Journal of Dairy Research (2011) **78** 15–22. © *Proprietors of Journal of Dairy Research 2010*
doi:10.1017/S0022029910000610

Effect of freeze-drying on viability and *in vitro* probiotic properties of a mixture of lactic acid bacteria and yeasts isolated from kefir

Patricia A Bolla[1,2], María de los Angeles Serradell[2]*, Patricio J de Urraza[2] and Graciela L De Antoni[1,2]

[1] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CCT CONICET La Plata, 47 y 116 (1900)
[2] Laboratorio de Microbiología, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina

Received 20 January 2010; accepted for publication 30 July 2010; first published online 8 September 2010

The effect of freeze-drying on viability and probiotic properties of a microbial mixture containing selected bacterial and yeast strains isolated from kefir grains (*Lactobacillus kefir*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*) was studied. The microorganisms were selected according to their potentially probiotic properties *in vitro* already reported. Two types of formulations were performed, a microbial mixture (MM) suspended in milk and a milk product fermented with MM (FMM). To test the effect of storage on viability of microorganisms, MM and FMM were freeze-dried and maintained at 4 °C for six months. After 180 days of storage at 4 °C, freeze-dried MM showed better survival rates for each strain than freeze-dried FMM. The addition of sugars (trehalose or sucrose) did not improve the survival rates of any of the microorganisms after freeze-drying. Freeze-drying did not affect the capacity of MM to inhibit growth of *Shigella sonnei in vitro*, since the co-incubation of this pathogen with freeze-dried MM produced a decrease of 2 log in *Shigella* viability. The safety of freeze-dried MM was tested in mice and non-translocation of microorganisms to liver or spleen was observed in BALB/c mice feed *ad libitum* during 7 or 20 days. To our knowledge, this is the first report about the effect of freeze-drying on viability, *in vitro* probiotic properties and microbial translocation of a mixture containing different strains of both bacteria and yeasts isolated from kefir.

Keywords: freeze-drying, kefir, LAB, yeast, probiotics, microbial translocation.

Kefir is a milk fermented product, originated in Caucasian mountains many centuries ago, that has become popular in many European countries and Canada and its consumption has been associated with several health-promoting properties such as antimicrobial, antitumoral, immunological and hypocholesterolemic effects (Farnworth, 2005). The milk is fermented with a mixed microflora confined to a matrix of discrete ''kefir grains'' in which yeasts and lactic acid bacteria (lactobacilli, lactococci, leuconostoc) coexist in a symbiotic association and are responsible for the acid-alcoholic fermentation (Angulo et al. 1993; Garrote et al. 2001). Because kefir composition is very complex, it is difficult to maintain a constant quality of fermented milk, and the shelf life of the product is limited (Zabala, 2004). Taking into account the health promoting properties attributed to kefir and the difficulty of industrial production and storage, it could promising to use a mixture of microorganisms isolated from kefir for the formulation of a new product.

Our workgroup have isolated and characterized more than 100 bacterial and yeast strains from kefir grains which showed differences in surface and probiotic properties such as bile salts and low pH resistance, adhesion to Caco-2 cells and inhibitory power against intestinal pathogens *in vitro* (Garrote et al. 2004; Golowczyc et al. 2007, 2008).

Among the microorganisms studied in the present work the yeasts *Saccharomyces cerevisiae* CIDCA 8112 and *Kluyveromyces marxianus* CIDCA 8154 showed a very high resistance to bile salts and were capable to down regulate pro-inflammatory response induced in intestinal epithelial cells *in vitro* and *in vivo* (Romanin et al. 2010). *Lactobacillus plantarum* CIDCA 83114 showed a high

*For correspondence; e-mail: maserr@biol.unlp.edu.ar

https://doi.org/10.1017/S0022029910000610 Published online by Cambridge University Press

inhibitory power against *Salmonella spp.* and *Shigella spp. in vitro* (Golowczyc et al. 2008) and prevented detachment of eukaryotic cells and minimized both F-actin rearrangements and morphological alterations mediated by enterohemorrhagic *Escherichia coli* O157:H7 on Hep-2 cells (Hugo et al. 2008). *Lactobacillus kefir* CIDCA 8348 through its S-layer inhibited the invasion of *Salmonella enterica* into Caco-2 cells (Golowczyc et al. 2007). *Lactococcus lactis* CIDCA 8221 has the ability to grow and acidify the milk faster than the other microorganisms already mentioned (Garrote 2000). In addition, other authors have demonstrated the ability of strains of *Lc. lactis* to inhibit pathogens by production of bacteriocins (Bravo et al. 2009).

The possibility to optimize the conditions to obtain a dehydrated microbial product with capacity to recover viability after rehydration would be the first step to increase the shelf life of a product and avoid the undesired changes in composition and properties that occur during preservation. Freeze-drying is one of the most commonly used procedures for preservation of microbial culture collections and concentrated starter cultures. However, during freeze-drying cells experience adverse environmental conditions, such as low temperature and low water activity, which reduces their viability. In this sense, the use of cryoprotectants has been demonstrated to improve the survival rates of different microorganisms and their effect seems to be one of the most important parameters during freezing and freeze-drying procedures (De Antoni et al. 1991; de Urraza & De Antoni, 1997; Carvalho et al. 2004; Blanquet et al. 2005). Different authors have demonstrated that carbohydrates and proteinaceous substances exert a protective effect on survival of bacterial or yeast strains during freeze-drying and storage. In particular, various sugars were found to be protective for different lactobacilli (Leslie et al. 1995; Linders et al. 1997; Selmer-Olsen et al. 1999; Carvalho et al. 2002, 2003, 2004). Among proteinaceous systems, milk or its proteins were commonly employed as cryoprotectant (Abraham et al. 1990; Abadias et al. 2001; Blanquet et al. 2005; Otero et al. 2007).

On the other hand, since the safety of probiotic is usually an issue of discussion in the health area, it is necesary to investigate not only the benefits but also the potential side effects of administration of probiotic microorganisms. A good safety indicator is the absence of microbial translocation through the epithelial barrier and the passage into sterile body sites. Although a degree of translocation in healthy hosts has been reported (Pavan et al. 2003), when the integrity of the intestinal mucosa is interrupted or when the immune system is unable to control the translocated microorganisms they could produce a local infection or reach the bloodstream causing sepsis (Berg, 1999).

The aim of this work was the development of a microbial mixture constituted of microorganisms isolated from kefir, the evaluation of freeze-drying for the preservation of the product in cryoprotected conditions, and the maintenance of the *in vitro* probiotic properties and the *in vivo* safety of the freeze-dried mixture. The results of this work will be essential in order to develop a freeze-dried microbial concentrate which could be used as a probiotic supplement of the diet or industrial starter culture.

Material and Methods

Microorganisms and culture conditions

Pure cultures used in this study comprised *Lactococcus lactis* CIDCA 8221, *Lactobacillus plantarum* CIDCA 83114, *Lactobacillus kefir* CIDCA 8348, *Kluyveromyces marxianus* CIDCA 8154 and *Saccharomyces cerevisiae* CIDCA 8112. These strains were previously isolated from kefir grains and have been identified and characterized by Garrote et al. (2001) and Delfederico et al. (2006). The original reference cultures were maintained in milk at −80 °C. Both lactobacilli and yeasts were propagated in MRS-broth (DIFCO, Detroit, USA) for 48 h at 30 °C. *Lactococcus lactis* was grown in 1.1.1 growth media (1% w/v of tryptone – Difco, Detroit, USA – 1% w/v of yeast extract – Biokard Diagnostic, Beauvais, France – and 1% w/v of lactose – Mann Research Laboratories, NY) (Abraham et al. 1990) for 24 h at 30 °C. *Shigella sonnei* (isolate n° 67212 from Hospital de Niños Sor Maria Ludovica de La Plata) was cultured in Tryptone soy broth (TSB) (Biokard Diagnostic, Beauvais, France) at 37 °C with shaking for 18 h.

Preparation of products for freeze-drying procedure

Each microorganism was cultured individually as described above. Microbial cultures were harvested by centrifugation at 10 000 ***g*** for 15 min and washed three times and resuspended in sterile phosphate buffered saline (PBS) pH = 7·2 (microbial suspensions). To obtain the microbial mixture (MM), a combination of 1 ml of each microbial suspension was centrifuged at 10 000 ***g*** for 15 min and resuspended in 1 ml sterile UHT milk or UHT milk with added trehalose (Mann Research Laboratories Division of Becton Dickinson & Co) (300 mM) or sucrose (Anedra, Argentina) (300 mM) or PBS. The final concentration of bacteria and yeasts in MM was 10^9 CFU/ml and 10^6 CFU/ml respectively. To obtain the milk product fermented with MM (FMM), 1 ml MM was added to 9 ml UHT milk and finally incubated at 30 °C during 48 h until pH = 4·90. The final concentration of microorganisms in the FMM was 10^9 bacterial CFU/ml and 10^6 yeast CFU/ml.

Freeze-drying and storage conditions

The ampoules with 200 µl of MM or FMM with or without cryoprotector were frozen at −80 °C for 8 h and freeze-dried for 24 h in air using a HETO 7D4 Lab Equipment.

https://doi.org/10.1017/S0022029910000610 Published online by Cambridge University Press

Dried cells were stored at 4 °C during a period ranged for 1 to 180 days. Immediately after freeze-drying and at different time intervals of storage, samples taken at random were rehydrated to the original volume with sterile deionised water and plated for enumeration of viable microorganisms.

Enumeration of microorganisms

The number of viable cells was determined as colony forming units per ml (CFU/ml). Serial dilutions of samples (from 10^{-1} to 10^{-9}) were prepared in 0·1% tryptone and plated on MRS agar, 1.1.1 agar and YGC agar (Biokard Diagnostic, Beauvais, France). Plates were incubated at 30 °C for 24–48 h in aerobic environment. The survival rate was calculated as CFU/ml after freeze-drying divided by CFU/ml before freeze-drying.

Plate count of *Shigella sonnei* was performed in Nutrient agar (Biokard Diagnostic, Beauvais, France).

Shigella inhibition assay

Overnight cultures of *Shigella sonnei* with approximately 10^8 CFU/ml were centrifuged (3000 ***g*** for 15 min) and the pellets were suspended at equal volume in TSB. Ten ml of either freeze-dried or fresh MM resuspended in TSB were added to *Shigella* culture at 1 : 1 ratio and incubated at 37 °C with shaking at 180 rpm. Enumeration of *Shigella* was performed at different time intervals (3, 8, 24, 48 h) as indicated. Cultures of *Shigella* in TSB and TSB with 30% v/v of UHT milk added were performed as controls.

Translocation assay

Seven-week-old BALB/c females were fed daily *ad libitum* with a 1/100 dilution of freeze-dried MM in sterile water (approximately 10^8 bacteria per ml and 10^6 yeasts per ml) for 7 or 20 consecutive days. Control group were offered milk (diluted 1/100 in PBS). Each experimental group consisted in 5 mice housed in cages kept in controlled environment of temperature and with a 12 h dark/light cycle. All animals received, simultaneously a sterile conventional balanced diet.

At the end of each feeding period, mice were killed by CO_2 chamber and liver, spleen and a 3-cm portion of small intestine were removed aseptically. Liver and spleen were homogenized in 0·1% sterile tryptone water (0·1 g per ml) and 100 µl of each organ homogenate was plated on VRBG Agar (Biokard Diagnostic, Beauvais, France) for enterobacteria, 1.1.1 Agar for lactococci, MRS agar for lactobacilli, YGC agar for yeasts and Nutritive Agar (AN). Plates were incubated under aerobic conditions for 24 h at 37 °C for VRBG and AN, and for 24–48 h at 30 °C for lactobacilli and lactococci. At the end of the incubation period, plates were examined. Translocation was considered to have occurred when colonies were observed on agar plates, since liver and spleen are organs normally devoided of microorganisms. Additionally, intestinal content was washed with 1 ml sterile PBS, and serial dilutions of samples were plated on the same culture media mentioned above.

Statistical analysis

Differences in survival rates of microorganisms were statistically tested by using Student t-test to determine any significant difference.

Results

Effect of cryoprotectants on freeze-drying resistance

The protective effects of the milk, and milk supplemented with 300 mM-trehalose or 300 mM-sucrose on the survival of each of all strain mentioned in Material and Methods were studied. The survival rates obtained with UHT milk as cryoprotectant were significantly higher ($P<0·05$) for all the microorganisms compared with those observed with PBS (Fig. 1). Addition of trehalose or sucrose did not increase the survival rates of lactobacilli (*Lb. kefir* CIDCA 8348 and *Lb. plantarum* CIDCA 83114), *Lc. lactis* CIDCA 8221 and *Sac. cerevisiae* CIDCA 8112, whereas survival rate of *K. marxianus* CIDCA 8154 decreased in presence of 300 mM-sucrose ($P<0·05$; Fig. 1).

All these results taken together, indicated that the addition of sugars to UHT milk did not improve the cryoprotective action of the milk. Therefore, UHT milk alone was used as cryoprotectant for all subsequent experiments.

Effect of storage and type of product on freeze-drying resistance

In order to study the capacity of each component of the freeze-dried MM to survive during storage after freeze-drying, the survival rates at different times of storage at 4 °C were analysed. When freeze-drying was performed with the MM all microorganisms suffered a reduction in survival rates between 0·5 and 1 log after 30 d storage (Fig. 2A). However, in the freeze-dried FMM there were a noticeable reduction of survival rates for all microorganisms (between 1·5 and 4·5 log) after the same time of storage (Fig. 2B).

Between 30 and 180 d storage, the viability of each microorganism in the MM decreased almost 2 log (Fig. 2A). However, during the same period of storage, the survival of each microorganism in the FMM showed a different behaviour. A significant reduction in survival rates of *Lb. plantarum* CIDCA 83114 and yeasts (*Sac. cerevisiae* CIDCA 8112 and *K. marxianus* CIDCA 8154) decreased almost 6 log after 180 d storage at 4 °C (Fig. 2B).

Since freeze-dried MM without previous fermentation showed a better stability than the FMM, we selected MM to perform all the subsequent experiments.

https://doi.org/10.1017/S0022029910000610 Published online by Cambridge University Press

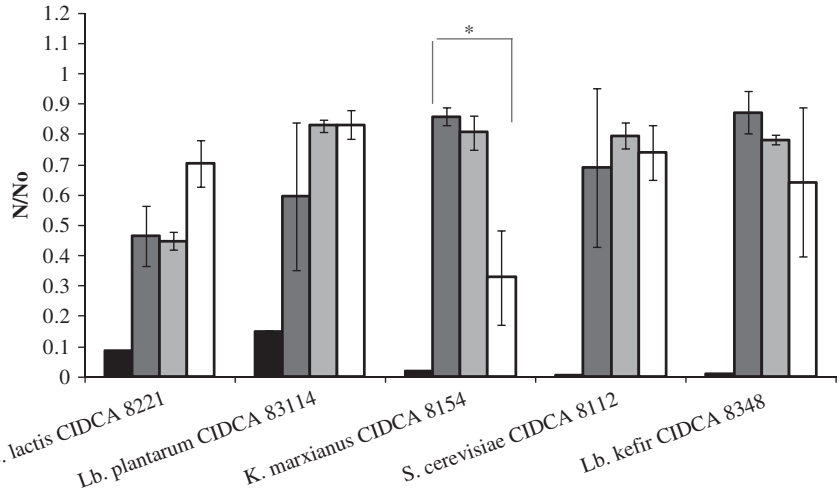

Fig. 1. Survival (N/No) of *Lc. lactis* CIDCA 8221, *Lb. kefir* CIDCA 8348, *Lb. plantarum* CIDCA 83114, *S. cerevisiae* CIDCA 8112 and *K. marxianus* CIDCA 8154 freeze-dried in PBS (■), UHT milk (▩), UHT milk supplemented with 300 mM trehalose (▩) or 300 mM sucrose (□) after freeze-drying procedure. No: counts of viable microorganisms (CFU/ml) before freeze-drying. N: counts of viable microorganisms (CFU/ml) after freeze-drying. *$P<0{\cdot}05$ vs UHT milk. In all cases, survival with the cryoprotectant is significantly different to survival in PBS buffer ($P<0{\cdot}05$).

Shigella inhibition assay

The incubation of *Shigella* with either the MM freeze-dried or not produced a bactericidal effect which is evident after 24 and 48 h of incubation, since a decrease of 2 log in *Shigella* viability was observed with both treatments (Fig. 3). The same figure shows that controls performed without MM did not affect *Shigella* viability. These results suggest that freeze-drying did not have a negative effect on the capacity of microbial mixture to inhibit *Shigella* growth *in vitro*.

The inhibitory effect of MM on *Shigella* viability could be attributed to the presence of acids, since a decrease of pH (from 6·0 to 5·0) during the first 24 h of incubation of *Shigella* with freeze-dried or fresh MM compared with controls in TSB was observed. According to this, when *Shigella* culture in TSB was acidified to pH 5·0 with lactic acid a decrease of 4 log in viability within 24 h incubation was observed (data not shown).

Translocation of microorganisms

In order to evaluate safety of feeding with freeze-dried MM, we performed a translocation assay in mice. The administration of freeze-dried MM during 7 or 20 d did not induce the translocation of microorganisms to liver or spleen, since no colonies were detected on any of five agar media selected to this assay (Table 1). Moreover, no cellular infiltration was observed by microscopic observation of haematoxylin-eosin stain of histological slices of small intestine from treated and control mice (Fig. 4).

Discussion

To the present, there are a small number of bibliographic references about the development of microbial concentrates or starters containing mixtures of bacteria and yeasts however they do not include studies about probiotic properties of those mixtures (Mugula et al. 2003; Loveness et al. 2007). Additionally, although the application of freeze-drying procedures to preserve microorganisms is well documented, most of the work was performed using individual strains of bacteria or yeasts, and there are only few reports including mixtures of bacterial strains (Champagne et al. 1991; Morgan et al. 2006).

This study shows the development of a probiotic mixture containing different strains of lactobacilli and yeasts isolated from kefir grains which can be preserved by freeze-drying. Given the safety profile assumed from the origin of selected strains, this product could be an attractive microbial mixture to develop a new probiotic supplement for human consumption. However, mechanisms of probiotic action *in vivo* are not yet elucidated. Based on the results of our study we hypothesise that the microbial mixture of lactic acid bacteria and yeast isolated from kefir grains could prevent or decrease the duration time and recurrence of gastrointestinal infections. These hypotheses are based on the evidence that *Lb. kefir* CIDCA 8348 through its S-layer decrease the invasion of Caco-2 cells by *Salmonella enterica* (Golowczyc et al. 2007) and *Lb. plantarum* CIDCA 83114 showed a high inhibitory power against enteropathogens *in vitro* (Golowczyc et al. 2008; Hugo et al. 2008). On the other hand, *K. marxianus* CIDCA 8154 is a lactose fermenter yeast (Echeverría et al. 2007) that could alleviate the lactose intolerance by the

https://doi.org/10.1017/S0022029910000610 Published online by Cambridge University Press

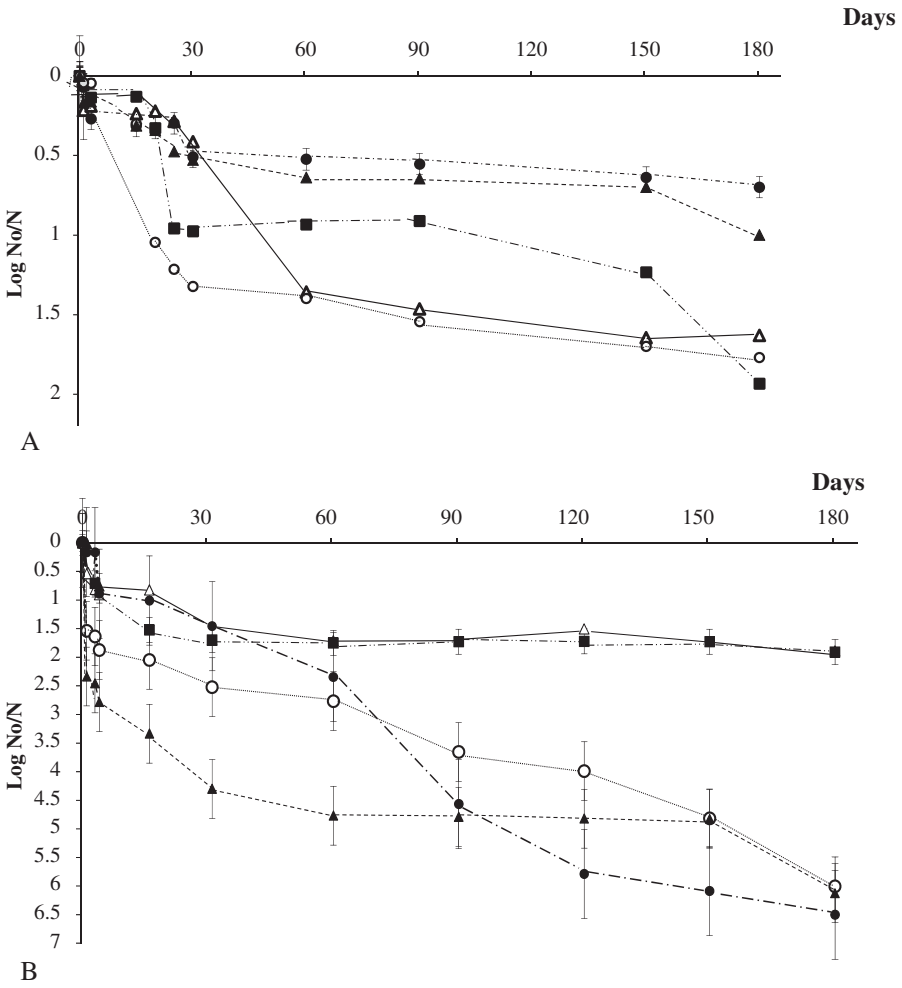

Fig. 2. Survival (log No/N) of each microorganism in the freeze-dried MM (A) or freeze-dried FMM (B) versus storage time at 4 °C. *Lc. lactis* CIDCA 8221 (●), *Lb. plantarum* CIDCA 83114 (△), *Lb. kefir* CIDCA 8348 (■), *Sac. cerevisiae* CIDCA 8112 (○) and *K. marxianus* CIDCA 8154 (▲). No: counts of viable microorganisms (CFU/ml) before freeze-drying. N: counts of viable microorganisms (CFU/ml) after freeze-drying.

increase of β-galactosidase at intestinal level. A large randomized placebo controlled trial is necessary to confirm these findings. In addition, both yeasts (*K. marxianus* CIDCA 8154 and *Sac. cerevisae* CIDCA 8112) have been shown to exert anti-inflammatory effects on stimulated intestinal epithelium *in vitro* and *in vivo* (Romanin et al. 2010).

Since all microorganisms included in the formulation are mesophiles, fermentation continues in refrigeration conditions. Like in the case of milk fermented with kefir grains, the shelf life of these products is less than one week because acidification and gas production increase during storage at 4–10 °C leading to a non acceptable product. For this reason, the alternative is the preservation in a dehydrated state. The comparison of survival of each strain in freeze-dried MM and freeze-dried FMM indicates that it was not possible to preserve the viability of yeasts in the last product. These results suggest that the acids produced during fermentation affect the capacity of microorganisms, especially yeasts, to survive freeze-drying in adverse environmental conditions. In contrast, after freeze-drying MM, the viability of all microorganisms (bacteria and yeasts) was compatible with future development of a probiotic mixture, since the final concentration of microorganisms was not lower than 10^7 CFU/ml after 6 months storage at 4 °C.

In our study, the protective effect of milk was evident for all the microorganisms analysed here, meanwhile the addition of sugars to the milk (trehalose or sucrose) did not increase the viability of any of the strains. These results agree with those reported by Carvalho et al. (2002) where the addition of sugars to 11% skim milk did not improve the viability of *Lb. plantarum* and *Lb. rhamnosus* during the freeze-drying process. However, conditions employed to perform freeze-drying (e.g. phase of growth, suspending fluid, cell concentration, volume of suspension, drying and

https://doi.org/10.1017/S0022029910000610 Published online by Cambridge University Press

Table 1. Translocation of microorganisms to spleen and liver after administration *ad libitum* of freeze-dried microbial mixture to BALB/c mice for 7 or 20 days.

	Counts of CFU in different culture media									
	Control mice					7- or 20-days treated mice				
Organ	MRS	YGC	VRBG	1.1.1	NA	MRS	YGC	VRBG	1.1.1	NA
Intestine†	$>10^9$	$>10^7$	$(2.3\pm0.3)10^7$	$>10^9$	$(7.0\pm0.9)10^7$	$>10^9$	$>10^7$	$(2.8\pm0.3)10^7$	$>10^9$	$(7.4\pm0.4)10^7$
Spleen‡	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Liver‡	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

† Counts are given in CFU per cm of small intestine
‡ Counts are given in CFU per 0·1 g of organ

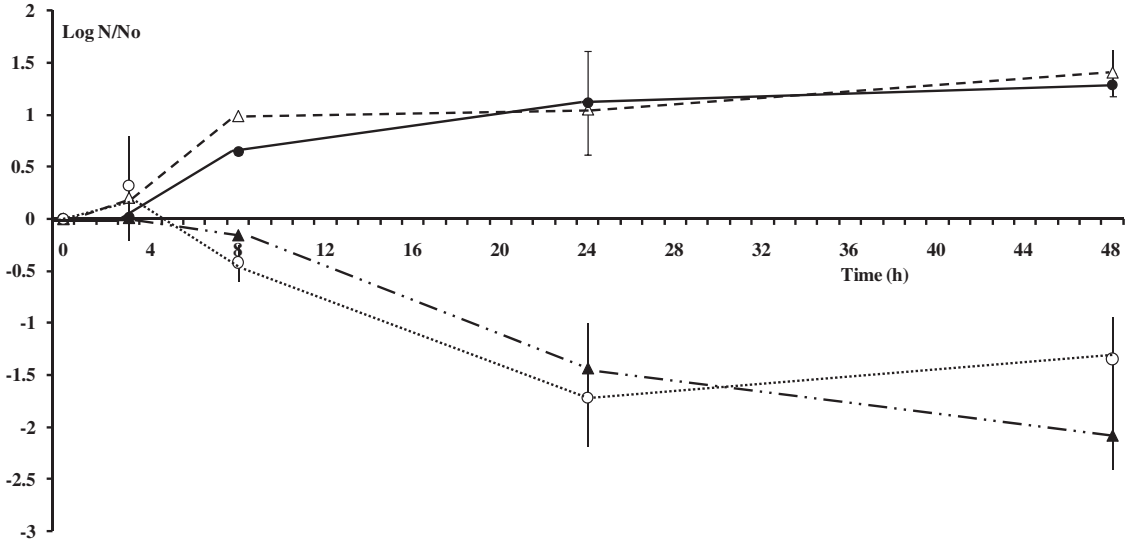

Fig. 3. Survival (Log N/No) of *Shigella sonnei* versus time of incubation in presence of freeze-dried (○) or fresh (▲) MM. Control of *Shigella* in TSB (△) and TSB with milk (●).

freeze-drying technique) could strongly affect microbial viability which has been reported by different authors (Tsvetkov & Brankova, 1983; Berny & Hennebert, 1991; Lodato et al. 1999). The ability of cells to remain viable and functionally active during long-term storage is an important requirement of potential probiotic strains (Sanders & Klaenhammer, 2001). In case of the MM, the inhibitory action against *Shigella* (mainly due to decrease of pH) did not change significantly after freeze-drying process. These results are in concordance with Juárez Tomás et al. (2009) who reported that different strains of lactobacilli maintain their probiotic properties (production of H_2O_2, lactic acid and bacteriocins) during storage at 4° C after freeze-drying process.

Several authors have been reported the possibility of bacterial translocation after administration of probiotic microorganisms (Kunz et al. 2004; Land et al. 2005). In this study, we found that the administration of the freeze-dried MM to mice for 7 to 20 d did not induce translocation of lactic acid bacteria, yeasts or indigenous enterobacteria. Similar results were reported by Vinderola et al. (2005) who have administrated several dilutions of kefir fermented milk to mice for 7 d and Yakabe et al. (2009) who evaluated one strain of *Lb. brevis* as a probiotic in rats. Moreover, there were no histopathologic changes in gastrointestinal tissue upon freeze-dried MM administration, consistent with results reported by other authors for different probiotic microorganisms (Zhou et al. 2000; Abe et al. 2009). Consequently with these results and taking into account the origin of the strains the microbial mixture proposed in this study can be considered a safe product.

In conclusion, freeze-drying in milk is a good procedure to preserve the microbial mixture viability, the *in vitro* capacity to inhibit *Shigella* and the *in vivo* non-translocation of the kefir-isolated microorganisms and enterobacteria. This study contributes to the knowledge of the effect of freeze-drying process and preservation of the behaviour of bacteria and yeasts in a mixture. To our knowledge, this is the first report regarding a safe freeze-dried microbial mixture potentially probiotic containing

https://doi.org/10.1017/S0022029910000610 Published online by Cambridge University Press

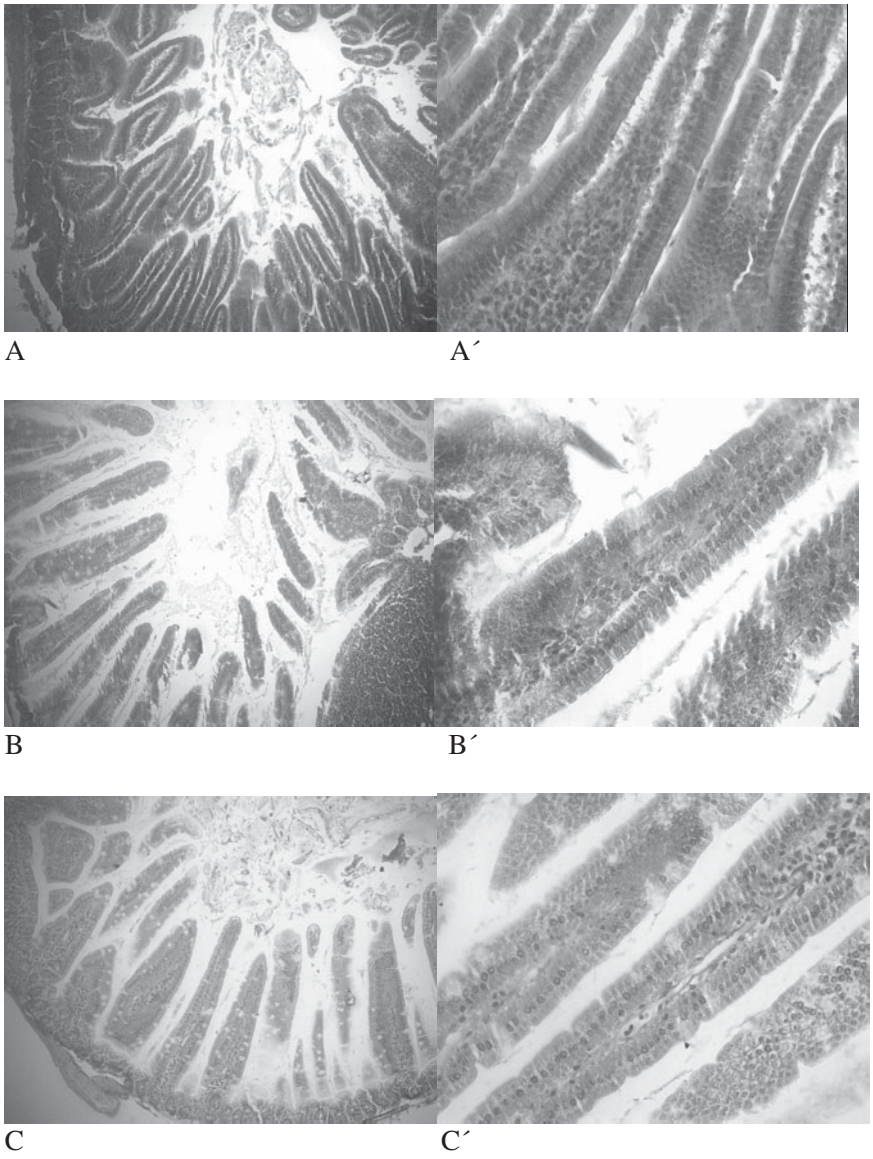

Fig. 4. Haematoxylin-eosin stain of histological slices of small intestine from treated and control mice. Panel A and A′: control mice; panel B and B′: mice treated daily with freeze-dried MM for 7 days; panel C and C′: mice treated for 20 days. Panels A, B and C: 10×. Panels A′, B′ and C′: 40×.

different strains of both kefir-isolated bacteria and yeasts with potential industrial application. These results will aid to the development of a new food-based product that could help in the prevention of infectious diseases.

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC-PBA) and Universidad Nacional de La Plata (UNLP). P. Bolla is a fellow of CONICET; M.A. Serradell is a member of the Carrera de Investigador Científico y Tecnológico of CONICET; G.L. De Antoni is a member of the Carrera de Investigador Científico of CIC-PBA and Professor of Microbiology of Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, UNLP; P. de Urraza is a Professor of Microbiology of Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, UNLP. Authors are grateful to Jorge Ventureira for technical assistance, Marina Golowczyc for critical reading of the manuscript and to the Hospital de Niños Sor María Ludovica de La Plata for provision of *Shigella sonnei* strain.

References

Abadias M, Benabarre A, Taixidó N, Usall J & Viñas IV 2001 Effect of freeze drying and protectants on viability of the biocontrol yeast *Candida sake*. *International Journal of Food Microbiology* **65** 173–182

Abe F, Muto M, Yaeshima T, Iwatsuki K, Aihara H, Ohashi Y & Fujisawa T 2009 Safety evaluation of probiotic bifidobacteria by analysis of mucin

https://doi.org/10.1017/S0022029910000610 Published online by Cambridge University Press

degradation activity and translocation ability. Anaerobe doi: 10.1016/j.anaerobe.2009.07.006

Abraham A, De Antoni G & Añón M 1990 Effect of calcium on the cryopreservation of *L. bulgaricus* in different freezing media. *Cryobiology* **27** 336

Angulo L, Lopez E & Lema C 1993 Microflora present in kefir grains of the Galician region north-west of Spain. *Journal of Dairy Research* **60** 263–267

Balcazar J, Vendrell D, de Blas I, Ruiz-Zarzuela I & Muzquiz J 2009 Effect of *Lactococcus lactis* CLFP 100 and Leuconostoc mesenteroides CLFP 196 on Aeromonas salmonicida Infection in brown trout (Salmo trutta). *Journal of Molecular Microbiology and Biotechnology* **17** 153–157

Berg R 1999 Bacterial translocation from the gastrointestinal tract. *Advances in Experimental Medical Biology* **473** 11–30

Berny J & Hennebert G 1991 Viability and stability of yeast cells and filamentous fungus spore during freeze-drying: Effects of protectants and cooling rates. *Mycologia* **83** 805–815

Blanquet S, Garrait G, Beyssac E, Parrier C, Denis S, Hébrard G & Alric M 2005 Effects of cryoprotectants on the viability and activity of freeze-dried recombinant yeast as novel oral drug delivery systems assessed by an artificial system. *European Journal of Pharmacautics and Biopharmaceutics* **61** 32–39

Bravo D, Rodriguez E & Medina M 2009 Nisin and lacticin 481 coproduction by *Lactococcus lactis* strains isolated from raw ewes' milk. *Journal of Dairy Science* **92** 4805–4811

Carvalho A, Silva J, Ho P, Teixeira P, Malcata F & Gibbs P 2002 Effect of additives on survival of freeze-dried *Lactobacillus plantarum* and *Lactobacillus rhamnosus* during storage. *Biotechnology Letters* **24** 1587–1591

Carvalho A, Silva J, Ho P & Teixeira P 2004 Relevant factors for the preparation of freeze-dried lactic acid bacteria. *International Dairy Journal* **14** 835–847

Carvalho A, Silva J, Ho P, Teixeira P, Malcata F & Gibbs P 2003 Effect of various growth media upon survival during storage of freeze-dried *Enterococcus faecalis* and *Enterococcus durans*. *Journal of Applied Microbiology* **94** 947–952

Carvalho A, Silva J, Ho P, Teixeira P, Malcata F & Gibbs P 2004 Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freeze-dried *Lactobacillus delbrueckii* ssp. bulgaricus. *Biotechnology Progress* **20** 248–254

Champagne C, Gardner N, Brochu E & Beaulieu Y 1991 The freeze-drying of lactic acid bacteria. A Review. *Canadian Institute of Food Science and Technology* **24** 118–128

De Antoni G, Pérez P, Abraham A & Añón C 1991 Trehalose, a cryoprotectant for *L. bulgaricus*. *Cryobiology* **26** 149–153

de Urraza P & De Antoni G 1997 Induced cryotolerance of *Lactobacillus delbrueckii* subsp. bulgaricus LBB by preincubation at suboptimal temperatures with a fermentable sugar. *Cryobiology* **35** 159–164

Echeverría I, Garrote G & De Antoni G 2007 Formulación de una leche fermentada probiótica y con bajo contenido de lactosa. *Tecnología Láctea Latinoamericana* **48** 44–47

Farnworth E 2005 Kefir – a complex probiotic. *Food Science and Technology Bulletin* **2** 1–17

Garrote G 2000 Bacterias lácticas y levaduras para la industria alimentaria: kefir. PhD Thesis Universidad Nacional de La Plata, Argentina

Garrote G, Abraham A & De Antoni G 2001 Chemical and microbiological characterisation of kefir grains. *Journal of Dairy Research* **68** 639–652

Garrote G, Delfederico L, Bibiloni R, Abraham A, Perez P, Semorile L & De Antoni G 2004 Lactobacilli isolated from kefir grains: evidence of the presence of S-layer proteins. *Journal of Dairy Research* **71** 222–230

Golowczyc M, Gugliada M, Hollmann A, Delfederico L, Garrote G, Abraham A, Semorile L & De Antoni G 2008 Characterization of homofermentative lactobacilli isolated from kefir grains: potential use as probiotic. *Journal of Dairy Research* **75** 211–217

Golowczyc M, Mobili P, Garrote G, Abraham A & De Antoni G 2007 Protective action of *Lactobacillus kefir* carrying S-layer protein against *Salmonella enterica* serovar Enteritidis. *International Journal of Food Microbiology* **118** 264–273

Hugo A, Kakisu E, De Antoni G & Perez P 2008 Lactobacilli antagonize biological effects of enterohaemorrhagic *Escherichia coli in vitro*. *Letters in Applied Microbiology* **46** 613–619

Kunz A, Noel J & Firchok M 2004 Two case of *Lactobacillus* bacteremia during probiotic treatment of short gut syndrome. *Journal of Pediatric Gastroenterology and Nutrition* **38** 457–458

Land M, Rouster-Stevens K, Woods C, Cannon M, Cnota J & Shetty A 2005 *Lactobacillus* sepsis associated with probiotic therapy. *Pediatrics* **115** 178–181

Leslie S, Israeli E, Lighthart B, Crowe J & Crowe L 1995 Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology* **61** 3592–3597

Linders L, Wolkers W, Hoekstra F & van't Riet K 1997 Effect of added carbohydrates on membrane phase behavior and survival of dried *Lactobacillus plantarum*. *Cryobiology* **35** 31–40

Lodato P, Segovia de Huergo M & Buera M 1999 Viability and thermal stability of a strain of *Saccharomyces cerevisiae* freeze-dried in different sugar and polymer matrices. *Applied Microbiology and Biotechnology* **52** 215–220

Nyanga L, Nout M, Gadaga T, Theelen B, Boekhout T & Zwietering M 2007 Yeasts and lactic acid bacteria microbiota from masau (Ziziphus mauritiana) fruits and their fermented fruit pulp in Zimbabwe. *International Journal of Food Microbiology* **120** 159–166

Morgan C, Herman N, White P & Vesey G 2006 Preservation of microorganisms by drying; a review. *Journal of Microbiological Methods* **66** 183–193

Mugula J, Narvhus J & Sørhaug T 2003 Use of starter cultures of lactic acid bacteria and yeasts in the preparation of togwa, a Tanzanian fermented food. *International Journal of Food Microbiology* **83** 307–318

Otero M, Espeche M & Nacer Macías M 2007 Optimization of the freeze-drying and survival throughout storage of fFreeze-dried *Lactobacillus gasseri* and *Lactobacillus delbrueckii* subsp. *delbrueckii* for veterinarian probiotic applications. *Process Biochemistry* **42** 1406–1411

Pavan S, Desreumaux P & Mercenier A 2003 Use of mouse models to evaluate the persistence, safety, and immune modulation capacities of lactic acid bacteria. *Clinical and Diagnostic Laboratory Immunology* **10** 696–701

Romanin D, Serradell M, González-Maciel D, Lausada N, Garrote G & Rumbo M 2010 Down-regulation of intestinal epithelial innate response by probiotic yeast isolated from kefir. *International Journal of Food Microbiology* **140** 102–108.

Rossland E, Andersen Borge G, Langsrud T & Sorhaug T 2003 Inhibition of *Bacillus cereus* by strains of *Lactobacillus* and *Lactococcus* in milk. *International Journal of Food Microbiology* **89** 205–212

Sanders M & Klaenhammer T 2001 Invited review: the scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. *Journal of Dairy Science* **84** 319–331

Selmer-Olsen E, Birkeland S & Sorhaug T 1999 Effect of protective solutes on leakage from and survival of immobilized lactobacillus subjected to drying, storage and rehydration. *Journal of Applied Microbiology* **87** 429–437

Tsvetkov T & Brankova R 1983 Viability of micrococci and lactobacilli upon freezing and freeze-drying in the presence of different cryoprotectants. *Cryobiology* **20** 318–323

Vinderola C, Duarte J, Thangavel D, Perdigon G, Farnworth E & Matar C 2005 Immunomodulating capacity of kefir. *Journal of Dairy Research* **72** 195–202

Yakabe T, Moore E, Yokota S, Sui H, Nobuta Y, Fukao M, Palmer H & Yajima N 2009 Safety assessment of *Lactobacillus brevis* KB290 as a probiotic strain. *Food and Chemical Toxicology* **47** 2450–2453

Zabala V 2004 Caracterización de leches fermentadas obtenidas con gránulos de kefir: composición y propiedades probióticas y tecnológicas. Master Thesis, Universidad Nacional de La Plata, Argentina

Zhou J, Shu Q, Rutherfurd K, Prasad J, Gopal P & Gill H 2000 Acute oral toxicity and bacterial translocation studies on potentially probiotic strains of lactic acid bacteria. *Food Chemical Toxicology* **38** 153–161

https://doi.org/10.1017/S0022029910000610 Published online by Cambridge University Press