Acknowledgements

This work was financed by the Spanish Plan Nacional de I + D (project AGL2001-2296). Luis Noriega was the recipient of a predoctoral fellowship from the Fundación para el Fomento en Asturias de la Investigación Científica Aplicada y la Tecnologia.

5. References

(1) ALONSO, A., SÁNCHEZ, P., MARTÍNEZ, J.L.: Antimicrob. Agents Chemother. **44** 1778-1782 (2000)
(2) BRETON, Y.L., MAZE, A., HARTKE, A., LEMARINIER, S., AUFFRAY, Y., RINCE, A.: Curr. Microbiol. **45** 434-439 (2002)
(3) CHARTERIS, W.P., KELLY, P.M., MORELLI, L., COLLINS, J.K.: Lett. Appl. Microbiol. **26** 333-337 (1998)
(4) DAVIS, I.J., RICHARDS, H., MULLANY, P.: Oral Microbiol. Immunol. **20** 191-194 (2005)
(5) DHAKEPHALKAR, P.K., CHOPADE, B.A.: Biometals **7** 67-74 (1994)
(6) GUEIMONDE, M., DELGADO, S., MAYO, B., RUAS-MADIEDO, P., MARGOLLES, A., DE LOS REYES-GAVILÁN, C.G.: Food Res. Int. **37** 839-850 (2004)
(7) GUEIMONDE, M., NORIEGA, L., MARGOLLES, A., DE LOS REYES-GAVILÁN, C.G., SALMINEN, S.: Int. J. Food Microbiol. **101** 341-346 (2005)
(8) KHEADR, E., BERNOUSSI, N., LACROIX, C., FLISS, I.: Int. Dairy J. **14** 1041-1053 (2004)
(9) KUMAR, M., UPRETI, R.K.: Ecotoxicol. Environ. Saf. **47** 246-252 (2000)
(10) LIM, K.S., HUH, C.S., BAEK, Y.J.: J. Dairy Sci. **76** 2168-2174 (1992)
(11) MARGOLLES, A., MAYO, B., DE LOS REYES-GAVILÁN, C.G.: Food Microbiol. **18** 67-73 (2001)
(12) MARGOLLES, A., GARCÍA, L., SÁNCHEZ, B., GUEIMONDE, M., DE LOS REYES-GAVILÁN, C.G.: Int. J. Food Microbiol. **82** 191-198 (2003)
(13) MIRANDA, C.D., CASTILLO, G.: Sci. Total Environ. **224** 167-176 (1998)
(14) NORIEGA, L., GUEIMONDE, M., SÁNCHEZ, B., MARGOLLES, A., DE LOS REYES-GAVILÁN, C.G.: Int. J. Food Microbiol. **94** 79-86 (2004)
(15) NORIEGA, L., DE LOS REYES-GAVILÁN, C.G., MARGOLLES, A.: J. Food Prot. **68** 1916-1919 (2005)
(16) PACHECO, S.V., MIRANDA, R., CERVANTES, C.: A. v. Leeuwenh. Int. Gen. Mol. Microbiol. **67** 333-337 (1995)
(17) RUAS-MADIEDO, P., HERNÁNDEZ-BARRANCO, A., MARGOLLES, A., DE LOS REYES-GAVILÁN, C.G.: Appl. Environ. Microbiol. **71** 6564-6570 (2005)
(18) SABRY, S.A., GHOZLAN, H.A., ABOUZEID, D.M.: J. Appl. Microbiol. **82** 245-252 (1997)
(19) SÁNCHEZ, B., NORIEGA, L., RUAS-MADIEDO, P., DE LOS REYES-GAVILÁN, C.G., MARGOLLES, A.: FEMS Microbiol. Lett. **235** 35-41 (2004)
(20) SILVER, S., PHUNG, L.T.: Ann. Rev. Microbiol. **50** 753-789 (1996)
(21) TANNOCK, G.W.: In Probiotics: a critical review. Horizon Scientific Press, Wymondham, UK (1999)
(22) TRAJANOVSKA, S., BRITZ, M.L., BHAVE, M.: Biodegradation **8** 113-124 (1997)
(23) UGUR, A., CEYLAN, O. Arch. Med. Res. **34** 130-136 (2003)
(24) YAZID, A.M., ALI, A.M., SHUHAIMI, M., KALAIVAANI, V., ROKIAH, M.Y., REEZAL, A.: Lett. Appl. Microbiol. **31** 57-62 (2000)
(25) YUKSEL, Z., EERDEM, Y.K.: J. Food Eng. **67** 301-308 (2005)

Relationship between solubility of freeze-dried skim milk and death of freeze-dried *Lactobacillus delbrueckii* ssp. *bulgaricus* during storage

A. Sofia CARVALHO[1], Joana SILVA[1], P. HO[2], Paula TEIXEIRA[1*], F.X. MALCATA[1] and P. GIBBS[1]

[1]Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal. *E-mail: pcteixeira@esb.ucp.pt
[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

To elucidate the relationship between death of freeze-dried cells of *Lactobacillus bulgaricus* during storage and decrease in powder solubility, the effect of milk precipitation during rehydration of freeze-dried *Lb. delbrueckii* ssp. *bulgaricus* upon death of these cells throughout storage, via addition of various carbohydrates to the drying medium was studied. The degree of survival of freeze-dried *Lb. bulgaricus* was found to be directly proportional to the square root of the absorbance of the supernatant. A darker powder resulted when most of the microorganism had died. A tentative explanation for the insolubility observed when the viability of the cells decreased is based on an increase in the extent of non-enzymatic browning (Maillard reactions), as a result of cell death. Results obtained throughout this study might represent a helpful tool towards development of a novel, straightforward, shortcut method for monitoring the viability of freeze-dried samples during storage.

Verhältnis zwischen der Löslichkeit getriergetrockneter Magermilch und dem Absterben von gefriergetrocknetem *Lactobacillus delbrueckii* ssp. *bulgaricus* während der Lagerung

Um das Verhältnis zwischen dem Absterben gefriertrockneter *Lactobacillus bulgaricus*-Zellen während der Lagerung und der Abnahme der Löslichkeit des Pulvers zu klären, wurde die Wirkung der Präzipitation der Milch während der Rehydrierung des gefriertrockneten *Lb. delbrueckii* ssp. *bulgaricus* auf das Absterben dieser Zellen während der

Lagerung untersucht, wenn verschiedene Kohlenhydrate dem Trocknungsmedium zugesetzt wurden. Der Überlebensgrad von gefriertrocknetem *Lb. bulgaricus* war direkt proportional der Quadratwurzel der Absorbanz des Überstandes. Ein dunkleres Pulver entstand, wenn die meisten Mikroorganismen abgestorben waren. Eine vorläufige Erklärung für die beobachtete Unlöslichkeit bei Abnahme der Lebensfähigkeit der Zellen könnte auf einer Zunahme des Umfangs einer nicht-enzymatischen Bräunung (Maillardreaktionen) als Ergebnis des Absterbens der Mikrooragnismen beruhen. Die Ergebnisse dieser Studie könnten für die Entwicklung eines direkten, abgekürzten Verfahrens zur Kontrolle der Lebensfähigkeit gefriertrockneter Proben während der Lagerung bedeutsam sein.

26 *Lactobacillus bulgaricus* (freeze-drying and death)

26 *Lactobacillus bulgaricus* (Gefriertrocknung und Absterben)

1. Introduction

Skim milk is regularly used as drying medium for lactic acid bacteria starter cultures because it is believed that it creates a porous structure in the freeze-dried product – thus making rehydration easier, and this provides a protective coating for cells (1, 3). The shelf life of milk powder depends on preheating treatment of milk, water activity (a_w) and storage temperature (9). The insolubilization undergone by skim milk powder during storage is generally attributed to Maillard reactions, which involve reducing sugars and proteins or free amino acids (5); they consist of a complex network of chemical reactions, the extent of which is influenced by such factors as temperature, a_w, moisture content and chemical composition of the matrix (7).

From an industrial viewpoint, a good starter culture should be able to retain high levels of viability during the freeze-drying process and for long periods of storage afterwards (2); nevertheless, bacterial cells are expected to partially (or totally) lose their viability and activity during storage (4).

To our knowledge, the extent to which milk precipitation during rehydration of freeze-dried samples relates to cell survival during storage has not been tackled so far. Therefore, the objective of this study was to elucidate the relationship between death of freeze-dried cells of *Lb. bulgaricus* during storage and decrease in powder solubility.

2. Materials and methods

2.1 Organism and culture preparation

Lactobacillus delbrueckii ssp. *bulgaricus* was obtained from the culture collection held at Escola Superior de Biotecnologia (Portugal). MRS broth was inoculated from the MRS agar slopes, and incubated at 37°C for 24 h. This culture was then inoculated, at the 1 % (v/v) level, into a second MRS broth, and again incubated at 37°C for 24 h. Cells from 100 ml-cultures were harvested by centrifugation at 7000 x *g* for 10 min, washed twice by centrifugation with sterile Ringer´s solution and then resuspended in the same volume of either sterile skim milk (control) containing 11 % (w/v) solids or sterile skim milk containing each of the following compounds: glucose (10 g l^{-1}), fructose (10 g l^{-1}), lactose (10 g l^{-1}), mannose (10 g l^{-1}) or sorbitol (10 g l^{-1}). Cellular suspensions were maintained for 1 h at room temperature (20°C) prior to freezing at -80°C, so as to allow for equilibration between cells and added compounds. All experiments were repeated twice.

2.2 Freeze-drying and storage

Samples of 20 ml were desiccated under vacuum (50 mtorr for 48 h) in a freeze-drier (Martin Christ, Osterad am Harz, Germany), at room temperature (20°C); the condenser was cooled at -55°C. Dried cells were stored in closed containers at 20 °C in air, and maintained in the dark.

2.3 Enumeration of survivors

At regular time 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. Three drops (20 µl each) of suitable dilutions were placed on each of three separate plates (thus providing triplicate results), which were examined following incubation for 48 h at 37 °C.

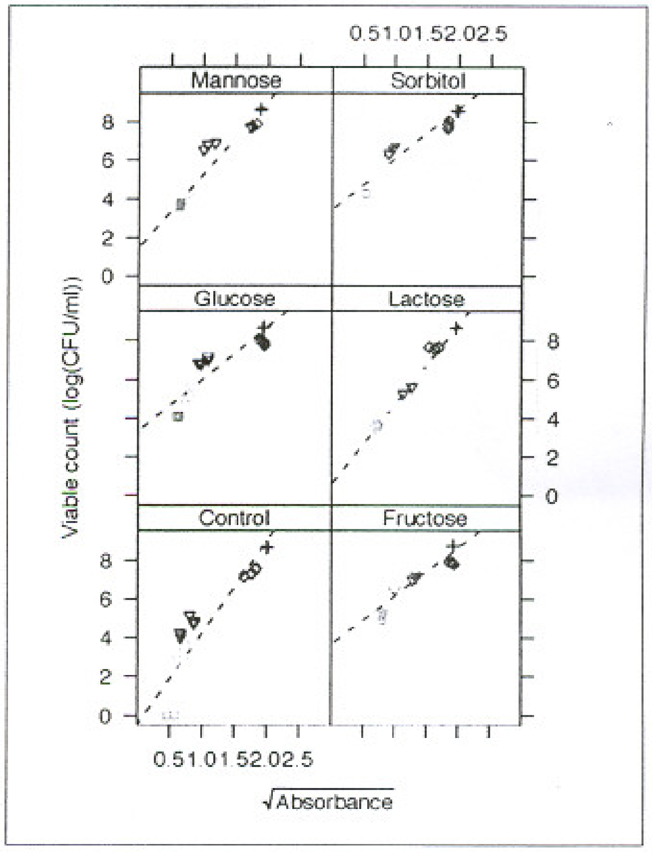

Fig. 1: Experimental data (symbols) and linear regression (dashed line) of log of viable counts and square root of absorbance of freeze-dried *Lb. bulgaricus*, during storage (○, 2 months; ∇, 3 months; x, 5 months; □, 6 months) in the presence of selected compounds added to skim milk (control). BFD and AFD represent the points before and after freeze-drying, respectively (+, BFD; ◊, AFD).

2.4 Measurement of optical density

During storage, freeze-dried samples were rehydrated with deionised water to their original volumes. The dried-rehydrated samples were then stored overnight at 4 °C, thus allowing precipitation of milk. The optical density of the supernatant was subsequently determined at 400 nm (Shimadzu UV-265, Japan).

2.5 *Statistical analysis*

Linear regression analyses were performed between viable counts (expressed as Log (CFU ml^{-1})) and absorbance (expressed as its square root) using the R language for programming (6).

3. Results

The degree of survival of freeze-dried *Lb. bulgaricus*, depicted in Figs. 1 and 2, was found to be directly proportional to the square root of the absorbance of the supernatant. Individual plots, each one prepared for a distinct carbohydrate, indicated that a linear relationship might possibly hold under certain conditions.

As observed in Fig. 2 a darker powder resulted when most of the microorganisms had died.

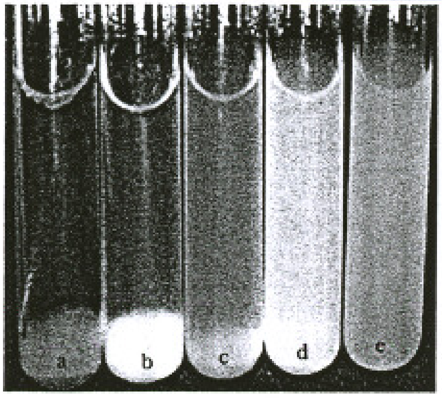

Fig. 2: Visual aspect of test tubes, corresponding to viable counts, in Log (CFU ml^{-1}): (a) 3.18, (b) 3.91, (c) 5.30, (d) 6.02 and (e) 6.59.

4. Discussion

An explanation for the insolubility observed when the viability of the cells decreased could be based on an increase in the extent of non-enzymatic browning (Maillard reactions), as a result of cell death. This hypothesis is corroborated by the similar behaviour observed when skim milk powder was heated to 100°C for 4 h; both insolubility and darkening were observed (data not shown).

Maillard reactions may be affected by the glass transition phenomenon (8); however, in our experiments rehydration during storage of skim milk powders that had been freeze-dried without *Lb. bulgaricus* but in the presence of the various compounds was significantly different from the behaviour observed when the powders had been dried and stored in the presence of the microorganism. In the absence of microorganisms, the precipitation of the skim milk powders after rehydration was much less extensive than in their presence (data not shown); hence the glass transition was likely not the main cause for the aforementioned precipitation.

It can also be concluded from this work that the colour of the powder was related to the various degrees of survival. The compounds produced during spray-drying of mixtures where Maillard reactions can occur, *e.g.* aqueous solutions of reducing sugars and free amino acids, produced coloured powders (10). In addition, MORALES and VAN BOEKEL (7) reported that colouring and brown compound formation in milk and milk products are accounted for mainly from the extent of Maillard reactions.

Our experimental observations indicate that analysis of bacterial cultures in the dairy and biotechnological industries at large may take advantage from a very simple and rapidly implemented protocol; hence, it might represent a helpful tool towards development of a novel, straightforward, shortcut method for monitoring the viability of freeze-dried samples during storage.

Acknowledgements

This work received partial financial support via project PRAXIS/P/Bio/12147/1998 (FCT, Portugal), jointly coordinated by Dr. Paula Teixeira and Dr. Paul Gibbs. Financial support for authors A. Sofia Carvalho and Joana Silva was provided by PhD fellowships, PRAXIS XXI /BD/18152/98 and PRAXIS XXI /BD/197131/99, respectively (FCT).

5. References

(1) ABADIAS, M., BENABARRE, A., TEIXIDÓ, N., USALL, J., VIÑAS, I.: Int. J. Food Microbiol. **65** 173-182 (2001)

(2) CARVALHO, A.S., SILVA, J., HO, P., TEIXEIRA, P., MALCATA, F.X., GIBBS, P.: Biotechnol. Lett. **24** 1587-1591 (2002)

(3) CARVALHO, A.S., SILVA, J., HO, P., TEIXEIRA, P., MALCATA, F.X., GIBBS, P.: Lait **83** 203-210 (2003)

(4) CASTRO, H.P., TEIXEIRA, P., KIRBY, R.: Biotechnol. Lett. **18** 99-104 (1996)

(5) FARKYE, N., SMITH, K., SCHONROCK, F.T.: In U.S. Dairy Export Council SMP Storage Fact Sheet, 2 (2001)

(6) IHAKA, R., GENTLEMAN, R.: J. Comp. Grap. Stat. **5** 299-314 (1996)

(7) MORALES, F.J., VAN BOEKEL, A.J.S.: Int. Dairy J. **8** 907-915 (1998)

(8) SCHEBOR, C., BUERA, D.P., KAREL, M., CHIRIFE, J.: Food Chem **65** 427-432 (1999)

(9) STAPELFELDT, H., NIELSEN, B.R., SKIBSTED, L.H.: Int. Dairy J. **8** 907-915 (1997)

(10) TOMLINSON, A.J., MLOTKIEWICZ, J.A., LEWIS, I.A.S.: Food Chem **48** 373-379 (1993)