Stability of freeze-dried vaginal *Lactobacillus* strains in the presence of different lyoprotectors

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Abstract: The industrial use of lactic acid bacteria as probiotic cultures depends on the preservation techniques employed, which are required to guarantee stable cultures in terms of viability and functional activity. The aim of this study was to evaluate the effects of 12% lactose and 12% sucrose suspended in water or reconstituted skim milk on the survival and expression of beneficial characteristics during freeze-drying and subsequent storage of 6 vaginal lactobacilli strains. A cubic polynomial model was also used for the first time to evaluate the effects of different protectors on survival behavior during storage. Different survival patterns were observed among the strains considered. The presence of both lactose and sucrose in water or in 6% skim milk as the suspension medium proved to be effective in maintaining a high degree of survival and expression of potentially probiotic characteristics (production of antimicrobial substances or auto-aggregation capabilities) of most strains after lyophilization and long-term storage. This study constitutes a valuable step to obtain concentrated cultures with the highest stability of microorganisms for pharmaceutical purposes.

Key words: vaginal lactobacilli, lyoprotectors, storage, probiotics.

Résumé : L'utilisation industrielle des bactéries lactiques comme cultures probiotiques dépend des techniques de préservation employées, lesquelles sont requises pour garantir des cultures stables en termes de viabilité et d'activités fonctionnelles. Le but de cette étude était d'évaluer les effets du lactose 12% et du sucrose 12% en suspension dans l'eau ou dans le lait écrémé reconstitué sur la survie et l'expression de caractéristiques bénéfiques lors de lyophilisation et l'entreposage subséquent de six souches de lactobacilles vaginales. Un modèle polynomial cubique a aussi été utilisé pour la première fois afin d'évaluer les effets de différents agents de protection sur la survie lors de l'entreposage. Différents patrons de survie ont été observés parmi les souches considérées. La présence des deux composés dans l'eau ou dans le lait écrémé 6% comme milieu de suspension s'est avérée efficace pour maintenir un haut niveau de survie et pour permettre l'expression de caractéristiques potentiellement probiotiques (production de substances antimicrobiennes ou capacité d'auto-agrégation) chez la plupart des souches après une lyophilisation et un entreposage à long terme. Cette étude constitue une étape importante dans l'obtention de cultures concentrées de microorganismes hautement stables à des fins pharmaceutiques.

Mots-clés : lactobacilles vaginales, lyoprotecteurs, entreposage, probiotiques.

[Traduit par la Rédaction]

Introduction

Lactobacilli are the predominant microorganisms of vaginal microbiota in healthy women. These bacteria exert a protective effect against pathogenic microorganisms through mechanisms such as adhesion, biofilm formation, production of lactic acid, hydrogen peroxide and bacteriocins, and immune system stimulation (Lepargneur and Rousseau 2002). Pharmaceutical probiotic products containing lactobacilli from the urogenital tract have shown quite good effects in preventing or treating genital infections in women, even when administered orally (Reid et al. 2003; Falagas et al. 2008).

To guarantee the beneficial effect of probiotic products, pharmaceutical companies should employ optimal preservation methods to assure high stability of the microorganisms during long-term storage (Sanders et al. 2005).

Freeze-drying has been the classical method used to produce dry bacterial powders because drying takes place at low temperatures, thus reducing heat degradation. However, freeze-drying may cause a different type of damage that may affect the viability and activity of many microorganisms (Castro et al. 1997; Van de Guchte et al. 2002). Cell injury can be attributed mainly to changes in the physical state of membrane lipids or in the structure of sensitive proteins (Leslie et al. 1995). Different compounds, such as sugars (sucrose, lactose, and trehalose), antioxidant substances (ascorbic acid and propyl gallate), amino acids (sodium glutamate and aspartate), and proteinaceous products (skim milk), have been tested to improve the survival of lactic

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acid bacteria during preservation (Carvalho et al. 2002; Huang et al. 2006; Martos et al. 2007).

Production of concentrated starter cultures for the food industry by freezing or freeze-drying has been extensively studied (Fonseca et al. 2000; Zhao and Zhang 2005), but there is little information available for microorganisms isolated from other ecological niches (Maggi et al. 2000; Zárate et al. 2005).

Most studies have analyzed the survival of microorganisms in the lyophilization process but not during storage (Leslie et al. 1995; Palmfeldt and Hahn-Hägerdal 2000; Schoug et al. 2008). However, the stability of functional activities and the viability of microorganisms immediately after freeze-drying and at intervals during the storage period has only been determined in a few studies (Tsvetkov and Brankova 1983; Johnson and Etzel 1995; Yao et al. 2008), many of them involving bacteria for food applications.

Few studies are available in the literature in which mathematical models (e.g., linear model) were applied to describe survival curves during the storage of lyophilized microorganisms (Miyamoto-Shinohara et al. 2006, 2008). Mathematical models allow the reduction of experimental data to a limited number of parameters of interest and the systematic analysis of the death rates for different microorganisms. As far as we know, a cubic polynomial model has not yet been used as a tool to objectively evaluate the survival behavior of freeze-dried microorganisms during storage.

Vaginal lactobacilli isolated from women in Tucumán, Argentina, were characterized in terms of their potentially probiotic properties (Ocaña et al. 1999*a*, 1999*b*, 1999*c*; Ocaña and Nader-Macías 2002) and growth performance (Juárez Tomás et al. 2002*a*, 2002*b*, 2003*a*, 2003*b*). The aim of the present study was to investigate the protective effects of selected agents on the probiotic properties and survival of vaginal lactobacilli during freeze-drying and 2 years of storage.

Materials and methods

Microorganisms and culture conditions

Six strains, isolated from the vaginal swabs of different women (Ocaña et al. 1999*a*), were obtained from the Culture Collection of Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina). The following strains were previously selected for their potentially probiotic and technological properties: *Lactobacillus paracasei* CRL 1289, *Lactobacillus acidophilus* CRL 1266 and *L. acidophilus* CRL 1251 (H_2O_2 producers) (Ocaña et al. 1999*c*; Juárez Tomás et al. 2003*a*); *Lactobacillus gasseri* CRL 1259 (uropathogen inhibition by lactic acid production) (Juárez Tomás et al. 2003*b*); *Lactobacillus johnsonii* CRL 1294 (remarkable auto-aggregating pattern) (Ocaña and Nader-Macías 2002; Juárez Tomás et al. 2005); and *Lactobacillus salivarius* CRL 1328 (bacteriocin producer) (Ocaña et al. 1999*b*; Juárez Tomás et al. 2002*b*).

Strains were subcultured in LAPTg broth (1.5% peptone, 1% tryptone, 1% glucose, 1% yeast extract, 0.1% Tween 80, pH 6.5) (Raibaud et al. 1961) at 37 °C 3 times just prior to experimental use.

Freeze-drying

Bacterial cells were grown in LAPTg broth at 37 °C for 16 h (stationary phase). Cells were centrifuged (10 000*g*, 15 min, 4 °C). The cell pellets were washed twice with distilled water and resuspended in solutions with the following protectors: 6% (*m*/*v*) reconstituted skim milk (RSM), 12% (*m*/*v*) sucrose, or 12% (*m*/*v*) lactose. Each carbohydrate was suspended either in water or 6% RSM. Aliquots (0.3 mL) of each bacterial suspension were frozen in sterile ampoules at –70 °C. The frozen samples were treated in a chamber-type freeze-drier (Lyovac GT2; Leybold, Köln, Germany) for 16 h at 0.3 mbar (1 bar = 100 kPa), which yielded products with <1% residual moisture. After the freeze-drying cycle was complete, the vials were heat-sealed under vacuum. Dried cells were stored at 4 °C. Two replications of 2 experiments were performed.

Cell viability

Lyophilized samples were rehydrated to the original volume with 0.1% peptone for 10 min at room temperature, and appropriate dilutions were poured in LAPTg agar (LAPTg broth containing 1% agar). Plates were incubated at 37 °C for 48 h, and the number of colony forming units (CFU) per mL (CFU/mL) from samples randomly taken before and after freeze-drying and every 6 months during the 2-year storage period were determined. The degree of survival was expressed as N_{AL}/N_{BL} or N_{AS}/N_{AL}, where N_{AL} and N_{BL} are the log(CFU/mL) after lyophilization and before lyophilization, respectively, and N_{AS} is the log(CFU/mL) after 24 months of storage.

Evaluation of potentially probiotic properties

The production of different inhibitory substances or the auto-aggregation ability of the selected strains was determined before and after freeze-drying and after 6, 12, and 24 months of storage. Rehydrated lyophilized cultures were grown in LAPTg broth for 24 h at 37 °C before testing. *Lactobacillus paracasei* CRL 1289, *L. acidophilus* CRL 1251, and *L. acidophilus* CRL 1266 were tested for H_2O_2 production by the quantitative spectrophotometric assay of *o*-dianisidine-peroxidase (Juárez Tomás et al. 2003*a*). Lactic acid production by *L. gasseri* CRL 1259 was determined by high performance liquid chromatography (Juárez Tomás and Nader-Macías 2007). Bacteriocin levels produced by *L. salivarius* CRL 1328 were studied according to the plate-diffusion method, by employing vaginal *Enterococcus faecalis* MP97 as an indicator microorganism (Juárez Tomás et al. 2002*b*). The auto-aggregation ability of *L. johnsonii* CRL 1294 suspended in phosphate saline buffer (8 g/L NaCl, 0.34 g/L KH_2PO_4, 1.21 g/L K_2HPO_4, pH 7) was assessed by the quantitative spectrophotometric method previously reported (Juárez Tomás et al. 2005).

Statistical analysis

Viability loss during freeze-drying (N_{AL}/N_{BL}) and after 24 months of storage (N_{AS}/N_{AL}) and changes in the beneficial properties during storage of each *Lactobacillus* strain in different protective media were evaluated by use of the Scheffé test of multiple comparisons. Differences were considered statistically significant at $p < 0.05$.

Analysis of variance with repeated measurements of viable counts after freeze-drying and at regular intervals during storage was carried out to compare the protective effect of

different agents. Lyoprotectors were evaluated as a fixed factor with 5 levels. The relationship between viability and storage time was considered as a polynomial model of the third order in the time adjusted for each protective agent, according to the following expression:

$$[1] \qquad [\log(\text{CFU/mL})]_t = A + Bt + Ct^2 + Dt^3$$

where $[\log(\text{CFU/mL})]_t$ is log(CFU/mL) at time t; t is the storage time in months; A is the log(CFU/mL) estimated by the model at initial time of storage; B, C, and D are lineal, quadratic, and cubic coefficients of variable time, respectively, which indicate death rates (months^{-1}). B is the slope of the straight line relating log(CFU/mL) to storage time, C is the asymptotic decrease or point of the curvature change in the survival curve, and D is the cubic death rate.

Results

Cell viability

Survival after freeze-drying and storage of 6 vaginal lactobacilli strains or species in different suspension media was determined by the plate dilution method and statistically evaluated by applying the Scheffé test of multiple comparisons.

Different degrees of cell viability were obtained after freeze-drying in the presence of RSM alone and in sucrose or lactose suspended in water or RSM (Table 1). A significant drop in cell viability was observed after lyophilization in almost all conditions. Cells dried in sucrose had N_{AL}/N_{BL} values of 0.75–0.93 after drying. The addition of this sugar to RSM significantly improved survival only for *L. acidophilus* CRL 1266, *L. paracasei* CRL 1289, and *L. johnsonii* CRL 1294. Best results for most lactobacilli were observed with lactose–RSM (N_{AL}/N_{BL} = 0.90–0.99) as the drying medium compared with lactose alone (N_{AL}/N_{BL} = 0.66–0.98), except for *L. gasseri* CRL 1259 and *L. salivarius* CRL 1328. Also, RSM was efficient in the lyophilization step (N_{AL}/N_{BL} = 0.87 – 0.91), except for *L. paracasei* CRL 1289 (N_{AL}/N_{BL} = 0.62).

Microbial cell survival of freeze-dried vaginal lactobacilli strains decreased after 24 months of storage (Table 1 and Fig. 1). Parameters estimated in the polynomial model are shown in Table 2. For most microorganisms except *L. acidophilus* CRL 1251 and *L. gasseri* CRL 1259, the estimated numbers of viable cells at the initial time of storage (A) were significantly different depending on the protectors employed; these values were similar to the experimental data (Fig. 1) and were higher in combinations of sucrose or lactose with RSM. On the other hand, the initial numbers of viable cells did not influence survival of most strains during storage, excepted *L. paracasei* CRL 1289 and *L. johnsonii* CRL 1294.

Negative values of the estimated lineal (B) and cubic (D) death rates indicate decreased viability during storage for all *Lactobacillus* strains and protective agents, except for *L. salivarius* CRL 1328 in lactose–RSM (B = 0.009 months^{-1}; D = 0.0001 months^{-1}). Most of the strains showed positive values for quadratic death rates (C), which means that, after the marked initial viability loss, the number of viable cells remained relatively stable. Analysis of survival parameters (Table 2) were coincident with the results shown in Fig. 1, which indicates that the cubic polynomial model accurately described the nonlinear variation of the experimental data (log(CFU/mL)) during storage.

The microorganisms tested showed statistically significant differences in the degree of survival (N_{AS}/N_{AL}) (Table 1) and in the B, C, and D coefficients (Table 2) in the presence of the different lyoprotectors tested. For most strains, the relationship of coefficient B with the protective media corresponded to that of the N_{AS}/N_{AL} values, except for *L. paracasei* CRL 1289 and *L. johnsonii* CRL 1294. Survival during the 2-year storage period was evaluated by eq. [1] and by N_{AS}/N_{AL}.

The results indicated that RSM was unable to maintain high viability during preservation (in general there were significantly higher lineal death rates and significantly lower N_{AS}/N_{AL}), except for *L. salivarius* CRL 1328. On the other hand, better results were obtained for most strains (lower lineal death rates, higher N_{AS}/N_{AL}) with lactose than with sucrose, suspended either in water or RSM. However, these differences were only significant for *L. salivarius* CRL 1328. The addition of RSM to sucrose or lactose improved survival only in half of the strains (*L. paracasei* CRL 1289, *L. johnsonii* CRL 1294, and *L. salivarius* CRL 1328).

The viable cell counts after lyophilization affected the viability only of *L. paracasei* CRL 1289 and *L. johnsonii* CRL 1294. For these microorganisms, lineal, quadratic, and cubic death rates were not significantly different under the different conditions evaluated (except for *L. johnsonii* CRL 1294 in RSM alone). However, only the combinations of carbohydrates and RSM promoted significantly higher recuperation of viable cells after the drying process and, consequently, higher degrees of survival (N_{AS}/N_{AL}) after storage.

L. johnsonii CRL 1294 was the most sensitive strain to storage in the freeze-dried form. On the other hand, *L. salivarius* CRL 1328 showed the best performance and robustness to the preservation technique applied.

Stability of probiotic properties

For most strains, probiotic characteristics (auto-aggregation or antimicrobial substance production capabilities) were maintained without significant changes after lyophilization (Figs. 2 and 3). However, the expression of beneficial properties of vaginal lactobacilli was affected to different extents during storage, depending on the strain, protective media, and storage time.

In general, the stability of probiotic properties during storage was higher in cultures from freeze-dried powder with lactose than in those with sucrose, both with water and RSM as the suspension media, except for *L. acidophilus* CRL 1266. Different results were obtained in the presence of RSM alone or added to the carbohydrates (Figs. 2 and 3).

The preservation of beneficial properties was independent of the number of viable cells in the lyophilized cultures, except for *L. acidophilus* CRL 1251 and CRL 1266. For these microorganisms, at a lower number of viable cells of lyophilized bacteria, lower growth of the cultures was observed (data not shown) and a greater decrease in H_2O_2 levels was recorded (Fig. 2). On the other hand, under the optimum conditions for each strain, the probiotic properties were well-preserved after 24 months of storage. For *L. salivarius* CRL 1328, bacteriocin production decreased abruptly after

Table 1. Viability of vaginal lactobacilli during freeze-drying and after 24 months of storage in the presence of different protective media.

		Lyophilization process	Storage
Microorganism	Protective media	N_{AL}/N_{BL} ± SD*	N_{AS}/N_{AL} ± SD†
Lactobacillus acidophilus CRL 1251	Sucrose	0.93±0.01a	0.71±0.00a
	Lactose	0.87±0.01b	0.78±0.01a
	RSM	0.87±0.00b	0.42±0.06b
	Sucrose–RSM	0.90±0.02a,b	0.70±0.02a
	Lactose–RSM	0.93±0.01a	0.76±0.02a
Lactobacillus acidophilus CRL 1266	Sucrose	0.88±0.01a	0.88±0.01a
	Lactose	0.82±0.02b	0.65±0.09b,c
	RSM	0.89±0.01c	0.50±0.02c
	Sucrose–RSM	0.96±0.01d	0.80±0.05a,b
	Lactose–RSM	0.93±0.00c,d	0.79±0.04a,b
Lactobacillus paracasei CRL 1289	Sucrose	0.76±0.02a	0.31±0.05a
	Lactose	0.66±0.04b	0.45±0.03b
	RSM	0.62±0.00b,c	0.38±0.01a,b
	Sucrose–RSM	0.91±0.01d	0.84±0.01c
	Lactose–RSM	0.93±0.00d	0.72±0.00d
Lactobacillus gasseri CRL 1259	Sucrose	0.93±0.01a,b	0.79±0.03a
	Lactose	0.97±0.01b	0.85±0.00a
	RSM	0.91±0.00a	0.58±0.00b
	Sucrose–RSM	0.94±0.02a,b	0.86±0.00a
	Lactose–RSM	0.97±0.01b	0.82±0.04a
Lactobacillus johnsonii CRL 1294	Sucrose	0.75±0.00a	0.08±0.11a
	Lactose	0.73±0.04a	0.15±0.05a
	RSM	0.86±0.00b	0.07±0.10a
	Sucrose–RSM	0.94±0.01c	0.55±0.04b
	Lactose–RSM	0.99±0.00c	0.66±0.04b
Lactobacillus salivarius CRL 1328	Sucrose	0.93±0.01a	0.27±0.04a
	Lactose	0.98±0.01a	0.77±0.02b
	RSM	0.92±0.03a	0.83±0.05b
	Sucrose–RSM	0.83±0.00b	0.86±0.01b,c
	Lactose–RSM	0.90±0.04a	0.96±0.01c

Note: Each value represents the mean of log(CFU/mL) for 2 replicates from 2 trials. Different letters after the values indicate significant differences ($p < 0.05$) for the effects of the protective media on N_{AL}/N_{BL} (viability loss during freeze-drying) or N_{AS}/N_{AL} (viability loss after 24 months of storage) for each *Lactobacillus* strain, according to the Scheffé test. RSM, reconstituted skim milk; CFU, colony forming units.

*log(CFU/mL) before (N_{BL}) and after (N_{AL}) lyophilization.

†log(CFU/mL) before storage (N_{AL}) and after 24 months storage (N_{AS}).

12 months of storage for bacterial cells suspended in lactose–RSM, whereas it progressively decreased for cells suspended in the other media.

Discussion

During industrial production of concentrated cultures, probiotic microorganisms are subjected to different types of adverse conditions, such as thermic, osmotic, mechanical, and oxidative stresses (Van de Guchte et al. 2002).

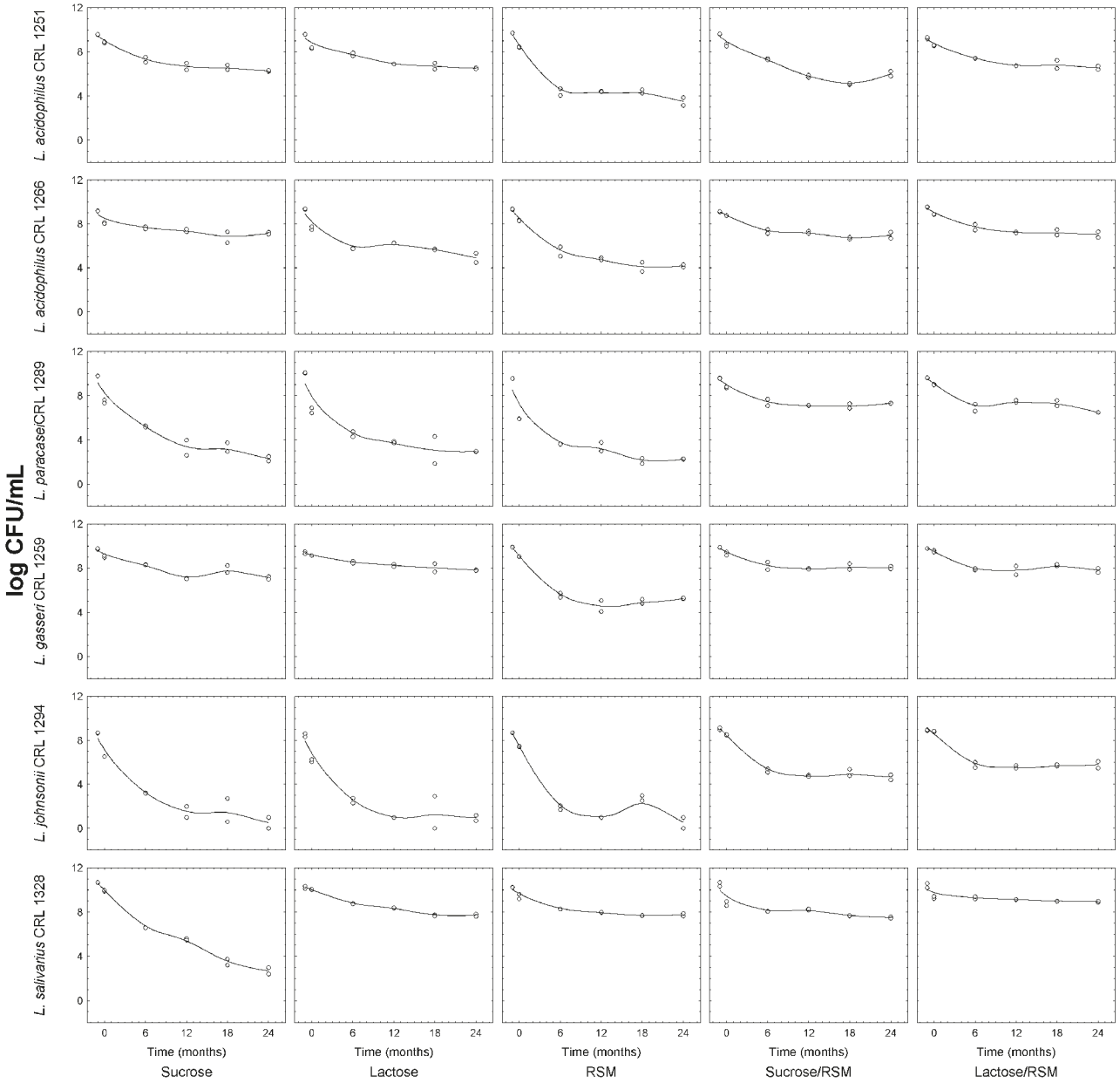
Lyophilization is a common process employed to preserve bacteria, either during concentrated culture production in the food and pharmaceutical industries or for culture collections. Many factors affect the survival of lactic acid bacteria dried by lyophilization, such as intrinsic tolerance of the cultures, growth conditions, stress response factors, cell-harvesting conditions, protective substances, drying media, rehydration, and storage conditions (Santivarangkna et al. 2007).

The use of suitable protective substances is an essential step during the freeze-drying process (Huang et al. 2006). Lactose and sucrose are employed because of their adequate properties and qualities for commercial preparation and storage of cultures (Gagné et al. 1993; Carvalho et al. 2004). Skim milk powder is usually selected as the drying medium for most of the lactic acid bacteria starters in the dairy industry (Abadias et al. 2001).

In the present study, the survival of microorganisms during lyophilization and storage was shown to be dependent on the strain and protective agent. All compounds tested were found to be effective in protecting the cells during the freeze-drying process. However, for most of the strains, the best results were obtained when carbohydrates, especially lactose, were suspended in RSM.

In this study, a cubic polynomial model was used for the first time to evaluate survival behavior during the storage of lactobacilli. The viability plots fit well to the model applied.

Fig. 1. Viability of freeze-dried vaginal lactobacilli during storage for 24 months at 7 °C in different protective media (reconstituted skim milk, RSM). Experimental data were adjusted by use of a polynomial model of the third order (eq. [1] , see the Material and methods section), and the parameters of model are shown in Table 2. The initial points in each graph represent the log(CFU/mL) in each cell suspension before lyophilization (N_{BL}).



Thus, the application of statistical analysis allowed us to determine the effects of different protectors on the survival parameters estimated for each strain. During storage, different survival behaviors were obtained among the strains evaluated; for half of them, maximum protection of the dried cells was obtained with either lactose or sucrose in RSM. However, *L. gasseri* CRL 1259 and both of the *L. acidophilus* strains did not show significant differences when water or RSM were used as the suspension medium.

The results obtained during the freeze-drying process were consistent with those reported by Font de Valdez et al. (1983), who found that skim milk (10% or 20%) alone was not a good protectant during lyophilization of 14 representative species of lactic acid bacteria isolated from dairy products. Also, Zayed and Roos (2004) showed that the combination of trehalose with RSM was more effective than trehalose alone in protecting *L. salivarius* during freeze-drying. However, Carvalho et al. (2002) reported that the addition of sugars, amino acids, or antioxidants to 11% skim milk did not improve the viability of *Lactobacillus plantarum* and *Lactobacillus rhamnosus* during the freeze-drying process but that these components preserved the cells during 10 months of storage. On the other hand, Tsvetkov and Brankova (1983) observed better results in preserving *L. plantarum* L4 with 8% RSM or 5% peptone than with 5% sucrose or 5% lactose during freeze-drying and 2 years of storage.

Different authors have suggested several mechanisms that

Table 2. Survival parameters estimated by the cubic polynomial model after freeze-drying and subsequent storage of vaginal lactobacilli in different protective media.

			Parameters estimated by cubic polynomial model†			
Microorganism	Protective media	$N_{BL} - N_{AL}$*	$A \pm$ SD	$B \pm$ SD	$C \pm$ SD	$D \pm$ SD
Lactobacillus acidophilus CRL 1251	Sucrose	0.71±0.06	8.87±0.25a	–0.384±0.111a	0.023±0.012a	–0.0005±0.0003a
	Lactose	1.25±0.07	8.38±0.25a	–0.116±0.111a	–0.001±0.012a	0.0001±0.0003a
	RSM	1.29±0.04	8.36±0.25a	–1.055±0.111b	0.082±0.012b	–0.0019±0.0003c
	Sucrose–RSM	1.00±0.16	8.63±0.25a	–0.151±0.111a	–0.016±0.012c	0.0007±0.0003b
	Lactose–RSM	0.68±0.06	8.61±0.18a	–0.306±0.079a	0.018±0.008a	–0.0004±0.0002a
Lactobacillus acidophilus CRL 1266	Sucrose	1.10±0.06	8.06±0.33a	–0.007±0.149a	–0.009±0.016a	0.0003±0.0004a
	Lactose	1.73±0.16	7.56±0.33a	–0.438±0.149a,b	0.037±0.016a,b	–0.0010±0.0004a
	RSM	1.00±0.11	8.28±0.33b	–0.594±0.149b	0.030±0.016b	–0.0005±0.0004a
	Sucrose–RSM	0.34±0.05	8.72±0.33b	–0.277±0.149a	0.014±0.016a,b	–0.0002±0.0004a
	Lactose–RSM	0.67±0.01	8.90±0.24b	–0.292±0.105a,b	0.017±0.011a,b	–0.0004±0.0003a
Lactobacillus paracasei CRL 1289	Sucrose	2.32±0.20	7.55±0.50a	–0.584±0.214a	0.029±0.023a	–0.0006±0.0006a
	Lactose	3.41±0.37	6.66±0.50b	–0.443±0.214a	0.020±0.023a	–0.0003±0.0006a
	RSM	3.64±(0.03	5.85±0.50b	–0.405±0.214a	0.016±0.023a	–0.0002±0.0006a
	Sucrose–RSM	0.84±0.11	8.75±0.50c	–0.309±0.214a	0.018±0.023a	–0.0003±0.0006a
	Lactose–RSM	0.64±0.03	8.96±0.35c	–0.557±0.151a	0.050±0.016a	–0.0013±0.0004a
Lactobacillus gasseri CRL 1259	Sucrose	0.70±0.06	9.19±0.32a	–0.313±0.142a,b	0.020±0.015a,b	–0.0004±0.0004a,b
	Lactose	0.24±0.14	9.16±0.32a	–0.133±0.142b	0.006±0.015b,c	–0.0001±0.0004b
	RSM	0.85±0.02	9.07±0.32a	–0.883±0.142c	0.055±0.015a	–0.0010±0.0004a
	Sucrose–RSM	0.58±0.22	9.34±0.32a	–0.300±0.142a,b	0.021±0.015a	–0.0004±0.0004a,b
	Lactose–RSM	0.25±0.11	9.57±0.22a	–0.488±0.100a	0.040±0.011a,b	–0.0010±0.0003a,b
Lactobacillus johnsonii CRL 1294	Sucrose	2.17±0.03	6.57±0.60a	–0.868±0.236a	0.053±0.025a	0.0011±0.0007a
	Lactose	2.30±0.36	6.20±0.60a	–0.954±0.236a	0.060±0.025a	–0.0001±0.0007a
	RSM	1.25±0.02	7.49±0.60b	–1.695±0.236b	0.139±0.025b	–0.0034±0.0007b
	Sucrose–RSM	0.56±0.09	8.50±0.60c	–0.854±0.236a	0.061±0.025a	–0.0013±0.0007a
	Lactose–RSM	0.1±0.02	8.80±0.42c	–0.774±0.167a	0.055±0.018a	–0.0012±0.0005a
Lactobacillus salivarius CRL 1328	Sucrose	0.75±0.09	9.69±0.18a,b	–0.564±0.091a	0.020±0.009a	–0.0004±0.0003a
	Lactose	0.19±0.13	9.96±0.18b	–0.215±0.091b	0.007±0.009a,b	–0.0001±0.0003a
	RSM	0.85±0.29	9.37±0.18a	–0.224±0.091b	0.011±0.009a,b	–0.0002±0.0003a
	Sucrose–RSM	1.74±0.01	8.66±0.18c	–0.100±0.091b,c	0.006±0.009b	–0.0002±0.0003a
	Lactose–RSM	1.07±0.42	9.34±0.13a	0.009±0.064c	–0.003±0.007b	0.0001±0.0002a

Note: Each value represents the mean of the parameters estimated from 2 replicates from 2 trials. Different letters indicate significant differences ($p < 0.05$) of the effects of the protective media on the individual parameter estimated by the model for each *Lactobacillus* strain according to analysis of variance. RSM, reconstituted skim milk.

*Decrease of survival between log(CFU/mL) before (N_{BL}) and after (N_{AL}) lyophilization.

†Parameters were estimated by applying the cubic polynomial model: A, log(CFU/mL) at the initial time of storage; *B*, lineal death rate (months^{-1}); *C*, quadratic death rate (months^{-1}); and *D*, cubic death rate (months^{-1}).

are responsible for the protective effect exerted by carbohydrates and proteinaceous substances on the survival of microorganisms during freeze-drying and storage (Leslie et al. 1995; Castro et al. 1997; Selmer-Olsen et al. 1999). Proteins in skim milk form a protective coating for the cells (Abadias et al. 2001). Carbohydrates could help to prevent or decrease the lethal effect of intracellular ice formation during freezing through hydrogen binding with water and cell structures (stabilizing the cell membrane and proteins), or they may protect against free radicals produced during the storage and rehydration of freeze-dried cultures (Leslie et al. 1995).

The ability of cells to remain viable and functionally active during long-term storage is an important requirement of potentially probiotic strains (Sanders et al. 2005). Under the optimum conditions, auto-aggregation or antimicrobial-substance production capabilities were retained without significant changes during the lyophilization process for most of the *Lactobacillus* strains tested. Also, most of the probiotic properties remained for the different storage times tested. However, bacteriocin production by freeze-dried *L. salivarius* CRL 1328 was significantly affected by the length of storage time, depending on the suspension media. Silva et al. (2002) reported that bacteriocin production by *L. salivarius* CTC 2197 and *Lactobacillus sakei* CTC 494 was not af-

Fig. 2. Production of H_2O_2 by *Lactobacillus acidophilus* CRL 1251 (*a*), *L. acidophilus* CRL 1266 (*b*), and *Lactobacillus paracasei* CRL 1289 (*c*) in cultures before lyophilization (control, black bar), after freeze-drying (light gray bars), and during storage at 6 (dotted bars), 12 (dark gray bars), and 24 months (open bars), in the presence of different protectors (reconstituted skim milk, RSM).

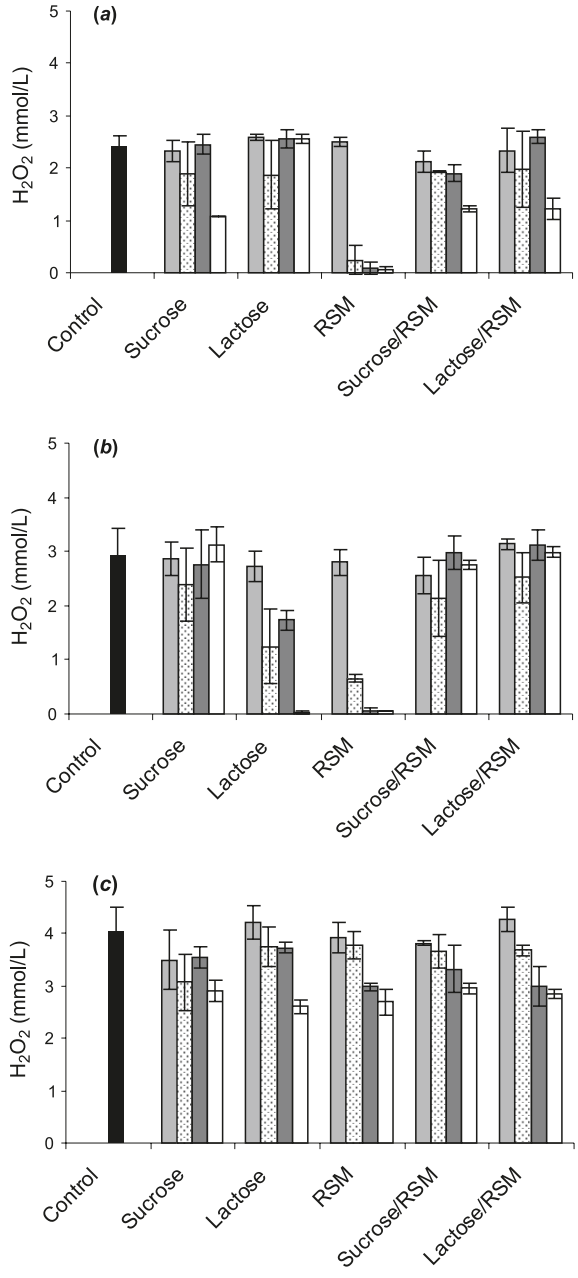
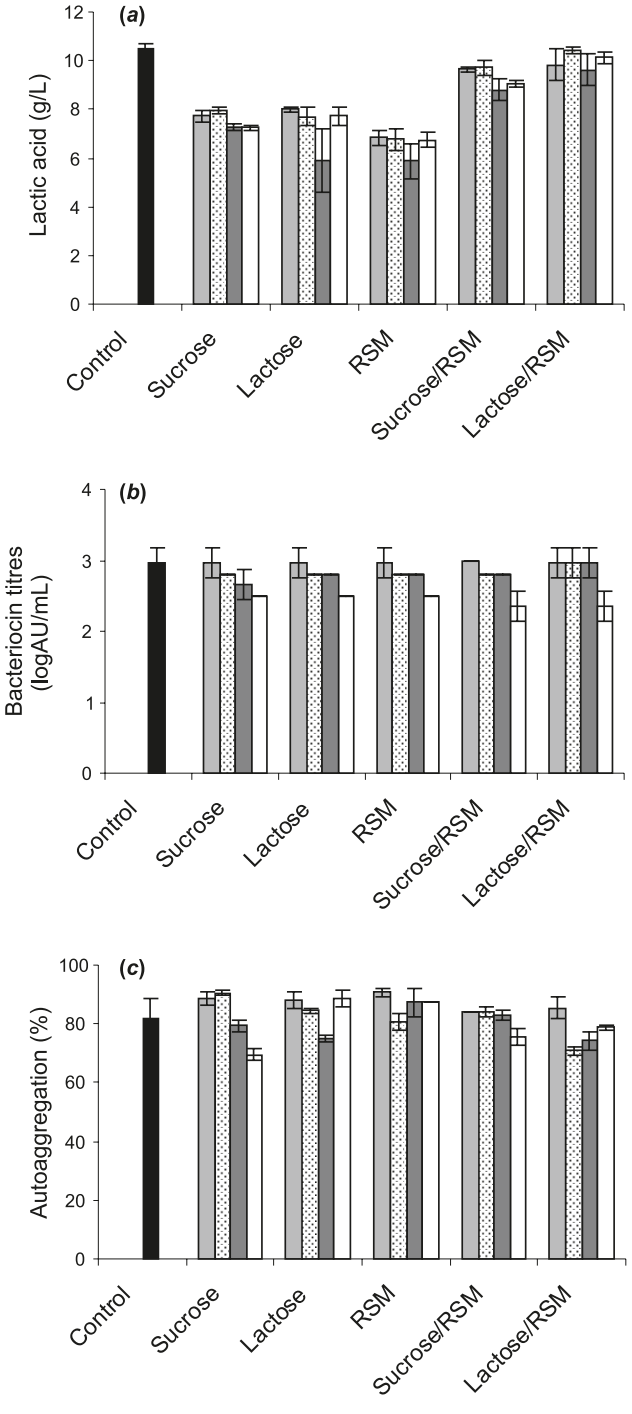


Fig. 3. Expression of the following beneficial properties: lactic acid production by *Lactobacillus gasseri* CRL 1259 (*a*), bacteriocin production by *Lactobacillus salivarius* CRL 1328 (*b*), and the auto-aggregation ability (percentages determined after 4 h of spectrophotometric assay) of *Lactobacillus johnsonii* CRL 1294 (*c*) in cultures before lyophilization (control, black bar), after freeze-drying (light gray bars), and during storage at 6 (dotted bars), 12 (dark gray bars), and 24 months (open bars), in the presence of different protectors (reconstituted skim milk, RSM).



fected during drying in 11% RSM with a short storage time of only 3 months; however, bacteriocin production was not quantified.

Lloyd (1975) classified the damage produced by freezing lactic acid bacteria in the dairy industry into 3 types: (*i*) a decrease in viability without an effect on acid production, (*ii*) no change in cell number with a decrease in acid production, and (*iii*) a decrease in both viability and acid production. These observations can also be related to the storage of freeze-dried vaginal lactobacilli; most of them belong to types (*i*) or (*iii*), except for *L. salivarius* 1328 in lactose–RSM because this microorganism had no significant

decrease in viability but with a loss of its probiotic properties.

The results of this work demonstrate that the resistance of microorganisms to lyophilization and storage is highly variable and strain-dependent, and that certain additives are more effective than others in protecting cells throughout drying and storage. The evaluation of both viability and expression of beneficial properties of lyophilized cultures, as well as the rational selection of protective agents, are valuable steps to optimize the stability of cells to be used in probiotic pharmaceutical products.

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