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ORIGINAL ARTICLE

Survival and beneficial properties of lactic acid bacteria from raniculture subjected to freeze-drying and storage

G. Montel Mendoza[1], S.E. Pasteris[1], M.C. Otero[2] and M.E. Fatima Nader-Macías[2]

1 Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Instituto Superior de Investigaciones Biológicas (INSIBIO-CONICET), Instituto de Biología "Dr. Francisco D. Barbieri", San Miguel de Tucumán, Argentina

2 Centro de Referencia para Lactobacilos (CERELA-CONICET), San Miguel de Tucumán, Argentina

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Correspondence
María E. Fatima Nader-Macías, Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, CP: T4000ILC San Miguel de Tucumán, Argentina.
E-mail: fnader@cerela.org.ar

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Abstract

Aim: To evaluate the effect of freeze-drying and storage conditions on the viability and beneficial properties of lactic acid bacteria (LAB) for raniculture.
Methods and Results: *Lactococcus lactis* CRL 1584, *L. lactis* CRL 1827, *Lactococcus garvieae* CRL 1828 and *Lactobacillus plantarum* CRL 1606 viability under different conditions was studied. 10% lactose and 5% skim milk + 5% lactose were excellent lyoprotectants, but 5% skim milk + 5% lactose and whey protein concentrated (WPC) or WPC + sugars were the lower cost lyoprotective options. The effect of temperature depended on both lyoprotectants and storage time. Thus, for *Lactococcus*, skim milk, skim milk + sucrose and WPC + sucrose were selected for lyophilization and storage at 4°C and skim milk + lactose for 25°C. For *Lact. plantarum* CRL 1606, the best lyoprotectants for lyophilization and storage at 4°C were milk + sugars and WPS + sucrose and, at 25°C, skim milk + sucrose.
Conclusions: Lactic acid bacteria viability after freeze-drying was strain-specific and depended on the lyoprotectant used. Highest viability was obtained when stored at 4°C, and the beneficial properties remained stable for 18 months independently of storage temperature.
Significance and Impact of the Study: The studies reported for the first time in this work are of primary interest to obtain dried bacteria to be included in beneficial products for raniculture.

Introduction

The autochthonous microbiota of human and animal ecosystems is constituted by a wide variety of micro-organisms recently termed microbioma that has physiological functions (beneficial effects) on the host, including maintenance of the ecological equilibrium, modulation of the immune system and protection or prevention against infectious diseases (Saulnier *et al.* 2011). In hatchery conditions, the microbioma can be affected by endogenous and exogenous factors and then the possibility of outbreaks of infectious disease increases. Treatment or prevention with therapeutics contributes to the spread of antibiotic resistant bacteria (Verschuere *et al.* 2000; Vine *et al.* 2004; Ringø *et al.* 2010) and to the presence of chemical residues in foodstuff without preventing recurrent episodes. Thus, valid and novel alternative strategies (probiotics) are being used instead of chemotherapeutics to maintain a well-balanced microbioma and thus prevent and control pathogen entry. In aquaculture, some beneficial effects were evidenced using lactic acid bacteria (LAB; Irianto and Austin 2002; Balcázar *et al.* 2007; Pérez-Sánchez *et al.* 2011; Pirarat *et al.* 2011), which also contributed to enhance productivity in hatchery conditions.

To restore the equilibrium of the microbioma, a probiotic or beneficial product must contain high amounts of viable micro-organisms when administered to the host. It must also maintain its viability and functional properties during storage to be able to exert its beneficial effects. Although there are numerous studies that deal with the viability and conservation of LAB to be used as food

preservatives, no reports were found on indigenous LAB for raniculture, which represents a new and promising aquaculture activity.

The recovery of high amounts of viable micro-organisms to be used in an ecosystem depends directly on their obtainment and storage methods. Low temperature (freezing or refrigeration) and freeze-drying (lyophilization) are the most common techniques used to stabilize probiotics. However, lyophilized cultures are more adequate than frozen ones based on both transport and storage costs (Berner and Viernstein 2006).

Lyophilization is a process that starts with the freezing of micro-organisms followed by sublimation (primary drying) and desorption (secondary drying) to reduce water content; then, neither microbial growth nor chemical reactions occur at this stage (Schoug Bergenholtz *et al.* 2012). This technique has been used to maintain the viability and functional properties of beneficial and probiotic micro-organisms to be applied in different ecological niches (Otero *et al.* 2007; Juárez Tomás *et al.* 2009; Bolla *et al.* 2011; Zhan *et al.* 2012). Many factors have been reported to affect the freeze-drying survival of LAB and their subsequent storage such as initial cell concentration, growth media, freezing rate and temperature and storage temperatures (Carvalho *et al.* 2004; Berner and Viernstein 2006; Schoug *et al.* 2006; Schoug Bergenholtz *et al.* 2012). The use of lyoprotectants is one of the most important factors to improve cell survival rate, and a range of mechanisms have been proposed to explain their protective effects (Schoug *et al.* 2006). Different substances such as sugars (sucrose, lactose, trehalose), proteinaceous compounds (skim milk), amino acids (sodium glutamate and aspartate) and antioxidants (ascorbic acid) have been used to improve the survival of micro-organisms during freeze-drying and subsequent storage (Huang *et al.* 2006; Juárez Tomás *et al.* 2009; Li *et al.* 2011). Among proteinaceous systems, milk and some of its proteins have proved to be very effective lyoprotectants for the LAB group (Otero *et al.* 2007; Bolla *et al.* 2011). Moreover, it has been suggested that freeze-drying and storage conditions are strain-dependent, so they should be evaluated and adjusted for each specific strain (Carvalho *et al.* 2004).

Our research group has studied the beneficial properties of LAB isolated from Argentinean *Lithobates catesbeianus* (bullfrog) hatcheries. Among them, *Lactococcus lactis* CRL 1584, *L. lactis* CRL 1827, *Lactococcus garvieae* CRL 1828 and *Lactobacillus plantarum* CRL 1606 were selected according to their surface properties (related to colonization ability) and antimicrobial activity against red-leg syndrome (RLS)-associated pathogens (*Citrobacter freundii* and *Pseudomonas aeruginosa*), and later evaluated for inclusion into a beneficial product for raniculture to prevent infectious disease outbreaks (Pasteris *et al.* 2009a, b, 2011; Montel Mendoza *et al.* 2012). Then, the aim of this work was to evaluate the efficacy of different lyoprotectants and storage conditions to maintain both cell viability and beneficial properties of selected LAB strains subjected to freeze-drying with a view to the design of beneficial products to be used during the *ex situ* breeding of amphibian species.

Materials and methods

Bacterial strains growth and cell preparations

Autochthonous *Lactococcus lactis* CRL 1584, *L. lactis* CRL 1827, *Lactococcus garvieae* CRL 1828 and *Lactobacillus plantarum* CRL 1606 were identified by phenotypic and genotypic approaches (Montel Mendoza *et al.* 2012) and deposited in the bacterial culture collection of CERELA (Centro de Referencia para Lactobacilos-CONICET, Tucumán, Argentina).

To obtain microbial cells for this study, *L. lactis* CRL 1584 was grown in LAPT (10 g l^{-1} yeast extract; 15 g l^{-1} peptone; 10 g l^{-1} tryptone and 1 ml l^{-1} tween 80; pH 6·8) broth supplemented with 1% (w/v) glucose (LAPTg; Raibaud *et al.* 1963), while *Lact. plantarum* CRL 1606, *L. lactis* CRL 1827 and *L. garvieae* CRL were grown in MRS broth (de Man *et al.* 1969). In all cases, strains were incubated (10 h) until the early stationary growth phase ($O.D._{540\ nm}$ = 1·2) at 37°C in microaerophilic conditions. Later, bacterial cells were harvested by centrifugation (8000 ***g***, 10 min at 4°C), washed twice with sterile distilled water and centrifuged. The pellets were resuspended in the following lyoprotectants (w/v): 10% lactose, 10% sucrose, 10% skim milk, 10% whey protein concentrate (WPC; Lacprodan 35, Arla-Foods Ingredients, Argentina), 5% lactose + 5% sucrose, 5% skim milk + 5% lactose, 5% skim milk + 5% sucrose, 5% WPC + 5% lactose and 5% WPC + 5% sucrose to obtain a concentration of approximately 10^{10} CFU ml^{-1}. Cells were also resuspended in distilled water (control). Finally, samples were frozen at −20°C overnight.

Freeze-drying and storage conditions of beneficial lactic acid bacteria strains

Samples stored at −20°C were incubated for 1 h at −70°C and later freeze-dried at a condenser temperature of −50°C at 110 militorr chamber pressure (Heto-FD4 freeze-dryer, Heto-Holten, Denmark) for 48 h.

Dried cells were later distributed in glycogelatin capsules at 25 ± 2°C, packed in plastic bottles with silica gel to maintain the dry state of the capsules and stored at both refrigerated (4°C) and room (25 ± 2°C) temperatures.

Determination of lactic acid bacteria cell viability

The number of viable cells (CFU) before and after freeze-drying was determined by the serial dilution method. Decimal dilutions were prepared from samples before freezing and plated on LAPTg agar (1·5% w/v). The weight of each lyophilized powder was calculated by determining weight differences between filled and empty capsules. All samples were rehydrated in 1 ml distilled water for 15 min at 25°C with gentle shaking. Then, samples were plated as described above and incubated for 48 h at 37°C in microaerophilia.

Cell viability obtained for each lyoprotectant was expressed as Survival Factor (SF), calculated using the following equation:

$$SF = 1 - (\log CFU_{before} - \log CFU_{after}) / \log CFU_{before}$$

$$CFU_{before} = CFUml^{-1} \times \text{total volume culture (ml) before the freeze} - \text{drying process}$$

$$CFU_{after} = CFUg^{-1} \times \text{total weight of the dry bacterial sample (g)}$$

Cell viability during storage was expressed as Survival Factor during t month of Storage (SFS_t) and calculated as follows:

$$SFS_t = 1 - (\log CFU_0 - \log CFU_t) / \log CFU_0$$

$$CFU_0 = \text{intial } CFUg^{-1} \times \text{total weight of the dry bacterial sample (g)}$$

$$CFU_t = \text{t time } CFUg^{-1} \times \text{weight of dry bacterial sample (g)}$$

Beneficial properties of selected lactic acid bacteria

To assess the maintenance of the beneficial properties of selected LAB strains, the degree of surface hydrophobicity and autoaggregation and the inhibitory activity against pathogens were determined before and after freeze-drying and during storage. Dried LAB cells were rehydrated as described previously and grown in LAPTg or MRS broth for 12 h. The degree of hydrophobicity was determined by the microbial adhesion to hydrocarbons (MATH) assay modified as described by Otero *et al.* (2004) and autoaggregation according to Montel Mendoza *et al.* (2012). Inhibitory activity was determined by the agar-well diffusion assay using *Pseudomonas aeruginosa* (an indigenous RLS-related pathogen) and *Listeria monocytogenes* Scott A (a food-borne bacterium) as indicator strains (Pasteris *et al.* 2009a).

Statistical analysis

All experiments were performed in duplicate. Cell viability data were analysed by an ANOVA-general linear model for analysis of residues to determine the effect of the variables (strain and drying medium) and the interactions of those effects on cell viability during the freeze-drying process.

ANOVA tests were used to quantify the effect of storage conditions (medium, temperature and time) on LAB viability during 18 months of storage. Significant differences between the mean values of each treatment were determined using Fisher's LSD test (95% confidence interval). Statistical analysis of the data was carried out with InfoStat 2008 (student version; National University of Córdoba, Córdoba, Argentina).

Results

Effect of the drying medium on survival to freeze-drying of lactic acid bacteria

The resistance of four selected beneficial LAB strains to the freeze-drying process was determined using nine lyoprotectants. Results showed significant differences in cell viability in all strains during the process ($P < 0·001$; Table 1). Thus, comparing the mean values of cell viability in water, *Lactococcus lactis* CRL 1584 was significantly (Fisher's LSD, $P \leq 0·05$) more susceptible (SF = 0·54 ± 0·0005) than the other LAB strains. *L. lactis* CRL 1827 and *Lactococcus garvieae* CRL 1828 showed intermediate values (SF = 0·73 ± 0·034 and 0·77 ± 0·006, respectively), while *Lactobacillus plantarum* CRL 1606 was the most resistant strain (SF = 0·81 ± 0·013). However, the behaviour of each strain was dependant

Table 1 ANOVA test applied for cell viability (Survival Factor, SF) of lactic acid bacteria freeze-dried in different protective media

Source of variation	Sum of squares	Degrees of freedom	Mean squares	*F*-statistical
Model	0·55	39	0·01	41·17*
Strain (S)	0·02	3	0·01	20·98*
Drying medium (DM)	0·37	9	0·04	120·68*
S × DM	0·16	27	0·01	16·91*
Residuals	0·01	40	0·00034	

*$P < 0·0001$.

on the lyoprotectant medium, as indicated by the significance of the interaction between strain and drying medium (Table 1) when the ANOVA test was applied to the Survival Factor (cell viability) during lyophilization. Therefore, the optimal drying condition for each strain must be evaluated individually. The mean cell viability values of each strain in different drying media are shown in Fig. 1. All LAB tested increased their resistance to lyophilization ($P \leq 0{\cdot}05$) when suspended in any of the lyoprotectants assayed, with the exception of *Lact. plantarum* CRL 1606, which showed a similar SF value ($P \leq 0{\cdot}05$) in water and in WPC.

In the case of *Lact. plantarum* CRL 1606, *L. lactis* CRL 1584 and CRL 1827, there were no differences ($P \leq 0{\cdot}05$) between the SF values obtained in the different sugars used as lyoprotective media when assayed individually or combined with milk, which represent best drying conditions (Fig. 1a,b,d). A similar behaviour was observed in *L. lactis* CRL 1584 with WPC and with its combinations (Fig. 1a).

With respect to *L. garvieae* CRL 1828, optimal values of cell viability were obtained when the LAB strain was suspended in lactose, milk + lactose, milk + sucrose and WPC + sucrose ($P \leq 0{\cdot}05$; Fig. 1c).

Viability of potentially beneficial lactic acid bacteria during storage

To determine the degree of survival of the four LAB strains freeze-dried in different lyoprotectants during their further storage at 4°C and at room temperature (25 ± 2°C), a full three-factor ANOVA test that includes medium, temperature and time was applied to each individual strain. In all cases, the effect of the interaction medium–temperature–time was significant ($P < 0{\cdot}001$; Table 2).

In all strains, SFS at 4°C was significantly higher ($P \leq 0{\cdot}05$) than at 25°C (mean $\Delta_{4°-25°C} = 0{\cdot}33 \pm 0{\cdot}02$). Moreover, ANOVA tests for each strain indicated that the decrease in SFS during the time period studied (18 months) was significant ($P \leq 0{\cdot}05$). However, due to the significance ($P < 0{\cdot}001$) of the interaction between the three factors under consideration, analysis of the SFS values in each individual treatment was performed to determine optimal storage conditions for each LAB strain. At 4°C *L. lactis* CRL 1584 showed mean SFS values higher than 0·80 for all lyoprotectants studied (Table 3). Best cell viability values were obtained in milk, milk + sugars and WPC + sugars, because there were no significant differences between them ($P \leq 0{\cdot}05$). At 25°C, there was a gradual loss of cell viability during storage in

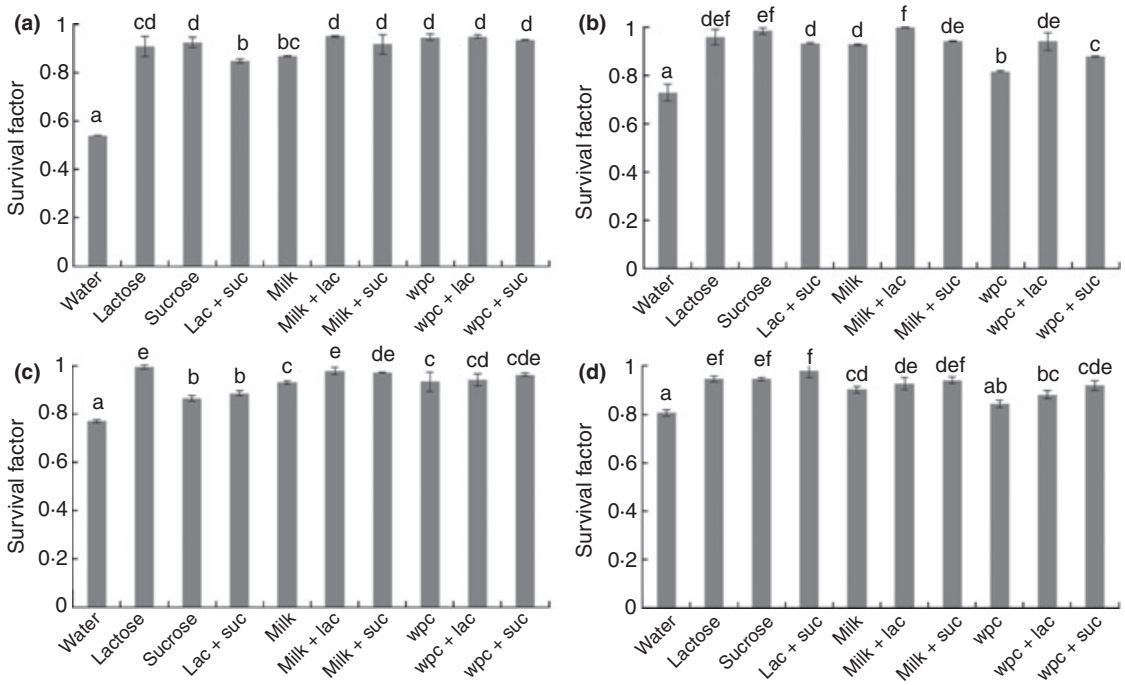

Figure 1 Survival of lactic acid bacteria suspended in different protective media after freeze-drying. *Lactococcus lactis* CRL 1584 (a), *L. lactis* CRL 1827 (b), *Lactococcus garvieae* CRL 1828 (c), *Lactobacillus plantarum* CRL 1606 (d). Survival was calculated as Survival Factor. Different letters indicate significant differences ($P \leq 0{\cdot}05$) in cell viability obtained with different protective media for each strain applying Fisher's LSD test. Lactose plus sucrose (lac + suc), skim milk plus lactose (milk + lac), skim milk plus sucrose (milk + suc), whey protein concentrate (WPC), whey protein concentrate plus lactose (WPC + lac), whey protein concentrate plus sucrose (WPC + suc).

Table 2 ANOVA test applied for the survival (SSF) of freeze-dried lactic acid bacteria during storage at different temperatures

	Lactococcus lactis CRL 1584				*L. lactis* CRL 1827			
Source of variation	SS	df	MS	*F*-stat	SS	df	MS	*F*-stat
Model	17·51	119	0·15	256·80*	15·88	119	0·13	351·65*
Drying medium (DM)	6·72	9	0·75	1303·00*	4·20	9	0·47	1230·36*
Temperature (T)	6·62	1	6·62	11560·39*	6·60	1	6·60	17390·79*
Time (t)	0·72	5	0·14	252·37*	0·90	5	0·18	471·89*
DM × T	2·67	9	0·30	517·70*	2·96	9	0·33	865·36*
DM × t	0·26	45	0·01	10·16*	0·49	45	0·01	28·66*
T × t	0·36	5	0·07	124·10*	0·43	5	0·09	227·10*
DM × T × t	0·16	45	0·0035	6·07*	0·31	45	0·01	17·99*
Residuals	0·07	120	0·00057		0·05	120	0·00038	
	Lactococcus garvieae CRL 1828				*Lactobacillus plantarum* CRL 1606			
	SS	df	MS	*F*-stat	SS	df	MS	*F*-stat
Model	12·49	119	0·10	194·07*	16·05	119	0·13	413·17*
Drying medium (DM)	1·81	9	0·20	372·19*	4·35	9	0·48	1479·80*
Temperature (T)	6·22	1	6·22	11498·72*	7·89	1	7·89	24174·20*
Time (t)	1·31	5	0·26	483·31*	0·61	5	0·12	375·99*
DM × T	1·45	9	0·16	297·61*	2·16	9	0·24	736·14*
DM × t	0·47	45	0·01	19·15*	0·33	45	0·01	22·28*
T × t	0·72	5	0·14	264·83*	0·41	5	0·08	249·48*
DM × T × t	0·52	45	0·01	21·46*	0·30	45	0·01	20·44*
Residuals	0·06	120	0·00054		0·04	120	0·00033	

SS, sum of squares; df, degrees of freedom; MS, mean squares; *F*-stat, *F*-statistical.
*$P < 0·0001$.

Table 3 Survival of freeze-dried lactic acid bacteria in different protective media during storage at 4ºC

	Strains			
Protective media	*Lactococcus lactis* CRL 1584	*L. lactis* CRL 1827	*Lactococcus garvieae* CRL 1828	*Lactobacillus plantarum* CRL 1606
Water	0·81 ± 0·09^{a}	0·87 ± 0·06^{a}	0·91 ± 0·04^{b}	0·82 ± 0·02^{a}
Lactose	0·80 ± 0·01^{a}	0·95 ± 0·02^{d}	0·86 ± 0·02^{a}	0·95 ± 0·02^{d}
Sucrose	0·82 ± 0·01^{b}	0·92 ± 0·02^{bc}	0·94 ± 0·02^{c}	0·91 ± 0·03^{c}
Lac + suc	0·89 ± 0·03^{c}	0·94 ± 0·02^{d}	0·94 ± 0·03^{c}	0·88 ± 0·03^{b}
Milk	0·97 ± 0·01^{e}	0·96 ± 0·02^{e}	0·97 ± 0·01^{ef}	0·91 ± 0·03^{c}
Milk + lac	0·98 ± 0·01^{e}	0·97 ± 0·01^{e}	0·95 ± 0·03 ^{cd}	0·98 ± 0·02^{e}
Milk + suc	0·98 ± 0·01^{e}	0·97 ± 0·02^{e}	0·98 ± 0·01^{f}	0·98 ± 0·01^{e}
WPC	0·91 ± 0·03^{d}	0·92 ± 0·05^{b}	0·97 ± 0·02^{ef}	0·97 ± 0·01^{e}
WPC + lac	0·98 ± 0·01^{e}	0·97 ± 0·02^{e}	0·94 ± 0·06^{c}	0·99 ± 0·01^{e}
WPC + suc	0·98 ± 0·01^{e}	0·94 ± 0·03 ^{cd}	0·96 ± 0·02de	0·98 ± 0·01^{e}

Lac, lactose; Suc, sucrose; WPC, whey protein concentrated.
Values are the mean ± standard deviation of the results obtained with different lyoprotective media during 18 months of storage. Different letters indicate significant differences ($P \leq 0·05$) in cell viability between protective media for each strain according to Fisher's LSD test.

most of the media assayed (Fig. 2a). In water (control) and sucrose, no viable cells were detected after 3 months of storage. Optimal SFS values were obtained in WPC + lactose and milk + lactose (mean SFS = 0·84 and 0·83, respectively; Fig. 2a).

In the case of *L. lactis* CRL 1827, mean SFS at 4°C was higher than 0·87 (Table 3). However, no viable cells were detected after 3 months of storage at 25°C when sucrose was used as a lyoprotectant (Fig. 2b). Similar results were obtained in water after 9 months of storage. Highest *L. lactis* CRL 1827 survival was observed in milk + lactose (mean SFS = 0·84; Fig. 2b).

Best cell viability values were obtained when *L. garvieae* CRL 1828 was lyophilized in milk, milk + sucrose and WPC and stored at 4°C. These values did not differ significantly ($P \leq 0·05$) for 18 months (Table 3). How-

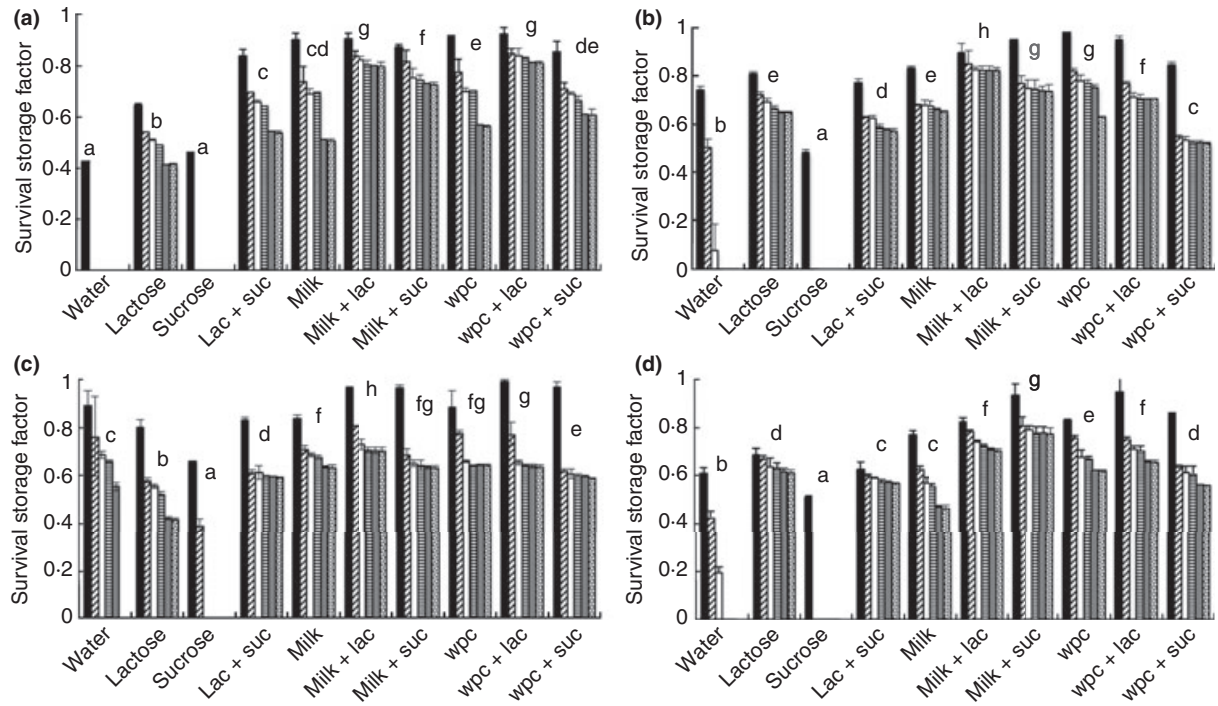

Figure 2 Survival of freeze-dried lactic acid bacteria in different protective media during storage at 25°C. *L. lactis* CRL 1584 (a), *L. lactis* CRL 1827 (b), *L. garvieae* CRL 1828 (c), *Lb. plantarum* CRL 1606 (d). Viability was determined at different storage periods: 1 month (■), 3 months (▨), 6 months (□), 9 months (▤), 12 months (▩) and 18 months (▦). Different letters indicate significant differences (P 0.05) in cell viability between protective media according to Fisher's LSD test. Lactose plus sucrose (lac+suc), skim milk plus lactose (milk+lac), skim milk plus sucrose (milk+suc), whey protein concentrate (wpc), whey protein concentrate plus lactose (wpc+lac), whey protein concentrate plus sucrose (wpc+suc).

ever, a significant decrease ($P \leq 0{\cdot}05$) in cell viability was observed between the first and the third month with all lyoprotectants during storage at 25°C (Fig. 2c). In addition, when using sucrose, a complete loss of cell viability after 6 months was observed. Moreover, SFS values obtained with lactose were significantly lower than with water at 12 months. No viable cells were detected in water at 18 months (Fig. 2c). For this strain, milk + lactose was the best lyoprotectant when stored at 25°C (mean SFS = 0·77; Fig. 2c).

When analysing *Lact. plantarum* CRL 1606, there was no significant loss of viability ($P \leq 0{\cdot}05$) in WPC, WPC + sugars and milk + sugars throughout storage at 4°C (Table 3). However, no viable cells were detected at 25°C after 3 and 9 months in sucrose and water, respectively (Fig. 2d), the optimal lyoprotectant being milk + sucrose (mean SFS = 0·81; Fig. 2d).

Impact of freeze-drying on beneficial properties

The degree of bacterial cell surface hydrophobicity and autoaggregation of the LAB strains and their inhibitory activity against pathogenic bacteria was evaluated. The beneficial properties of the rehydrated strains were not different from the original cells (before freeze-drying), and this behaviour was maintained during 18 months of storage (Table 4).

Discussion

Bullfrog production is an intensive process and the stress produced by crowding increases the risk of epizootics by opportunistic micro-organisms that belong to the normal microbiota. Among bacterial infectious diseases, RLS is the main cause of bullfrog mass mortality and therefore responsible for high economic losses (Densmore and Green 2007).

The inclusion of beneficial LAB strains in veterinary products or formulas to be used in intensive farm cultures requires a methodology to preserve high cell viability and beneficial properties during product elaboration and subsequent storage. In this work, the efficacy of nine lyoprotective media during the freeze-drying process and the later storage conditions (temperature and time) was evaluated. Their effect on bacterial viability and maintenance of the beneficial properties of indigenous *Lactococcus lactis* CRL 1584 and CRL 1827, *Lactococcus garvieae* CRL 1828 and *Lactobacillus plantarum* CRL 1606, benefi-

Table 4 Maintenance of the beneficial properties of freeze-dried lactic acid bacteria after rehydration and growth

Strains	Hydrophobicity (%)*	Autoaggregation (%)*	Inhibitory activity	
			Halo (mm)†	Pathogenic bacteria
Lactococcus lactis CRL 1584	2·2 ± 1·3	11·2 ± 2·3	7 ± 1	*Listeria monocytogenes*
L. lactis CRL 1827	60·4 ± 4·5	6·4 ± 3·4	10 ± 1	*Pseudomonas aeruginosa*
Lactococcus garvieae CRL 1828	50·5 ± 4·7	95·5 ± 3·7	4 ± 1	*Ps. aeruginosa*
Lactobacillus plantarum CRL 1606	12·1 ± 3·5	4·1 ± 2·5	9 ± 1	*Ps. aeruginosa*

*Values are the mean ± standard deviation of the results obtained under different conditions at 18 months of storage.
†Halos represent the inhibitory activity due to organic acids + hydrogen peroxide and/or bacteriocin (for *L. lactis* CRL 1584).

cial and potential probiotic candidates for raniculture (Pasteris *et al.* 2009a,b; Montel Mendoza *et al.* 2012), was studied. The results obtained evidenced that LAB resistance to lyophilization was dependent on both micro-organism and lyoprotective agent used, as demonstrated by the significance of their interaction (Table 1). Similar observations were reported for other LAB strains isolated from a wide variety of sources, such as *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus collinoides*, *Lactobacillus brevis*, *Oenococcus oeni* and *L. lactis* used in different biotechnological processes (Fonseca *et al.* 2004; Zhao and Zhang 2005; Berner and Viernstein 2006; Huang *et al.* 2006; Schoug *et al.* 2006).

Moreover, the selected LAB strains showed different SF values when dried in water that could be explained by the intrinsic resistance of each micro-organism to lyophilization, as shown in two potentially probiotic *Lactobacillus gaserii* strains for cows (Otero *et al.* 2007). Water was used as control not only to evaluate strains resistance to the freeze-drying process but also because the potentially probiotic LAB should be added to the water of boxes containing bullfrogs in different growth stages, especially tadpoles, which are more susceptible to RLS (Mauel *et al.* 2002).

Bacterial cell survival during freeze-drying depends on different factors such as cell density, physiological status of micro-organisms and rehydration conditions (Zhao and Zhang 2005). Thus, to eliminate the effects of these factors and highlight those associated with the drying medium, prelyophilization (initial cell concentration, age of the cultures) and rehydration (medium, temperature, volume and time) conditions were standardized for all strains evaluated in this work.

Different lyoprotectants have been used to decrease cell damage and maintain LAB strains viability during lyophilization. Skim milk, which contains a mixture of macromolecules (lactoalbumine and casein) and a saccharide, is one of the selected lyoprotectants for many LAB strains because it prevents cellular injury by stabilizing the cell membrane constituents and provides a protein-protective coating for the cells (Castro *et al.* 1996; Selmer-Olsen *et al.* 1999; Tan *et al.* 2007). Moreover, skim milk creates a porous structure in the freeze-dried product that makes further rehydration easier (Abadias *et al.* 2001a). Different sugars have been reported to provide good levels of protection for bacteria during freeze-drying. These sugars replace structural water in membranes after dehydration (Clegg 1986; Crowe and Crowe 1986; Chen *et al.* 2006) and prevent unfolding and aggregation of proteins by hydrogen bonding with polar groups of proteins (Hanafusa 1985; Carpenter *et al.* 1990). In this study, the effect of the different lyoprotective agents was statistically significant in all cases and depended on specific strain (Table 1), 10% lactose and 5% skim milk + 5% lactose being the best lyoprotective agents for all LAB strains assayed. Therefore, this combination could be considered an appropriate medium for the drying process applied to micro-organisms to be included in a probiotic product because skim milk is less expensive than lactose. Skim milk could also represent an extra protein source for bullfrog feeding because balanced feed provides 40% of proteins mainly from fish and meat flours and from powder milk. A strain-specific behaviour was also reported for *Lactobacillus* sp. (Juárez Tomás *et al.* 2009) and *L. lactis* (Berner and Viernstein 2006) of different origin, where carbohydrates (lactose or sucrose) + skim milk were the best lyoprotectants for most of the strains studied. Different authors have reported that skim milk is an efficient lyoprotective agent for related micro-organisms (Zamora *et al.* 2006). Specifically, Bolla *et al.* (2011) reported a high recovery of cell viability when *L. lactis* CIDCA 8221 was lyophilized using milk + sucrose as a lyoprotective medium.

It should be noted that more than one of the lyoprotective agents used here was efficient for all LAB strains. However, other aspects (e.g. production costs and maintenance of cell viability during storage) must be taken into account to select best drying conditions. Therefore, some low-cost options were assayed for each strain, which did not show differences in SF values compared with 5% skim milk + 5% lactose such as WPC alone or supplemented with lactose or sucrose (Fig. 1). It is

important to remark that most of the lyoprotectants evaluated (sucrose, milk and WPC) are produced by regional industries.

Previous studies showed that temperature is a critical parameter in microbial survival during storage (Teixeira *et al.* 1995; Gardiner *et al.* 2000; Abadias *et al.* 2001b). Thus, in this work, the stability of beneficial LAB strains during storage at 4 and 25°C was evaluated. As expected, the results showed that SFS values were significantly higher at 4°C. However, the effect of temperature was dependant on both lyoprotective medium and storage period. This behaviour was previously observed for other LAB (Carvalho *et al.* 2004; Zamora *et al.* 2006).

The high SFS values found for the *Lactococcus* species stored at 4°C enables the selection of lower cost lyoprotective media that can be applied to the whole technological process (lyophilization and storage). For example, skim milk, skim milk + sucrose and WPC + sucrose could be appropriate media for the selected LAB. However, when stored at 25°C, different lyoprotective media could be selected for the freeze-drying process, as for example skim milk + lactose and, in the case of *L. lactis* CRL 1584, WPC + sucrose.

With respect to *Lact. plantarum* CRL 1606, the best lyoprotective media for both lyophilization and storage at 4°C were milk + sugars and WPC + sucrose, while skim milk + sucrose was the best medium at 25°C. A previous report indicates that the supplementation of skim milk with other lyoprotective agents can enhance its intrinsic protective effect during storage depending on the compound added (Font de Valdez *et al.* 1983).

The capability of indigenous LAB strains to remain viable and functionally active during long-term storage is an important requirement for beneficial micro-organisms (Sanders and Klaenhammer 2001). In our freeze-drying conditions, the surface properties and the ability of LAB strains to inhibit the growth of *Pseudomonas aeruginosa* (a specific pathogen from raniculture) and *Listeria monocytogenes* Scott A (a food-borne bacterium) remained stable during 18 months of storage. Similar results were observed by Bolla *et al.* (2011), who reported that a mixture of probiotic cell suspensions (*Lact. plantarum*, *Lactobacillus kefir*, *L. lactis* and yeast strains) from kefir grains maintained their antimicrobial activity against *Shigella sonnei*.

Moreover, the beneficial properties of the LAB strains under study were expressed during the 18-month storage at different temperatures after freeze-drying with various lyoprotective media. These results are in agreement with the ones reported by Silva *et al.* (2002) for the production of bacteriocins by lactobacilli when dried in 11% reconstituted skim milk and stored for 3 months, while Juárez Tomás *et al.* (2009) reported that bacteriocin production by an *Lactobacillus salivarius* strain was affected by storage time, depending on the lyoprotectant used.

The results obtained, reported for the first time for selected LAB from bullfrog hatcheries, are of primary interest to obtain dried bacteria for their inclusion in different products, formulas or adjuncts for raniculture to be added at different stages of the biological cycle of *Lithobates catesbeianus*. Thus, further studies are needed to evaluate the maintenance of the probiotic properties of selected LAB strains administered either in water for larval and tadpole specimens or in balanced feed for juvenile and adult animals.

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Conflict of interest

No conflict of interest declared.

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