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Preservation of probiotic strains isolated from kefir by spray drying

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Abstract

Aims: This work aims to investigate the survival of *Lactobacillus kefir* CIDCA 8348, *Lactobacillus plantarum* CIDCA 83114 and *Saccharomyces lipolytica* CIDCA 812, all isolated from kefir, during spray drying and subsequent storage.
Methods and Results: Micro-organisms were grown in De Man, Rogosa, Sharpe (MRS) or yeast medium (YM) medium and harvested in the stationary phase of growth. The thermotolerance in skim milk (*D* and *Z* values), the survival of spray drying at different outlet air temperatures and subsequent storage in different conditions during 150 days were studied. The resistance to the heat treatments was higher in *Lact. plantarum* compared to *Lact. kefir* and *S. lipolytica*. The three micro-organisms studied varied considerably in their ability to survive to spray drying processes. *Lactobacillus plantarum* showed the highest survival rate for all the tested outlet air temperatures and also to the further storage in the dried state. The survival rates of *Lact. kefir* and *S. lipolytica* through drying and subsequent storage in the dried state decreased when the drying outlet air temperatures increased.
Conclusions: Spray drying is a suitable method to preserve micro-organisms isolated from kefir grains. A high proportion of cells were still viable after 80 days of storage at refrigerated temperatures
Significance and Impact of Study: It is the first report about spray-dried probiotic strains isolated from kefir grain and contributes to the knowledge about these micro-organisms for their future application in novel dehydrated products.

Introduction

The preservation of micro-organisms by different drying methodologies has been used for decades. Among these methodologies, spray drying (SD) has the advantage that can be used to produce large amounts of dairy ingredients in a relatively inexpensive way; the spray-dried powders can be transported at a low cost and can be stored in a stable form for prolonged periods. However, previous studies have shown that during and after spray drying and also through subsequent storage in the dried state, cells can suffer from a variety of stresses including heat, osmotic and oxidative stress that result in the loss of cellular viability and activity, especially when they are stored at room temperature (Teixeira *et al.* 1995a,b; Gardiner *et al.* 2000; Silva *et al.* 2002, 2005). The use of SD has been investigated as a convenient method for producing large quantities of some probiotic bacteria (Gardiner *et al.* 2000; Desmond *et al.* 2001; Silva *et al.* 2002; Corcoran *et al.* 2004) and more recently, yeasts (Abadias *et al.* 2005). From a functional ingredient perspective, the generation of dehydrated probiotic bacteria is a challenge in terms of retaining viability and functionality during powder manufacture and storage (Meng *et al.* 2008).

Kefir is the product of fermentation of milk by micro-organisms confined in a matrix of discrete 'kefir

grains'. Several health promoting properties are associated with kefir consumption (Lopitz-Otsoa *et al.* 2006) such as stimulation of the immune system (Vinderola *et al.* 2005), inhibition of tumour growth (Liu *et al.* 2002) and inhibition of pathogenic micro-organisms (Garrote *et al.* 2000; Kakisu *et al.* 2007) among others. This beneficial effect is ascribed to the presence of a complex microbiota formed by yeasts and lactic acid bacteria (LAB) (Garrote *et al.* 2001; Santos *et al.* 2003). In this sense, among CIDCA collection of micro-organisms isolated from kefir, some strains showed very promising probiotic properties: *Lact. kefir* CIDCA 8348 adheres strongly to Caco-2 cells and antagonizes *Salmonella* invasion (Golowczyc *et al.* 2007), *Lact. plantarum* CIDCA 83 114 inhibits *Salmonella* and *Escherichia coli in vitro* (Golowczyc *et al.* 2008) and *S. lipolytica* CIDCA 812 adheres to Caco-2 cells and showed high co-aggregation to *E. coli* (M.A. Golowczyc and G. Garrote, unpublished data).

The conservation of these strains in a dried state seems to be an attractive way to preserve its viability for their inclusion into a probiotic product. However, little, if any, is known about the effect of SD on these micro-organisms, because no previous studies about survival of *Lact. kefir* or *S. lipolytica* to SD were published before. In the present work, the capacity of these micro-organisms to survive the SD process and the subsequent conditions of storage was evaluated.

Materials and methods

Strains and growth conditions

Strains were previously isolated and identified from kefir grains of three different origins (Garrote *et al.* 2001). Working cultures were cultivated in De Man, Rogosa, Sharpe broth (MRS; Difco, Detroit, MI, USA) or Yeast Medium (YM; Difco, Detroit, MI, USA) at 30°C for 24 h under static conditions. To prepare the inoculum, strains were inoculated in MRS or YM broth and incubated at 30°C for 24 h, and these cultures were then used to inoculate another MRS or YM broth (1% v/v).

Cells were harvested by centrifugation at 7000 ***g*** at 4°C for 10 min and washed twice with sterile Ringer's solution.

Heat treatment

The wet cell pellets were resuspended to the original volume in reconstituted skim milk powder (11% w/v; Oxoid, Hampshire, UK). Aliquots (1 ml) were transferred to 49 ml of skim milk previously equilibrated at the heating temperature, namely, 45, 50, 55, 60 and 65°C. At regular intervals, samples were taken and immediately diluted (10-fold) in sterile Ringer's solution at room temperature. Survivors were enumerated on MRS or YM agar by the drop count technique (Miles and Misra 1938). The D values (time required to reduce the viable count 1 log cycle at a constant temperature) and Z values (temperature increase required to reduce the D value by 1 log) were calculated.

Spray drying and storage

The cellular pellets obtained as described earlier were resuspended to the original volume in reconstituted skim milk powder (11% w/v) at room temperature. Each sample was spray dried in a pilot scale apparatus (Niro Atomizer, Copenhagen, Denmark). Spray drying conditions were: outlet air temperature 70, 75, 80 and 85°C, inlet air temperature 180°C and atomizing air pressure 5 bar. Powder was collected in a single cyclone separator. Samples of the spray-dried powder were placed in sealed glass bottles (ambient atmospheric condition) and vacuum packed in polyethylene bags in a Multivac-Gastrovac (A300/41/42; Multivac Sepp Haggenmüller KG, Germany) (vacuum condition) and stored at 6 and 20°C.

Enumeration of survivors

To calculate the survivors after spray drying and during storage in the dried state, samples were rehydrated to the original volume with sterile Ringer's solution. Samples were homogenized for 1 min in a vortex mixer and maintained at room temperature for 10 min and then, serially diluted. Survivors were enumerated on MRS or YM agar by the drop count technique.

Water activity measurements

The water activity (a_w) in the spray-dried powders was measured at room temperature in duplicate using an Aqualab Model Series 3 TE instrument (Decagon Devices, Inc.).

Statistical analysis

Results were expressed as means ± standard deviation of at least two independent experiments. For statistical comparisons, analysis of variance (ANOVA) of viable counts before and after spray drying and at regular time intervals during storage was performed using the statistical program INFOSTAT Software (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). Statistical differences were considered at the 0·05 level of significance.

Results

Thermotolerance

Table 1 lists the D and Z values of *Lact. plantarum* 83114, *Lact. kefir* 8348 and *S. lipolytica* 812 in skim milk. The D_{55}, D_{60} and D_{65} values for *Lact. kefir* CIDCA 8348 were significantly higher ($P < 0{\cdot}05$) compared with *Lact. Plantarum*; however, the Z value was higher for *Lact. plantarum*. *Saccharomyces lipolytica* showed D and Z values lower than each parameter for the *Lactobacillus* isolates.

Resistance during spray drying

Survival of *Lact. plantarum* 83114, *Lact. kefir* 8348 and *S. lipolytica* 812 during spray drying at different outlet air temperatures is shown in Table 2. The ability to survive the spray drying process varied considerably among the three investigated micro-organisms. For each outlet temperature studied, *Lact. plantarum* 83114 showed a reduction of 1 log CFU ml^{-1}, and no significant differences ($P < 0{\cdot}05$) were observed in the number of viable cells obtained among the outlet air temperature after spray drying. *Lact. kefir* 8348 and *S. lipolytica* 812, however, showed a greater reduction in viability compared with the *Lact. Plantarum*, and survival decreased with an increase in the outlet air temperature.

As was expected, the water activity (a_w) of spray-dried powders decreased with increasing outlet air temperature (Table 2).

Survival during storage

The survival of spray-dried *Lact. plantarum* 83114, *Lact. kefir* 8348 and *S. lipolytica* 812 during 150 days of storage, when the drying process was performed at different outlet temperatures (70, 75, 80 and 85°C) and the powders stored in atmospheric conditions at 20 and 6°C, is shown in Fig. 1. Survival of *Lact. plantarum* 83114 during the storage period (Fig. 1a) was not dependent on the outlet air temperatures studied during spray drying. During storage at 6°C, there was no significant decrease

Table 1 D_T (min) and Z values (°C) of *Lactobacillus plantarum* CIDCA 83114, *Lactobacillus kefir* CIDCA 8348 and *Saccharomyces lipolytica* CIDCA 812 heated in skim milk.

	D_{45}	D_{50}	D_{55}	D_{60}	D_{65}	Z values
Lact. plantarum CIDCA 83 114	nd	nd	7·50 ± 0·84	3·00 ± 0·14	0·70 ± 0·09	9·4
Lact. kefir CIDCA 8348	nd	nd	21·4 ± 0·71	3·57 ± 0·18	1·30 ± 0·13	7·8
S. lipolytica CIDCA 812	60·20 ± 1·31	14·2 ± 0·82	8·29 ± 0·14	0·12 ± 0·04	nd	6·0

nd, not determined.
Values are the mean ± SD of three independent assays.

Table 2 Survival and water activity (a_w) of *Lactobacillus plantarum* CIDCA 83114, *Lactobacillus kefir* CIDCA 8348 and *Saccharomyces lipolytica* CIDCA 812 after spray drying at different air outlet temperatures

	Log CFU ml^{-1}				
Strain	Before spray drying	Outlet temperature (°C)	After spray drying*	Log reduction	a_w
Lact. plantarum CIDCA 83 114	11·0 ± 0·25†	70	10·0 ± 0·17	1·0	0·440
		75	9·9 ± 0·17	1·1	0·361
		80	9·9 ± 0·19	1·1	0·290
		85	9·9 ± 0·25	1·1	0·236
Lact. kefir CIDCA 8348	10·7 ± 0·01	70	9·0 ± 0·13	1·7	0·467
		75	8·9 ± 0·05	1·8	0·397
		80	8·5 ± 0·15	2·2	0·293
		85	7·9 ± 0·03	2·8	0·266
S. lipolytica CIDCA 812	9·28 ± 0·02	70	7·0 ± 0·07	2·2	0·380
		75	6·0 ± 0·00	3·2	0·295
		80	6·3 ± 0·19	3·0	0·254
		85	5·1 ± 0·03	4·2	0·224

*The values in the column 'After spray-drying' are those obtained after rehydration of the powder back to its original volume.
†Values are the mean ± SD of two independent assays. The Log of CFU for *Lact. plantarum* was recorded in De Man, Rogosa, Sharpe (MRS) (Difco, Detroit, MI, USA).

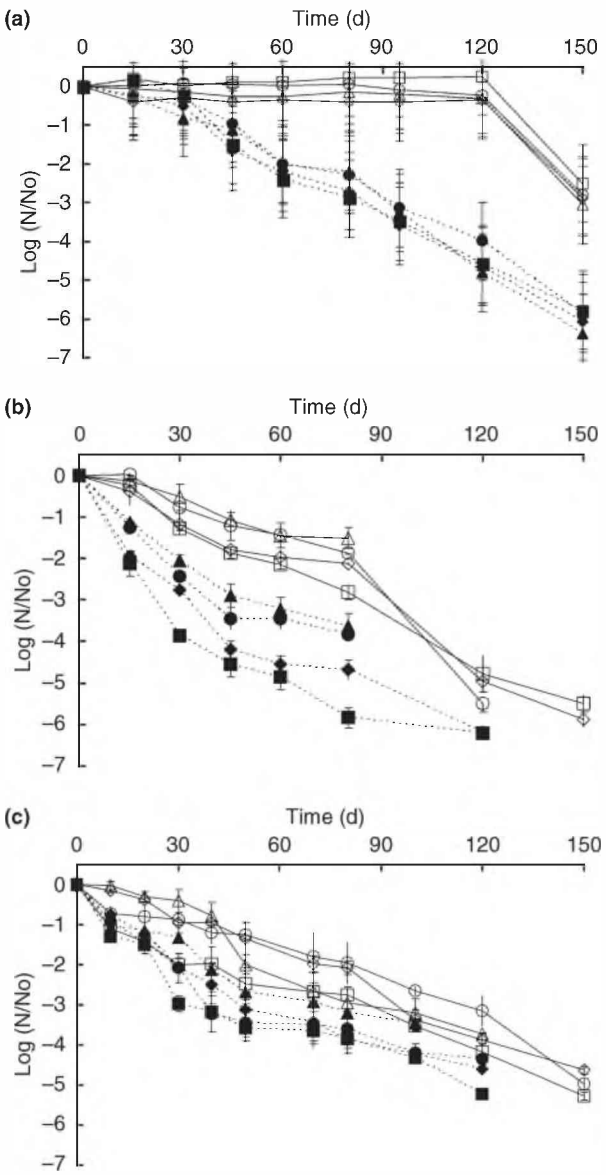

Figure 1 Survival of *Lactobacillus plantarum* CIDCA 83114 (a), *Lactobacillus kefir* CIDCA 8348 (b) and *Saccharomyces lipolytica* CIDCA 812 (cultivated in yeast medium) (c), in spray-dried skim milk obtained at different outlet temperatures during storage at 20°C (dash line, closed symbols) and 6°C (solid line, open symbols) in atmospheric conditions: (■) and (□) outlet temperature 70°C; (◆) and (◇) outlet temperature 75°C; (●) and (○) outlet temperature 80°C; (▲) and (△) outlet temperature 85°C. Viabilities were evaluated up to 150 days. Where no data is plotted, the number of viable cells was less than 10^2 CFU ml^{-1}.

in survival during 120 days, and after this period, the viability declined 3 log in 30 days, while at 20°C the viability decline was more dramatic. On the other hand, *Lact. kefir* 8348 showed a greater and constant decrease in viability during storage at both temperatures (Fig. 1b). Spray-dried samples obtained at 75, 80 and 85°C outlet temperatures and stored at 6°C showed high survival during the first 80 d but after this time, a significant decrease was observed. However, the survival of the micro-organism stored during 120 days at 20°C and 150 days at 6°C was higher when the outlet air drying temperature was 70 and 75°C. The spray-dried *S. lipolytica* CIDCA 812 obtained at outlet air temperatures of 75 and 80°C and stored at 6°C showed the highest survival during all the storage period (Fig. 1c). Results show that the number of viable cells after 80 days was 10^{11} CFU g^{-1} for *Lact. plantarum*, 10^7–10^8 CFU g^{-1} for *Lact. kefir* and 10^5–10^6 CFU g^{-1} for *S. lipolytica*.

No significant differences were observed between the survival rates of the three micro-organisms at 6°C in the storage condition (atmospheric air or vacuum conditions) during the whole storage period (data not shown).

Discussion

Spray drying has been investigated as a method of preservation of LAB to be used as starter and probiotic cultures (Teixeira *et al.* 1995a,b; Desmond *et al.* 2001; Corcoran *et al.* 2004). Cellular damage because of simultaneous thermal and dehydration stresses is a major disadvantage of the spray drying process (Gardiner *et al.* 2000; Desmond *et al.* 2001; Silva *et al.* 2005). In this work, three probiotic strains isolated from kefir grains were dried to investigate spray drying as a method for their preservation.

Spray drying can produce stable powders of certain bacteria and yeast species; however, with the high temperatures involved in this process, the species require a certain level of thermotolerance. In addition, the degree of survival or destruction of bacteria during spray drying depends on the temperature–time combinations used (Teixeira *et al.* 1995a,b). Although it is not possible to directly extrapolate from the death kinetics of a culture in solution toward performance during spray drying, we studied the thermotolerance of the strains in skim milk, the spray drying medium, as a first step in the characterization. The present results show a correlation between Z values and resistance to the drying process for the three strains studied.

One of the most important prerequisites for the use of probiotics is that they survive throughout the production process and storage time until the end of shelf life. In this sense, we studied the survival of three micro-organisms isolated from kefir during the spray drying process and subsequent storage in different condition. Results of the present study confirm previous findings whereby the outlet air temperature or the temperature at which the product leaves the drying chamber is the major drying parameter affecting viability of spray-dried cultures (Teixeira *et al.* 1995a; Gardiner *et al.* 2000; Abadias *et al.*

2005). The lowest outlet air temperature studied (70°C) was associated with the highest survival rate for the three micro-organisms during drying and subsequent storage. *Lactobacillus plantarum* showed the highest survival at all outlet air temperatures investigated, demonstrating that it is a strain highly resistant to drying/temperature. However, results obtained with *Lact. kefir* and *S. lipolytica* highlighted the importance of the optimization of drying process parameters for each micro-organism because these micro-organisms showed a decrease in survival with increased outlet air temperatures.

Water activity is an important factor affecting the stability of dry and dehydrated products during storage. In this work, not surprisingly, the a_w of the probiotic powders decreased with increasing outlet temperature, an observation previously reported for probiotic powders (Teixeira *et al.* 1995b; Desmond *et al.* 2002). Some researches consider that dehydrated micro-organisms are compatible with a long period of storage when the a_w is lower than 0·20 (Mille *et al.* 2005). In the present work, the lowest a_w values obtained were 0·28. Therefore, survival of these cultures during storage could be improved under controlled a_w (Teixeira *et al.* 1995b).

The storage conditions (atmosphere, temperature, etc.) can greatly affect the stability of dehydrated products. In accord with other reports (Teixeira *et al.* 1995b; Gardiner *et al.* 2000; Desmond *et al.* 2001), our results showed that the storage temperature was a critical parameter affecting the survival of micro-organisms, and for all the strains studied, survival rates were higher at the lower storage temperature. During storage, membrane lipid oxidation seems to be a detrimental factor for cellular survival (Teixeira *et al.* 1996). Therefore we studied the survival of spray-dried cells stored in atmospheric and vacuum conditions and, unexpectedly, no significant differences were observed. This result might be because the relative reaction rate for oxidation is minimal at a_w about 0·3, the a_w values obtained in our dried samples.

The number of viable micro-organisms in powders decreased gradually during storage, but with a high number of viable micro-organisms initially of the process, the final number of *Lactobacillus* in the spray-dried powder remains still high. However, from an economical standpoint, survival level below 10% might be considered unacceptable.

Of the organisms investigated, *Saccharomyces lypolitica* was the most damaged during the drying process. Similar results were observed by Abadias *et al.* (2005) that studied the survival of *Candida sake* to spray drying and concluded that it was not a good dehydration method. Some authors have observed that some solutes have a protecting role on the cytoplasmic membrane and keep the cell wall intact (Lievense and Van't Riet 1993; Carvalho *et al.* 2004). Also, cells exposure to predrying stress treatments, i.e. sublethal temperatures, are described to be more resistant to the subsequent lethal treatments i.e. drying (Carvalho *et al.* 2004; Silva *et al.* 2005). Growing and drying conditions, i.e. composition of the growth media, the addition of cryoprotectants to the growth or to the drying media, the temperature and the pH of growth and the growth phase, are also to be considered as variables that influence the viability of LAB cells to drying and subsequent storage in the dried state (Teixeira *et al.* 1995b; Carvalho *et al.* 2004; Silva *et al.* 2005). These reports suggested that the addition of processing aids may improve the viability during spray drying of micro-organisms, although there are not sufficient studies about dehydration of yeast.

In conclusion, in this study we found that three micro-organisms isolated from kefir grains, which were selected on the basis of their probiotic properties, varied considerably in their ability to survive during the spray drying process; nevertheless, a high number of viable cells were obtained after 80 days storage at refrigerated temperatures. To our knowledge, although the behaviour of many spray-dried lactobacilli strains have been studied, this is the first report about spray-dried probiotic strains isolated from kefir grain. In addition, this study contributes to the knowledge about the *Saccharomyces lypolitica* behaviour during spray drying process and storage. However, more work is still needed to obtain a stable product for the application of these strains at the industrial level.

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