Review

Relevant factors for the preparation of freeze-dried lactic acid bacteria

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Abstract

The industrial exploitation of lactic acid bacteria (LAB) as starter and/or probiotic cultures depends strongly on the preservation technologies employed, which are required to guarantee long-term delivery of stable cultures in terms of viability and activity. Freeze-dried preparations exhibit advantages relative to preparations made with other techniques in terms of long-term preservation, coupled with convenience in handling, storage, marketing and application. Degrees of survival of LAB cultures as high as possible, during drying and subsequent storage, are thus of nuclear importance, both technologically and economically.

This review covers several factors, deemed relevant for preservation of freeze-dried LAB. The state of the art of the knowledge focussed on the improvement of LAB survival during freeze-drying and subsequent storage is presented, including specific discussion of the effects of (i) intrinsic factors, (ii) growth factors, (iii) sub-lethal treatments, (iv) drying media and (v) storage and rehydration.

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Introduction

The commercial significance of the dairy fermentation industry, which encompasses production of e.g. cheese, yoghurt and sour cream, is well recognised worldwide, and ranks second only to the production of alcoholic beverages. It has long been established that a few lactic acid bacteria (LAB), in particular those belonging to the genera *Lactococcus*, *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Pediococcus*, play a central role in the aforementioned processes of production (Daly, Fitzgerald, O'Connor, & Davis, 1998). Furthermore, an increasing interest exists for dairy products containing specific bacterial species with potential health improving properties (Banina et al., 1998). On industrial settings, LAB may be preserved and distributed in liquid, spray-dried, frozen or lyophilised forms. All these preparations are suitable for inoculation of bulk starter tanks. However, expanding interest in ready-to-use culture concentrates for direct inoculation of milk vats has placed greater emphasis on starter production and preservation methods, that promote high cell viability and activity (Broadbent & Lin, 1999).

The industrial use of LAB as starter cultures for the food industry depends on the concentration and preservation technologies employed, which are required to guarantee long-term delivery of stable cultures in terms of viability and functional activity (Carvalho et al., 2003b). Freezing and freeze-drying have commonly been used for this purpose, but these techniques bring about undesirable side effects, such as denaturation of sensitive proteins and decreased viability of many cell types (Leslie, Israeli, Lighthart, Crowe, & Crowe, 1995). The steps of manufacture, storage and eventual use of LAB impose environmental stresses on the bacterial cells, such as freezing and drying, long-term exposure to low a_w, and low pH during fermentations, or low temperature and high salt concentration during cheese ripening, respectively (Bunthof, van den Braak, Breeuwer, Rombouts, & Abee, 1999). Research on the behaviour of *Lactobacillus bulgaricus* during drying and storage has identified factors, such as storage temperature and water activity of the dried powders, to be critical parameters that affect survival (Teixeira, Castro, Malcata, & Kirby, 1995b). The viability of dried cultures depends also on the method employed to rehydrate them, as survival is increased after slow rehydration (Teixeira, Castro, & Kirby, 1995a). Finally, heat shock increases survival of exponential phase cells during spray-drying. However, exponential phase cells are not as resistant to spray-drying, even after heat shock, as cells harvested in the stationary phase (Teixeira et al., 1995a). Loss of viability of dried cultures is a consequence of cell damage at several target sites, namely the cell wall, the cell membrane and the DNA (Teixeira et al., 1995a, b), as well as a result of membrane lipid oxidation (Teixeira, Castro, & Kirby, 1996; Castro, Teixeira, & Kirby, 1997).

Maximization of survival of LAB cultures during drying and subsequent storage for long periods is of vital importance, both technologically and economically (Selmer-Olsen, Birkeland, & Sørhaug, 1999). Microbial cell survival throughout drying and storage is dependent on many factors, including initial concentration of microorganisms (Costa, Usall, Teixidó, Garcia, & Viñas, 2000), growth conditions (Palmfeldt & Hahn-Hägerdal, 2000), growth medium (Carvalho et al., 2003a, d, e), drying medium (Font de Valdez, de Giori, de Ruiz Holgado, & Oliver, 1983; Leslie et al., 1995; Hubalék, 1996; Linders, Wolkers, Hoekstra, & van't Riet, 1997c; Linders, de Jong, Meerdink, & van't Riet, 1997a; Abadias, Benabarre, Teixidó, Usall, & Viñas, 2001a; Carvalho et al., 2002) and rehydration conditions (Teixeira et al., 1995a; Abadias, Teixidó, Usall, Benabarre, & Viñas, 2001b). This paper reviews the research done on the improvement of LAB survival during freeze-drying and subsequent storage, including specific sections on (i) intrinsic factors, (ii) growth factors, (iii) sub-lethal treatments, (iv) drying medium and (v) storage and rehydration.

Intrinsic factors

Distinct species of one given genus may often exhibit rather different behaviours during freezing, drying and subsequent storage (Hubalék, 1996; Mauriello, Aponte, Andolfi, Moschetti, & Villani, 1999; Fonseca, Béal, & Corrieu, 2000; Gardiner et al., 2000; Carvalho et al., 2002). Previous work (Bazoğlu, Özilgen, & Bakir, 1987; Fonseca et al., 2000) reported on effects of bacterial cell size upon survival during freezing and freeze-drying: enterococci (i.e. small spherical cells) are apparently more resistant to freezing and freeze-drying than lactobacilli (rods). According to Fonseca et al. (2000),

the higher the surface area of the cell, the higher the membrane damage owing to extracellular ice crystal formation during freezing. A similar relation also appears to exist for survival during storage of LAB in the freeze-dried state (Carvalho et al., 2003c).

Under comparable circumstances or in standard conditions, distinct strains of the same species can differ in their behaviour during drying (Lian, Hsiao, & Chou, 2002) and storage in the dried state (Carvalho et al., 2003a, c). Although such an inter-strain variability of performance has been hardly explained, a few hypotheses have been put forward, namely: (i) differences in genetic constitution may lead to differences in phenotype between various *Lactococcus lactis* strains (O'Callaghan & Condon, 2000); and (ii) differences in cell wall and membrane composition, with different melting points of its phospholipids, may cause differences between strains (Selmer-Olsen et al., 1999). Although elucidation of cause and effect mechanisms still requires further investigation, it is important to realise that, for each LAB strain of interest, the influence of various environmental conditions on survival in the dried state is specific for that species, and likely for that strain.

Growth factors

Although major emphasis has been placed on the effect of the drying medium, the growth medium is also a critical parameter, which is likely to play a role upon survival subsequent to freeze-drying. Several factors have been identified which may explain the protection afforded by each of various growth media, e.g. accumulation of compatible solutes (which involves type of sugar substrate present and occurrence of osmotic stress, among other factors), production of exopolysaccharides, and altered fatty acid profile of the membrane. Each of these factors will be detailed in the following subsections.

Accumulation of compatible solutes

It is widely accepted (Bâati, Fabre-Ga, Auriol, & Blanc, 2000) that, when used in industrial food processing, LAB are exposed to a number of stress conditions, such as low temperature, low pH and low water activity, which cause membrane and cell wall damage, inhibition of active transport, retention of nutrients, morphological changes and loss of viability. Bacteria have meanwhile developed adaptive strategies to face the challenges of changing environments, and to survive under conditions of stress (Abee & Wouters, 1999). For instance, the response of bacteria to hyperosmolarity encompasses two aspects: their ability to develop multitolerance towards other environmental stresses, and their ability to accumulate osmoprotective compounds (Pichereau, Hartke, & Auffray, 2000).

Under osmotic stress conditions, organic solutes (which are often osmoprotectant molecules themselves) can accumulate intracellularly to high amounts and become major osmolytes (or osmotically active solutes), hence allowing the organism to re-establish its osmotic balance; in addition to osmoregulation, these compatible solutes help the cell to cope with osmotic stress (Bayles & Wilkinson, 2000). Since either freezing or drying processes subject cells to low water stress conditions, mechanisms of adaptation involving accumulation of osmotic stress compounds may enhance survival during those processes.

Compatible solutes are small organic molecules, which share a number of common properties: they are very soluble and can be accumulated to high levels in the cytoplasm of osmotically stressed cells. They are either neutral or zwitterionic molecules, and specific transport systems available in the cytoplasmic membrane allow the controlled accumulation of these compounds. They do not alter enzyme activity, and may even protect enzymes from denaturation brought about by salts, as well as protect them against freezing and drying. Compounds such as betaine and carnitine have proven protective during drying of LAB (Kets & de Bont, 1994; Kets, Teunissen, & de Bont, 1996b). Since the microorganisms will unlikely be able to accumulate compatible solutes during the short drying process, these solutes should be accumulated prior to drying.

It was suggested (Carvalho et al., 2003d) that addition of NaCl to the growth medium (Fig. 1), as well as different concentrations of undefined components that are sources of compatible solutes (e.g. peptones, tryptone, and meat and yeast extracts) (Carvalho et al., 2003a), may lead to increased production/accumulation of compatible solutes, and therefore might promote survival of LAB throughout storage in the dried state. Linders, Meerdink, and van't Riet (1997b) reported that the presence of NaCl during growth of *Lactobacillus plantarum* resulted in a lower residual activity, after drying by convection or in fluidised bed. Evidence has been presented (Carvalho et al., 2003d) that rising the medium osmolarity through addition of an electrolyte (NaCl) or of a non-electrolyte (sucrose) has distinct consequences upon *Lb. bulgaricus* survival during storage in the dried state. Higher survival rates during storage in dried form were indeed observed only when these bacteria were previously grown in MRS supplemented with NaCl. Other studies (Glaasker, Tjan, Steeg, Konings, & Poolman, 1998; Gouesbet, Jan, & Boyaval, 2001) have shown that sucrose- and NaCl-stressed *Lb. plantarum* and *Lb. bulgaricus* do not accumulate the same compounds in response to osmotic stress.

The exact mechanism by which compatible solutes protect cells during drying and subsequent storage is still

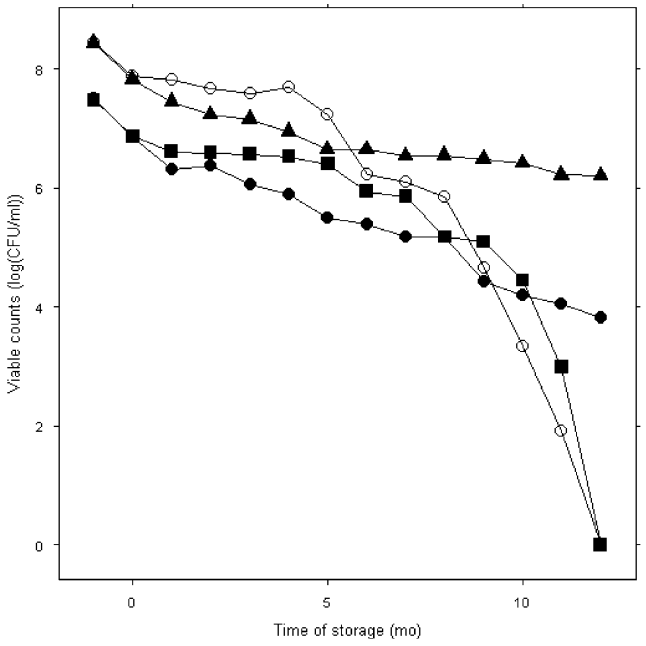

Fig. 1. Effect of various growth media composition: MRS containing $20\,g\,L^{-1}$ glucose (O), MRS containing $10\,g\,L^{-1}$ glucose $+10\,g\,L^{-1}$ sucrose (■), MRS containing $10\,g\,L^{-1}$ glucose $+10\,g\,L^{-1}$ sucrose $+5\,g\,L^{-1}$ NaCl (●), and MRS containing $5\,g\,L^{-1}$ NaCl (▲) on survival during storage of freeze-dried *Lb. bulgaricus* (from Carvalho et al., 2003d).

unknown. However, it is reasonable to suggest that the protection mechanism is similar to those underlying cryoprotection, i.e. (i) prevent aggregation and maintain solubility of cellular proteins and (ii) alter the physical properties of the cell membrane (Ko, Smith, & Smith, 1994).

Sugar substrates present in the growth medium

Chervaux, Ehrlich, and Maguin (2000) reported that different sugars are taken up by *Lb. bulgaricus* via distinct transport systems. The evidence made available strongly suggests that growth in the presence of various sugar substrates produces cells with distinct morphological and physiological traits, thus reflecting distinct resistances to the various stress treatments tested. Certain modifications of prevailing conditions or unusual conditions during growth of LAB may alter the fermentation profile to a significant degree, hence leading to the formation of other metabolites (Hugenholtz & Kleerebezem, 1999; Torino, Taranto, Sesma, & Font de Valdez, 2001). Studies by Hofvendahl and Hahn-Hägerdal (2000) have shown that, in addition to lactic acid, other compounds are formed by homofermenters during growth on sugars other than glucose. A few homofermentative LAB were found to produce small amounts of mannitol, which in most cases remains inside the cell (Wisselink, Weusthuis, Eggink, Hugenholtz, & Grobben, 2002). It was also claimed (Kets, Galinski, de Wit, de Bont, & Heipieper, 1996a) that accumulation of mannitol, sorbitol and glutamate in bacteria depends on the carbon sources in the growth medium. It is, therefore, possible that mannitol (or other compounds) could be produced during fermentation of specific sugars, and might be responsible for the distinct survival behaviours during storage of freeze-dried LAB. The protective effect of sorbitol and glutamate during storage in the freeze-dried state was described later (Carvalho et al., 2002, 2003c). The favourable effect of mannitol, sorbitol and glutamate as compatible solutes on microorganisms subjected to low water activity—as detailed in the next subsection—may lead to enhanced viability of dried starter cultures.

Studies by Carvalho et al. (2003a) have shown that survival of *Enterococcus* spp. during storage in the dried state is dependent on the commercial medium (MRS, M17 and Lee's) (Table 1) selected to grow the cells. A possible explanation for the differences observed is based on the different carbon sources present in the three media tested. Posterior studies by the same group (Carvalho et al., 2003e) have demonstrated that survival of *Lb. bulgaricus* during and after freeze-drying depends on the sugars included in both the growth and the drying medium. The lowest decrease in viability of *Lb. bulgaricus* cells after freeze-drying was obtained when that organism was grown in MRS containing mannose; the same tendency was observed during storage. The log reduction observed in MRS containing mannose indicated that this sugar would be effective for growth of *Lb. bulgaricus* cells if the objective were preservation of viability throughout storage. There were significant differences in cell survival during dried storage when growth was in either standard MRS containing glucose, or alternatively in MRS containing fructose or lactose. These observations indicate that a sugar other than glucose in the standard MRS should be used in order to extend the viability of the dried cells during storage at room temperature.

Table 1
Effect of various growth media (MRS, M17 and Lee's) on survival during storage at room temperature for 7 mo, of several strains of freeze-dried *Ent. faecalis* and *Ent. durans* (from Carvalho et al., 2003a)

		Growth medium		
Species	Strains	MRS	M17	Lee's
Ent. durans	ED 1	++	+	+
	ED 2	++	+	+
Ent. faecalis	EF 1	++	++	++
	EF 2	–	++	+
	EF 3	–	–	++
	EF 4	+	+	++
	EF 5	–	++	++
	EF 6	–	+	++

Key: ++ less than 2 log cycle reduction; + more than 2 but less than 3 log cycle reduction; – more than 3 log cycle reduction.

Exopolysaccharide production

Exopolysaccharide (EPS)-producing LAB are commonly used as starter cultures, namely in yoghurt manufacture, because EPS improves the viscosity and texture of said matrix, and consequently reduces its susceptibility to syneresis (i.e. spontaneous loss of whey) (Low et al., 1998). The production of EPS by LAB has been the topic of considerable research (Grobben et al., 1998; Kimmel, Roberts, & Ziegler, 1998), much of which has dealt with the effects of medium composition and growth conditions on the extent of EPS produced. In general, the term EPS refers to two types of secreted polysaccharides; the first type is attached to the cell wall as a capsule (capsular polysaccharides, or CPS), whereas the other is produced as loose, unattached material (slime EPS, or EPS proper) (Degeest, Vaningelgem, & de Vuyst, 2001b). The total amount of EPS formed is influenced by the medium composition (e.g. the quantitative and qualitative carbohydrate of the growth medium) (Mozzi, Savoy de Giori, Olivier, & Font de Valdez, 1995a; Looijesteijn, Boels, Kleerebezem, & Hugenholtz, 1999a; Degeest, Janssens, & de Vuyst, 2001a; Hassan, Frank, & Shalabi, 2001), the temperature of growth (Mozzi, Savoy de Giori, Olivier, & Font de Valdez, 1995b; Mozzi, Savoy de Giori, Olivier, & Font de Valdez, 1996), the rate of microbial growth (Grobben et al., 1998; de Vuyst, de Vin, Vaningelgem, & Degeest, 2001) and the stress conditions applied (Looijesteijn & Hugenholtz, 1999b). It seems that EPS has some sort of biological function, since it is rather unlikely that microorganisms use both substrate and energy for the synthesis of useless metabolites (Looijesteijn, Trapet, de Vries, Abee, & Hugenholtz, 2001). It has indeed been suggested (Torino et al., 2001) that EPS formation is part of a survival strategy in harmful environments. Most functions proposed for EPS are of a protective nature, e.g. protection against desiccation, phagocytosis, phage attack, antibiotics, toxic compounds and osmotic stress (Ruas-Madiedo, Hugenholtz, & Zoon, 2002).

According to Billi and Potts (2002), EPS may exhibit properties similar to those described for glass-forming polymers; however, no correlation was found between the amount of EPS produced and the subsequent resistance during freezing and freeze-drying (Looijesteijn et al., 2001), as well as during storage of freeze-dried cells (Carvalho et al., 2003e).

Altered membrane profile

The cytoplasmic membrane, which provides the boundary between the cytoplasm and the external environment, regulates the flow of nutrients and metabolic products into and out of the cell, thereby permitting homeostasis of the cytoplasmatic environment (Annous, Kozempel, & Kurantz, 1999). Modifications in the cell environment may even alter the composition of the membrane. It has been shown (Bâati et al., 2000) that cell membrane composition of microorganisms can be modified in a number of ways, and that some of those modifications may play a role in protection against freezing. Conditions prevailing during growth, e.g. composition of the growth medium (Annous et al., 1999), growth phase of the cells (Rees, Dodd, Gibson, Booth, & Stewart, 1995), incubation temperature (Hazeleger et al., 1995; Broadbent & Lin, 1999; Murga, Cabrera, Font de Valdez, Disalvo, & Seides, 2000), presence of salt and other solutes (Russell et al., 1995) and pH (Russell et al., 1995), do strongly affects the composition of membrane lipids. Variations in the prevailing environmental conditions induce variation of the membrane lipid structure, which in turn affect its fluidity; the major way through which bacteria maintain the ideal membrane fluidity is by changing its fatty acid composition (Annous et al., 1999). Changes in the structural and dynamic characteristics of the membrane affect the functions of proteins associated thereto via alteration of the lipid environment of enzymes, and may even be involved in the cellular response to stress (Beney & Gervais, 2001).

It has previously been described that cultivation in the presence of Tween 80 brings about changes of the fatty acid composition of LAB cells, and that these changes influence their subsequent resistance to freezing (Smitle, Gilliland, Speck, & Walter, 1974; Goldeberg & Eschar, 1977) and tolerance to bile salts (Kimoto, Ohmomo, & Okamoto, 2002). For *Enterococcus* strains exposed to several components of the growth media, e.g. presence or absence of Tween 80 and ascorbic acid, it was suggested that different fatty acid profiles and different degrees of oxidation of membrane lipids could occur. The fatty acid composition and the degree of oxidation is apparently related to survival of cells during freezing (Goldberg & Eschar, 1977; Murga et al., 2000), drying and subsequent storage in the dried state (Teixeira, Castro, & Kirby, 1996; Castro, Teixeira, & Kirby 1996). The addition of ascorbic acid to the drying medium had already been demonstrated (Teixeira et al., 1995b) to have a protective effect on spray-dried cell concentrates of *Lb. bulgaricus* during storage.

Sub-lethal treatments

Bacteria respond to changes in their immediate surroundings via metabolic reprogramming, which leads to a cellular state of enhanced resistance (Desmond, Stanton, Fitzgerald, Collins, & Ross, 2001). Resistance encoded by defense systems can be tentatively divided into two classes. The first comprises a specific system induced by a sublethal dose of a chemical or physical

stress, which permits survival against a challenge dose of the same agent (Teixeira et al., 1994; Desmond et al., 2001; Gouesbet et al., 2001). The second comprises more general systems, which prepare the cells to survive against very different environmental stresses, without the need for cultures to have been previously exposed to that stress (Hartke et al., 1996; Flahaut, Laplace, Frère, & Aufray, 1998; Pichereau et al., 2000; Desmond et al., 2001; Gouesbet et al., 2001).

Recall that microorganisms have developed signal transduction systems which, in response to environmental stresses, control the coordinated expression of genes involved in cellular defense mechanisms. A common regulatory mechanism involves the modification of sigma (σ) factors, whose primary role is to bind to core RNA polymerase hence conferring promoter specificity (Abee & Wouters, 1999).

Considerable efforts have been targeted at understanding how cells cope with environmental stresses, and many studies (Lorca & Font de Váldez, 1999; Bâati et al., 2000) have in fact demonstrated that microorganisms can adapt to environmental stresses by inducing the synthesis of proteins and other products. Stress proteins prevent alteration of membrane-bound enzymes, and may influence the physical characteristics of the membranes themselves. Recent studies (Beney & Gervais, 2001) have shown that stress proteins may, in turn, be involved in a feedback process, with a stabilizing effect on membrane proteins and lipids.

Synthesis of stress proteins is often growth phase-dependent. It was claimed (Teixeira et al., 1994; Kim et al., 2002) that only exponential phase cells are capable of adaptive response, but other studies (Guzzo et al., 1997) reported synthesis of stress proteins during the stationary phase.

Carvalho et al. (2003d) also observed that starvation in the stationary phase of *Lb. bulgaricus* improved resistance during storage in the dried state; however, the effect of such a stress treatment was growth medium-dependent because, in the presence of NaCl, starvation was rather inadequate in protecting dried cells during subsequent storage. As suggested by Broadbent and Lin (1999) for the effect of heat and cold shocks on *L. lactis*, the positive effect of starvation upon resistance of LAB during storage in the freeze-dried state can be attributed to (i) stress-induced membrane changes (that may contribute to enhance freeze and freeze-drying resistance) and (ii) stress protein synthesis (that could act as macromolecular stabilizers, strengthen the hydrogen-bonded structure of water, and hence increase the level of non-freezable water surrounding those macromolecules).

Variations in growth kinetics of LAB were observed for various commercial media (Carvalho et al., 2003a), or when the composition of the growth medium had been changed (Carvalho et al., 2003d, e). It was suggested that said variations might lead to cells with distinct survival patterns during storage in the dried state. The relationship between final pH of growth of *Lb. bulgaricus* and survival through spray-drying and storage of the dried cells was evaluated by Silva et al. (2003). These authors have shown that, in non-controlled pH fermentation runs (bearing a final pH of 4.5), the cells were more resistant to heat stress, spray drying and storage in the dried state than those from cultures under controlled pH (set to 6.5). These findings seem to indicate that acid shock, or adaptation thereto may yield cells which exhibit alternative physiological states, and hence potentially different tolerances to other stresses (Abee & Wouters, 1999). Previous studies (Giard et al., 1996) have indeed shown that starvation of *Enterococcus faecalis* caused by glucose exhaustion results in development of a multiresistant state that protects the starved cells from other stresses; however, Rince, Flahaut and Auffray (2000) reported that adaptation of *Ent. faecalis* to acidic pH resulted in only a small effect upon other challenges.

Further research is still needed to understand the mechanisms of stress, which may eventually lead to the development of cultures with improved capacity to survive and function under industrial production conditions.

Drying medium

Freeze-drying has for long been considered as a suitable dehydration process for bacteria, with the ultimate goal of achieving a solid and stable final formulation. The choice of an appropriate drying medium is thus very important in the case of LAB, so as to increase their survival rates during dehydration itself and subsequent storage. For most LAB cultures of commercial interest for the dairy industry, skim milk powder is selected as drying medium because it (i) prevents cellular injury by stabilizing the cell membrane constituents (Castro et al., 1996; Selmer-Olsen et al., 1999), (ii) creates a porous structure in the freeze-dried product that makes rehydration easier and (iii) contains proteins that provide a protective coating for the cells (Abadias et al., 2001a). Supplementing skim milk with protective agents may enhance its intrinsic protective effect during storage, to different degrees depending on the compound added. The ability of a compound to preserve the viability of cells during periods of desiccation has been implicated either with the presence of an amino group, a secondary alcohol group or both (Font de Valdez et al., 1983). The compounds are differently permeable to the cells, which in turn affects the mechanism of their protective effect. Three categories may thus be identified: (i) penetration of both the cell wall and the cytoplasmatic membrane (e.g. DMSO and glycerol); (ii) penetration of the cell wall, but not the

membrane (e.g. oligosaccharides, amino acids and low molecular weight polymers); and (iii) no penetration of the cell wall, or no direct interaction with the cell wall or membrane (e.g. polymers with high molecular weight, such as proteins and polysaccharides). According to Hubalék (1996), distinct properties of the active compounds added result in different protection features; e.g. permeable compounds make the cell membrane more plastic and bind water colligatively—which suppresses excess dehydration, reduces salt toxicity and prevents formation of ice crystals within the cell during freezing. Semi-permeable compounds do in turn induce plasmolysis of cells prior to freezing, and concentrate between the cytoplasmatic membrane and the cell wall as a buffer layer against ice growing, thus providing mechanical protection for the membrane. On the other hand, non-permeable compounds adsorb on the surface of microorganisms where they form a viscous layer, hence, inhibiting the rate of ice growth via increasing the solution viscosity, and keeping the amorphous structure of ice in the close proximity of the cell.

Sugars and sugar derivatives present in the drying medium

Various sugars (e.g. glucose, fructose, lactose, mannose and sucrose), sugar alcohols (e.g. sorbitol and inositol) and non-reducing sugars (e.g. trehalose) have been tested for their protective effect during drying and subsequent storage (Leslie et al., 1995; Linders et al., 1997a, c; Carvalho et al., 2002, 2003c, e). These compounds were in most cases found to be effective toward protection of various LAB. Among those assayed for, the sugars that are metabolised were not significantly more effective than those that cannot be metabolised, thus indicating that the effect in stake is of a physicochemical nature. In agreement with results published by Font de Valdez et al. (1983), no significant correlation was unfolded between the protective efficiency of a sugar or a sugar alcohol on a bacterium and its fermentability thereby.

Results by Carvalho et al. (2003e) have shown that the effect of the various sugars in the drying medium has a marked dependence on the type of sugar previously included in the growth medium (Table 2). The characteristics of the transporters involved in sugar uptake, which may result in an efficient or inefficient import of a sugar (Chervaux et al., 2000), could accordingly lead to differences in their performance as protectants during storage in the dried state. It was also observed (Garro, Font de Valdez, Olivier, & de Giori, 1996) that the level of enzymes was affected by the carbon source made available in the growth medium. Both factors could (at least partially) account for and elucidate the growth medium-dependence exhibited in the case of certain sugars (Carvalho et al., 2003e).

Carvalho et al. (2003c) demonstrated that sorbitol has a strong protective effect upon survival of *Lb. bulgaricus*, *Lb. plantarum*, *Lactobacillus rhamnosus*, *Ent. faecalis* and *Enterococcus durans* during storage (Table 3), even though no significant differences were observed in terms of viability of cells during freeze-drying in the presence or absence of sorbitol. Similar results were previously obtained by Fonseca et al. (2000), who have claimed no effect of glycerol during freezing of *Streptococcus thermophilus*, despite a positive effect during frozen storage. An increase in the residual activity and viability during freeze-drying after addition of sorbitol to the drying medium had been previously reported (Linders et al., 1997c; Abadias et al., 2001a, b) for various organisms, including *Lb. plantarum*. Damage to biological systems derived from freeze-drying has been attributed to two primary causes: (i) changes in the physical state of the membrane lipids and (ii) changes in the structure of sensitive proteins in the cell (Leslie et al., 1995). The mechanisms underlying sorbitol protection of dried cells would thus be: (i) prevention of damage to the membrane via interaction therewith (Linders et al., 1997a), and prevention of lipid oxidation owing to its anti-oxidant properties (Linders et al., 1997c); (ii) stabilisation of the protein structure, and hence preservation of functionality associated with formation of sorbitol–protein complexes (Wisselink et al., 2002); and

Table 2
Influence of distinct sugars added to the growth medium on the effect of drying medium compounds, during prolonged storage of freeze-dried *Lb. bulgaricus* (from Carvalho et al., 2003e)

Growth medium	Drying medium				
MRS containing	Skim milk (SM)(11%)	SM + Glu	SM + Fru	SM + Lac	SM + Man
Glucose (Glu)	•	−−	−−	•	•
Fructose (Fru)	•	−	−	•	•
Lactose (Lac)	•	−	+	−−	•
Mannose (Man)	•	++	+	•	−

Key: ++ less than 2 log cycle reduction; + more than 2 but less than 3 log cycle reduction; − more than 3 but less than 4 log cycle reduction; −− more than 4 log cycle reduction; • no survival by end of the storage period tested.

Table 3
Protective agents added to the drying medium that improve viability (or activity) of bacteria during drying (and subsequent storage)

Species	Drying process	Phase of the process analysed	Drying medium	Protective compound(s)	Reference
Candida sake	Freeze-drying	After freeze-drying	H_2O	Galactose[a] Raffinose[a] Glutamate[a]	(Abadias et al., 2001a)
			Skim-milk	Lactose[a] Adonitol[b]	
LAB	Freeze-drying	During freeze-drying and storage	Skim-milk	Sorbitol[c] Monosodium glutamate[c]	(Carvalho et al., 2003c)
LAB	Freeze-drying	After freeze-drying	H_2O	Skim-milk[d] Trehalose[b]	(Castro et al., 1997)
Pantoea agglomerans	Freeze-drying	After freeze-drying	H_2O	Trehalose[b] Sucrose[a]	(Costa et al., 2000)
LAB	Freeze-drying	After freeze-drying	Skim-milk	Adonitol[e]	(Font de Valdez et al., 1983)
LAB	Air-drying	After air-drying	H_2O	Sorbitol[f]	(Linders et al., 1997a)
E. coli *B. thuringiensis*	Freeze-drying	After freeze-drying	H_2O	Trehalose[g] Sucrose[g]	(Leslie et al., 1995)

Note: Concentration of the protective compound — [a]10% (w/v); [b]5% (w/v); [c]1% (w/v); [d]11% (w/v); [e]2 M; [f]0.3 g g^{-1} fresh cell pellet; [g]100 mM.

(iii) maintenance of turgor, owing to accumulation of said additive (Yoo & Lee, 1993).

Crowe, Reid, and Crowe (1996) suggested that trehalose is more effective than other sugars in protecting dry biomaterials, whereas Leslie et al. (1995) claimed that trehalose can protect liposomes, isolated biological membranes and some intact cells from the adverse effects of freezing and drying (Table 3). Such an increase in survival has been attributed to: protection of protein functionality, owing to formation of a glassy matrix during freeze-drying, that possesses high viscosity and low mobility (Franks, Hatley, & Mathias, 1991; Bell & Hageman, 1996); and/or to solute binding to the dried protein, thus serving as a water substitute when the hydration shell of the proteins is disrupted (Carpenter, Arakawa, & Crowe, 1991). In addition to protection of both the structure and the function of proteins during drying, trehalose and other carbohydrates are able to lower the transition temperature of dry membranes, via replacement of water between the lipid headgroups. This phenomenon prevents phase transition, and its accompanying leakage upon rehydration (Leslie et al., 1995; Castro et al., 1997). Leslie et al. (1995) claimed that trehalose protects both *Escherichia coli* and *Bacillus thuringiensis* during freeze-drying and storage, whereas Linders et al. (1997c) reported that trehalose exhibits no positive effect on *Lb. plantarum* activity following fluidised bed drying. Despite all these features, studies by Carvalho et al. (2002) demonstrated that trehalose is not significantly more effective in protecting freeze-dried *Lb. plantarum* than the other carbohydrates investigated. In the case of *Lb. rhamnosus*, it even behaves significantly worse, especially by the end of the storage period considered. Crystallization of trehalose during sublimation could decrease the availability of the sugar to form hydrogen bonds with the protein (Carpenter et al., 1991), and thus enable the sugar to protect bacterial cells during drying and subsequent storage. Additionally, the cost of this compound would likely restrict its large scale industrial use. Sucrose, which bears properties similar to those entertained by trehalose, was found efficient when added to the drying medium during storage of dried cells of *Lb. bulgaricus*, but this protection was once again growth medium-dependent (Carvalho et al., 2003d).

Other compounds

The ability of monosodium glutamate (MSG) to protect viability and activity of distinct microorganisms during cryopreservation and freeze-drying was described by a number of researchers (Porubcan & Sellars, 1975; Font de Valdez et al., 1983; Font de Valdez, de Giori, de Ruiz Holgado, & Oliver, 1985; Hubalék, 1996; Martos, Ruiz-Holgado, Olivier, & de Valdez, 1999). Abadias et al. (2001a) claimed that MSG, when used alone, was very effective in preserving *Candida sake* cells during lyophilization; however, combination of MSG with skim milk destroyed that favourable effect. Carvalho et al. (2003c) found, for the majority of LAB tested, that increased survival during storage (Table 3) is obtained when cells were freeze-dried in the presence of MSG.

The stabilisation of their protein structure via reactions between the amino group of the protectant and the carboxyl groups of the microorganism proteins, coupled with the ability to retain greater amounts of residual moisture have been pointed out (Font de Valdez et al., 1983) as a possible rationale for protection by MSG during freeze-drying.

Antioxidants can be defined as substances which interfere with the normal oxidation processes in oils and fats, thus delaying them (Ahmad, 1996). The autoxidation of membrane phospholipids may be delayed by natural antioxidants, but the effect achieved is closely related to their concentration. Previous works (Teixeira et al., 1995a; Castro et al., 1996; Teixeira et al., 1996) have demonstrated that cell membrane damage occurs during spray-drying and freeze-drying processes, and some antioxidants were reported (Teixeira et al., 1995b; Hubalék, 1996) to protect membrane lipids against damage. Such phenolic antioxidants as propyl-gallate are commonly added to foods so as to inhibit lipid oxidation; however, their degree of effectiveness is often difficult to predict because there are distinct pathways by which phenolic compounds influence the lipid oxidation rate (Mei, McClements, & Decker, 1999). Depending on the nature of the additive employed, a low concentration may either have an anti- or a pro-oxidant effect (Bruun-Jensen, Skovgaard, & Skibsted, 1996): e.g. addition of propyl-gallate has been found (Carvalho et al., 2002) quite effective in protecting dried cells of *Lb. plantarum*, but its effect vanishes by the end of the storage period in the case of *Lb. rhamnosus*. Phenolics are believed to interrupt the free-radical chain of oxidative reactions, by contributing hydrogen from the phenolic hydroxyl groups themselves, thus forming stable free radicals which do not initiate nor propagate further oxidation of lipids (Dziezak, 1986); they may also chelate transition metal ions, hence reducing metal-induced oxidative reactions (Mei et al., 1999).

Storage and rehydration

Previous studies (Teixeira et al., 1995a; Gardiner et al., 2000; Abadias et al., 2001b) have shown that temperature is a critical parameter for microbial survival during storage. The stability of dried samples decreases during storage, and higher survival rates are recorded at lower storage temperatures. In addition to the factors already described, subsequent storage conditions, including temperature, atmosphere, exposure to light and relative humidity appear to be very important for the recovery of freeze-dried cells (Abadias et al., 2001b). It has been demonstrated (Castro et al., 1996; Teixeira et al., 1996) that changes in the lipid profile occur during storage of spray-dried and freeze-dried concentrates of *Lb. bulgaricus*, hence suggesting membrane lipid oxidation. In order to prevent/reduce such an oxidative phenomenon, thus providing increased survival during storage, dried powders should be stored under vacuum (Castro et al., 1995) or under controlled water activity (Teixeira et al., 1995b).

An organism which survives the various steps of freezing, drying and storage may, nevertheless, lose its viability during rehydration (Sinha, Shukla, Lal, & Ranganathan, 1982). Therefore, rehydration is a critical step in the recovery of freeze-dried microorganisms, because cells that were subjected to sublethal injury may not be able to repair said damage if they are rehydrated under inappropriate conditions (Costa et al., 2000). The rehydration solution itself (in terms of osmolarity, pH and nutritional energy source), as well as the rehydration conditions (in terms of rehydration temperature and volume) may significantly affect the rate of recovery to the viable state, and thus influence survival rates (Teixeira et al., 1995a; Poirier, Maréchal, Richard, & Gervais, 1999; Selmer-Olsen et al., 1999). It was suggested (Costa et al., 2000) that an environment characterised by a high osmotic pressure could control the rate of hydration, and thus avoid osmotic shock; Abadias et al. (2001b) has indeed demonstrated a significant increase in viability of *C. sake* cells when the same solution tested as protectant was used to rehydrate dried samples.

Practical recommendations for preparation of freeze-dried LAB

Optimum protocols for the preparation of freeze-dried LAB vary widely between species, and even between strains. However, in view of the results reviewed above, a basic (necessarily general) methodology for the preparation, storage and rehydration of freeze-dried LAB is suggested.

Growth conditions

The first step should be the selection of a few commercial growth media available for that particular LAB (Carvalho et al., 2003a); the performance of each medium on survival during freeze-drying and subsequent storage should then be investigated. In particular, the effect of adding NaCl to each such medium should also be assessed (Carvalho et al., 2003d). The results of these preliminary steps should permit one to choose the best medium (with or without NaCl) for the preservation of a given LAB during prolonged storage.

Drying conditions

In the absence of other relevant information on the specific LAB cultures, skim milk powder should be

selected as drying medium. The sugar (or sugars) present in the growth medium selected should be the first compound(s) to be tested as protective agent(s) (Carvalho et al., 2003e). Other compounds, e.g. sorbitol, MSG, adonitol and trehalose, which were previously claimed as effective in protecting bacteria during drying and subsequent storage (Table 3), also deserve to be investigated.

Storage conditions

Previous studies (Castro et al., 1995; Teixeira et al., 1995a; Gardiner et al., 2000; Abadias et al., 2001b) have shown that storage conditions are critical for recovery of freeze-dried cells. Hence, the dried powders should be stored under vacuum, maintained in controlled water activity and exposed to darkness.

Rehydration conditions

The positive effect of any compounds added to the drying medium seems to relate directly to the growth medium composition; e.g. Abadias et al. (2001b) demonstrated a significant increase in the viability of *C. sake* when the same solution tested as protectant in the drying medium was used to rehydrate dried samples. (Despite the eukaryotic nature of this specific microorganism, Abadias et al. (2001a) had reported large similarities between its behaviour and those of several LAB during drying and subsequent storage.) Therefore, the use of the same compound in the growth, the drying and the rehydration medium should be investigated.

Conclusions

Full comparison of the experimental data from distinct studies is difficult for a number of reasons. Most reports have indeed focused on survival during the process of drying and not during the process of storage afterwards. Also, water was used as drying medium instead of a complex medium like skim milk, and activity rather than viability of cells was considered. Other sources of discrepancy between the results available in the literature arise from the different microorganisms or model systems employed (e.g. membranes, liposomes or enzymes), as well as the alternative drying methods or the distinct concentrations of protective agents employed.

The mechanisms underlying damage and protection by freezing, drying and storage are indeed complex, and not fully understood to date. However, evidence has been made available which proves that suitable selection of the composition of the growth and drying media is essential to afford protection during storage of freeze-dried cells. It should also be emphasized that the influence of each protective agent on the survival of each LAB strain in the dried state should be determined on a case-to-case basis. In addition, information on the sites of impact and the nature of the injury produced by a variety of stressful conditions (e.g. freezing, drying, storage or rehydration), together with knowledge of the induction of stress proteins (particularly those which provide resistance during drying and subsequent storage) are definitively important towards production of dried starter cultures, which will be characterized by high survival rates even after extended storage.

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