS-glucose). For bifidobacteria the MRS medium were supplemented with 1 g/L ascorbic acid (Bioshop, Burlington, Ontario, Canada). This was carried out by sterilizing by filtration (0.22 μm pore size) a 100 g/L ascorbic acid solution and aseptically adding 1% (v/v) to the various media. The lyophilisation medium was composed of 200 g/L (w/w) skim milk powder (Agropur, Granby, Québec, Canada) and 50 g/L sucrose (LabMat).

All media were sterilized at 121 °C for 10 min with temperature probe (real retention times at the sterilization temperature) except for the lyophilisation medium which was sterilized at 100 °C for 5 min.

2.3. Production of laboratory-grown free and spray-coated bacteria

An intermediate culture was prepared by adding 5 mL of the fresh inocula to 500 mL of MRS medium and incubating at 37 °C until a pH of 4.5 was reached. The larger fermentations were made in 8 jars of 5 L, each containing 3 L of MRS-glucose (w/o ascorbic acid) medium. They were inoculated at 1% (v/v) with the intermediate culture and incubated for 24 h at 37 °C with pH control at 6.2 using KOH 6 N. The cell suspensions were then centrifuged (Beckman, Fullerton CA, USA) at 6000 g for 20 min at 4 °C and resuspended at 20% of their initial volume with lyophilisation medium at 4 °C. The cell concentrate was lyophilised (FTS, Stone Ridge, NY USA) under the following program: −40 °C for 4 h at atmospheric pressure, 16 h at 0 °C and 100 mTorr vacuum, 16 h at 20 °C and 100 mTorr vacuum, 56 h at 20 °C at 10 mTorr vacuum. The powder was grinded using a Ultra Centrifugal Mill ZM-1 unit (Retsch Inc. Newtown, PA, US) equipped with a sieve size of 1 mm, and then filtered in stainless steel mesh (W.S. Tyler Canada Ltd, St. Catharines, Ontario, Canada) to only retain the particles having between 53 and 250 μm. The powders were subsequently placed in hermetic glass bottles and kept at 4 °C. The powders of laboratory-grown free-cell will be referred to as LF-Lr when they are of *L. rhamnosus* R0011 and LF-Bl when of *B. longum* ATCC 15708.

The spray-coating was carried out as described by Durand and Panes (2003) in a STREA-1 fluid bed system (GEA, Columbia, Maryland, USA) equipped with a bottom-coating Würster vessel assembly. The air used for fluidization was previously dried (relative humidy of approximately 5%) and injected at room temperature. The spraying air was injected at a velocity of 30 L/min. The fat used for coating was the DP108 blend of fractionated palm kernel oil and palm oil from Aarhus United (Port Newark, NJ, USA). The fat was heated at 80 °C and distributed at 10 mL/min over 400 g of the freeze-dried free-cell cultures (LF-Lr or LF-Bl). In all, 160 g of fat was sprayed over the 400 gram of powder. The resulting laboratory-ME (spray-coated) products of the *L. rhamnosus* R0011 and *B. longum* ATCC 15768 cultures will be referred to as LM-Lr and LM-Bl.

2.4. Hydration rate of free and microencapsulated powders

Free cell or ME (spray coated) bacterial powders (5 g) were added to 45 mL of water at 25 °C in a 125 mL beaker. Agitation was carried out with a magnetic bar and was strong enough to create a small vortex. Samples were taken periodically, and the degree of dissolution was estimated by measuring the Brix (soluble solids as equivalent sucrose) using a Fisherbrand UriSystems refractometer (Fisher).