View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Repositório Institucional da Universidade Católica Portuguesa

# Survival of freeze-dried *Lactobacillus plantarum* and *Lactobacillus rhamnosus* during storage in the presence of protectants

*Key words:* additives, preservation, processing, starter cultures, viability

A. Sofia Carvalho[1], Joana Silva[1], Peter Ho[1,2], Paula Teixeira[1,*], F. Xavier Malcata[1] & Paul Gibbs[1]
[1]*Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal*
[2]*Departamento de Ciências de Engenharia e Tecnologia, Escola Superior de Tecnologia e Gestão, Instituto Politécnico de Viana do Castelo, Apartado 574. P-4901-908 Viana do Castelo, Portugal*
[*]*Author for correspondence (Fax: +351 225090351; E-mail: paula@esb.ucp.pt)*

## Abstract

No significant differences were observed in the viability of *Lactobacillus plantarum* and *Lactobacillus rhamnosus* cells during freeze-drying in the presence or absence of inositol, sorbitol, fructose, trehalose, monosodium glutamate and propyl gallate. However, survival was higher during storage when drying took place in the presence of these compounds. Sorbitol produced more significant effects than the other compounds toward maintaining viability of freeze-dried *L. plantarum* and *L. rhamnosus*.

## Introduction

Lactic acid bacteria (LAB) have been widely studied owing to their widespread application in many fermentation processes in the form of starter cultures. Those type of cultures can be obtained by growing selected strains of LAB under controlled conditions, and preserving them by freezing and/or drying (Kets & de Bont 1994); although freeze-drying is often used for long term storage of biological samples, it produces undesirable side effects that hamper viability of many species. Damage to biological systems resulting from freeze-drying can be attributed primarily to changes in the physical state of membrane lipids or to changes in the structure of sensitive proteins (Leslie *et al.* 1995); the activity of proteins tends in fact to be substantially lowered during freeze-drying and subsequent storage, unless the product is kept below −20 °C (Franks *et al.* 1991). In attempts to reduce such adverse changes on functional properties, several compounds have been examined as protective agents during drying (Font de Váldez *et al.* 1983, Leslie *et al.* 1995, Hubalék 1996, Linders *et al.* 1997a, b, Abadias *et al.* 2001). From the industrial standpoint, a good starter culture should maintain high levels of viability during the freeze-drying process and for long periods of storage afterwards.

The objective of the research work reported here was to assess the influence of selected compounds upon survival of *Lactobacillus plantarum* and *Lactobacillus rhamnosus* during freeze-drying and subsequent storage.

## Material and methods

### *Organism and culture preparation*

*Lactobacillus plantarum* (LR-ESB) and *Lactobacillus rhamnosus* (LR-ESB) were obtained from the culture collection held at Escola Superior de Biotecnologia (Portugal). MRS broth was inoculated from the MRSa slopes, and incubated at 37 °C for 24 h. This culture was then inoculated, at the level of 1% (v/v) in a second MRS broth, and again incubated at 37 °C for 24 h. Cells were harvested by centrifugation at 7000 × *g*

for 10 min, and washed twice with sterile Ringer's solution. Cells were then suspended in sterile reconstituted skimmed milk containing 11% (w/v) solids, and reconstituted skimmed milk containing each of the compounds to be tested: myo-inositol (12.5 g $l^{-1}$), sorbitol (12.5 g $l^{-1}$), fructose (12.5 g $l^{-1}$), trehalose (3.5 g $l^{-1}$), monosodium glutamate (MSG) (12.5 g $l^{-1}$) and propyl gallate (7 g $l^{-1}$). Cellular suspensions were maintained for 1 h at room temperature (20 °C) prior to freezing at −80 °C to allow for equilibration between cells and added compounds. The experiments were repeated twice.

*Freeze-drying and storage*

Samples were desiccated under vacuum (50 mtorr) in a freeze-drier (Martin Christ, Deutschland). Dried cells were stored in closed containers at 20 °C in air, under darkness.

*Enumeration of survivors*

At regular intervals during storage, freeze-dried samples taken at random were rehydrated to the original volume with deionised water, and suitable dilutions were plated on MRS agar by the drop count technique. Plates were examined after incubation at 37 °C for 48 h.

*Statistical analysis*

Analysis of variance (ANOVA) of viable counts after freeze-drying and at regular intervals during storage was carried out using the statistical program R (Ihaka & Gentleman 1996), taking 5% as level of significance. Multiple comparison of treatment means (2 replicates × 2 experiments), using 95% confidence intervals, was estimated via Tukey's honestly significant difference (HSD); the results were plotted using the Trellis display (Becker *et al.* 1996).

## Results and discussion

Statistical analysis of the viable counts was performed considering three factors: experimental replication, medium additive and storage time. Experimental replication, and its two-way interactions with the other two factors were not significant; however the medium additive and the storage time, and their two-way mutual interaction were statistically significant ($p < 0.05$). The experimental results pertaining to survival of freeze-dried *L. plantarum* and *L. rhamnosus* throughout storage at room temperature, in the presence of each of the four sugars (myo-inositol, sorbitol, fructose and trehalose), the amino acid sodium salt (MSG) and the antioxidant (propyl-gallate) are shown in Figure 1. This plot indicates that the addition of every of those compounds to the drying medium increases significantly survival of *L. plantarum* and *L. rhamnosus* during storage, but not during the freeze-drying process.

Various sugars, sugar alcohols (e.g. sorbitol and inositol), non-reducing sugars (e.g. trehalose) and monosaccharides (e.g. fructose) have been tested for their protective effect during drying (Leslie *et al.* 1995, Linders *et al.* 1997a, b). All compounds tested in our work were found to be effective in protecting both *L. plantarum* and *L. rhamnosus*. Among those tested, fructose and trehalose, which are metabolised by those lactobacilli, were not significantly more effective than inositol, which cannot be metabolised. This result suggests that the effect is of a physicochemical nature. In agreement with the results published by Font de Valdéz *et al.* (1983), no correlation was found between the protective efficiency of a sugar or sugar alcohol on a bacterium and its fermentability.

Sorbitol was the most effective protectant for both LAB during storage. An increase in residual activity and viability during freeze-drying following addition of sorbitol to the drying medium had been previously reported for various organisms, including *L. plantarum* (Linders *et al.* 1997a, Abadias *et al.* 2001). Mechanisms proposed in attempts to rationalize protection by sorbitol during storage include preventing membrane damage by interaction with the membrane (Linders *et al.* 1997b), and stabilising protein functionality and structure (Yoo & Lee 1993).

No significant differences were observed in our study pertaining to viability of cells during drying in the presence or absence of inositol or fructose; however survival during storage was higher in their presence. Font de Valdéz *et al.* (1983) also found that inositol has no protective effect upon viability of LAB during freeze-drying, whereas Linders *et al.* (1997) showed that addition of fructose to the drying medium does not increase the residual activity after fluidised bed-drying of *L. plantarum*.

In the present work trehalose was not significantly more effective in protecting freeze-dried *L. plantarum* than the other carbohydrates investigated; in the case of *L. rhamnosus*, it behaves even significantly worse, especially by the end of the storage period. Addi-

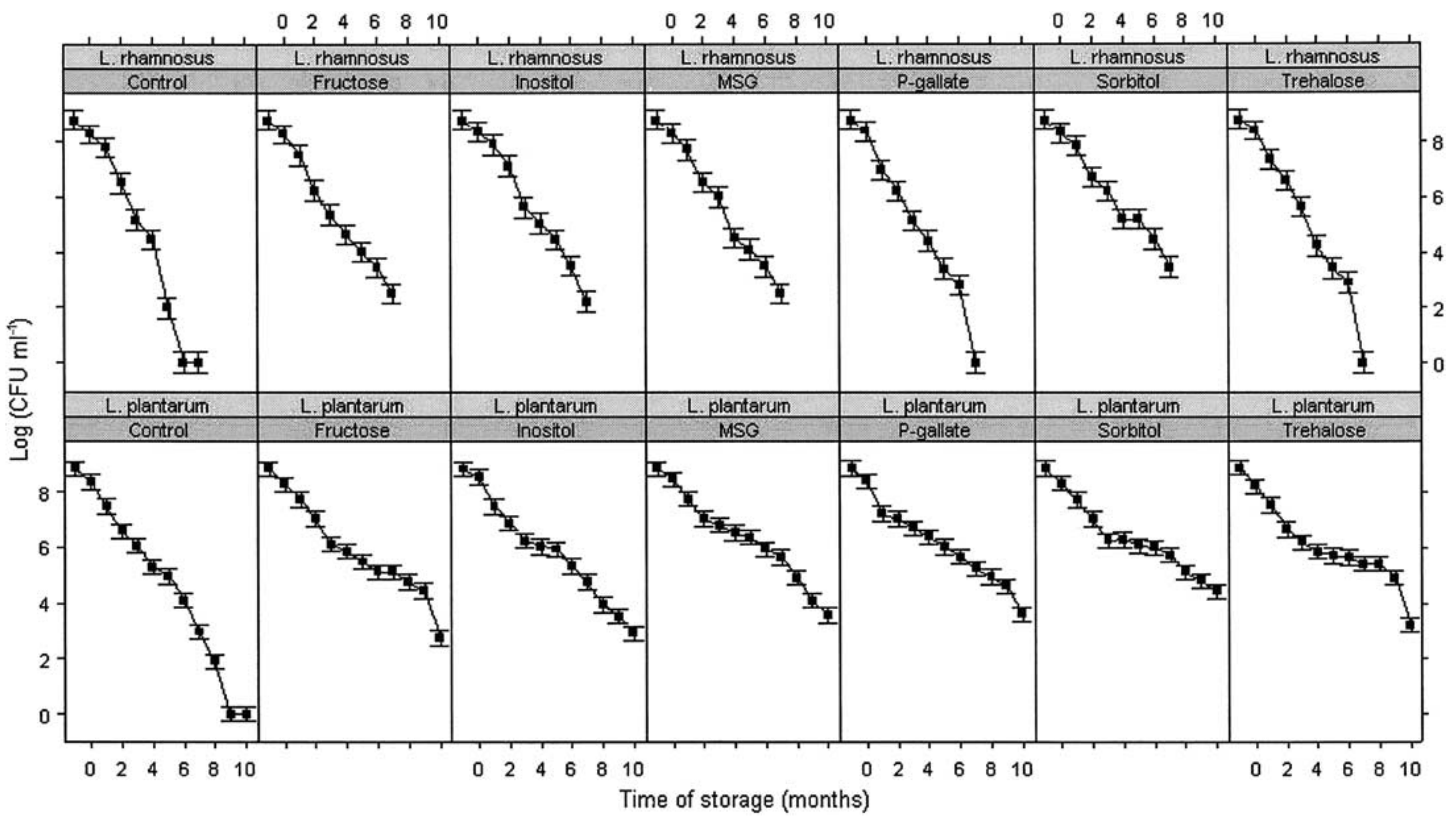


*Fig. 1.* Survival of freeze-dried *Lactobacillus plantarum* and *Lactobacillus rhamnosus* during storage in the presence of selected compounds added to the drying medium (fructose, inositol, MSG, *p*-gallate, sorbitol and trehalose). Bars represent the 95% confidence intervals of treatment means, based on Tukey's honestly significant difference (HSD).

tionally, the cost of this compound would restrict its large industrial use. Linders *et al.* (1997a) reported that trehalose had no positive effect on *L. plantarum* activity following fluidised bed drying. Leslie *et al.* (1995) however, claimed that trehalose protects both *Escherichia coli* and *Bacillus thuringiensis* during freeze-drying and storage.

For both LAB, our study conveys experimental evidence that cells freeze-dried in the presence of MSG undergo significantly higher survival during storage. The cryoprotection associated with glutamic acid, sodium glutamate and MSG towards different microorganisms had already been reported (Porbucan & Sellars 1975, Hubálek, 1996). Abadias *et al.* (2001) found that MSG, when used alone, produced one of the best results in preservation of *Candida sake* cells during lyophilization; however, MSG combined with skimmed milk could not increase cell viability. The stabilisation of the protein structure by the reactions between amino groups of the protectant compound and the carboxyl groups of the microorganism proteins, and the ability to retain greater amounts of residual moisture were put forward as tentative explanations of the protection by MSG during freeze-drying (Font de Valdéz *et al.* 1985).

Addition of propyl-gallate has been found quite effective in protecting dried cells of *L. plantarum*, but its effect is varnished by the end of the storage period in the case of *L. rhamnosus*. Phenolic antioxidants such as propyl gallate (PG) are commonly added to foods so as to inhibit lipid oxidation. Previous work (Castro *et al.* 1996, Teixeira *et al.* 1996) suggested that lipid oxidation and survival of dried cells during storage may be related. Some antioxidants were reported to protect membrane lipids against damage (Teixeira *et al.* 1995, Hubalék 1996).

Full comparison of the data obtained in the present work with those from previous 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 (Font de Valdéz *et al.* 1983), water was used as drying medium (Castro *et al.* 1997) instead of a complex medium like reconstituted skimmed milk, and activity of cells rather than viability was considered (Linders *et al.* 1997a). Other sources of discrepancy between our results and some data available in the literature arise from the different microorganisms or model systems employed (e.g. membranes, liposomes or enzymes), as well as the different drying methods or the distinct concentrations of protective agents used.

The killing mechanisms associated with freeze-drying and storage, and with protection of microorganisms from injury, are complex. Our results cannot fully explain the exact mode of action of each agent; however, there is no doubt that suitable selection of the protecting agent used as additive to the drying medium is essential affording strong protection during storage of dried LAB. Several compounds added to the microbial suspensions before drying were found to affect the stability of the cells during long term storage, so their use is recommended for production of freeze-dried cultures that will eventually be used as starters.

## Acknowledgements

This work received partial financial support via project PRAXIS/P/BIO/12147/1998 (FCT, Portugal), co-ordinated by authors P. Teixeira and P. Gibbs. Financial support for authors A. S. Carvalho and J. Silva was provided by PhD fellowships (PRAXIS XXI/BD/18152/98 and PRAXIS XXI/BD/197131/99, respectively).

## References

Abadias M, Benabarre A, Teixidó N, Usall J, Viñas I (2001) Effect of freeze-drying and protectants on viability of the biocontrol yeast *Candida sake*. *Int. J. Food Microbiol.* **65**: 173–182.

Becker RA, Cleveland WS, Shyu M-J (1996) The visual design and control Trellis display. *J. Comp. Graphic Stat.* **5**: 123–155.

Castro HP, Teixeira PM, Kirby R (1996) Changes in cell membrane of *Lactobacillus bulgaricus* during storage following freeze-drying. *Biotechnol. Lett.* **18**: 99–104.

Castro HP, Teixeira PM, Kirby R (1997) Evidence of membrane damage in *Lactobacillus bulgaricus* following freeze-drying. *J. Appl. Microbiol.* **82**: 87–94.

Font de Valdéz G, de Giori G, de Ruiz Holgado AP, Oliver G (1983) Protective effect of adonitol on lactic acid bacteria subjected to freeze-drying. *Appl. Environ. Microbiol.* **45**: 302–304.

Font de Valdéz G, de Giori G, de Ruiz Holgado AP, Oliver G (1985) Effect of drying medium on residual moisture content and viability of freeze-dried lactic acid bacteria. *Appl. Environ. Microbiol.* **49**: 413–415.

Franks F, Hatley RHM, Mathias SF (1991) Materials science and the production of shelf-stable biologicals. *Biopharm* **4**: 38–42.

Hubalék Z (1996) Cryopreservation of microorganisms. Academia, Prague, Check Republic.

Ihaka R, Gentleman R (1996) R: A language for data analysis and graphics. *J. Comp. Graphic. Stat.* **5**: 299–314.

Kets EPW, de Bont AM (1994) Protective effect of betaine on survival of *Lactobacillus plantarum* subjected to drying. *FEMS Microbiol. Lett.* **116**: 251–256.

Leslie SB, Israeli E, Lighthart B, Crowe JH, Crowe LM (1995) Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Appl. Environ. Microbiol.* **61**: 3592–3597.

Linders LJM, Wolkers WF, Hoekstra FA, van't Riet K (1997a) Effect of added carbohydrates on membrane phase behaviour and survival of dried *Lactobacillus plantarum*. *Cryobiology* **35**: 31–40.

Linders LJM, de Jong GIW, Meerdink G, van 't Riet K (1997b) Carbohydrates and the dehydration inactivation of *Lactobacillus plantarum*: the role of moisture distribution and water activity. *J. Food Eng.* **31**: 237–250.

Porubcan RS, Sellars RL (1975) Stabilized dry cultures of lactic acid-producing bacteria. US Patent 3 897 307, July 9.

Teixeira P, Castro H, Kirby R (1996) Evidence of membrane lipid oxidation of spray-dried *Lactobacillus bulgaricus* during storage. *Lett. Appl. Microbiol.* **22**: 34–38.

Teixeira P, Castro H, Malcata FX, Kirby R (1995) Survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* following spray-drying. *J. Dairy Sci.* **78**: 1025–1031.

Yoo B, Lee CM (1993) Thermoprotective effect of sorbitol on proteins during dehydration. *J. Agric. Food Chem.* **41**: 190–192.