International Journal of Dairy Technology SDT SOCIETY OF DAIRY TECHNOLOGY

doi: 10.1111/1471-0307.12038

ORIGINAL RESEARCH

Study of the effects of spray-drying on the functionality of probiotic lactobacilli

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Three probiotic lactobacilli strains were spray-dried in 20% (w/v) skim milk and submitted to a simulated gastrointestinal digestion. Fresh or spray-dried cultures were administered to mice for 5 and 10 days, and Immunoglobulin A (IgA)-producing cells were enumerated in the small intestine by immunohistochemistry. Spray-drying significantly enhanced the resistance of Lactobacillus paracasei *A13 and* Lactobacillus casei *Nad to a simulated gastrointestinal digestion (0.96 and 1.95 log orders, respectively), compared with fresh cultures. Also, a significant higher number of IgA-producing cells were induced by spray-dried cultures compared with fresh cultures. Spray-drying is a suitable, but strain-dependent, technological process for the development of probiotic cultures in skim milk with increased functionality.*

Keywords Spray-drying, Viability, Lactobacilli, Microscopy, *In vivo*, Immunomodulation.

INTRODUCTION

The FAO/WHO defined probiotic bacteria as 'live micro-organisms which when consumed in adequate numbers confer a health benefit on the host' (FAO/WHO 2002). These healthy micro-organisms are commercialised mainly as frozen or freeze-dried cultures for direct food addition. The production of dried cell cultures has attractive and strategic advantages because dehydrated cultures demand less storage capacity and lower transport and refrigeration costs compared with frozen cultures. Nowadays, the industrial manufacture of commercially available dried probiotic bacterial cultures is carried out almost exclusively by freeze-drying, a discontinuous and expensive process with low yields, time and energy demanding (Knorr 1998; Meng *et al.* 2008), but having as the main advantage the high levels of viable cells that can be achieved, almost strain independently. Spray-drying has not only cost advantages over freeze-drying as a technological tool for probiotic culture development (Peighambardoust *et al.* 2011), but also allows the continuous production of large amounts of dried cells in short periods of time (Gardiner *et al.* 2000). However, the different stress factors bacteria are exposed to during spray-drying (osmotic, heat, oxidative) due to the quite harsh conditions of temperature required for product dehydration, can cause membrane damage and inactivation depending on the technological conditions applied. In a previous work, we found that certain probiotic strains can resist spray-drying and long-term storage in a strain-dependent manner (Páez *et al.* 2012), in accord with previous reports (Gardiner *et al.* 2000; O'Riordan *et al.* 2001; Desmond *et al.* 2002a; Lian *et al.* 2002; Corcoran *et al.* 2004; Ananta *et al.* 2005). The food matrix and many technological variables involved in the manufacture of probiotic cultures have been pointed out as responsible for changes in the functionality of probiotic bacteria (Ranadheera *et al.* 2010). For instance, technological factors such as the pH at which cells are grown (Saarela *et al.* 2009), the time at which biomass is harvested during bulk production (Saarela *et al.*

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2005), the protectants used for culture storage (Desmond *et al.* 2002a,b; Saarela *et al.* 2005; Vinderola *et al.* 2011) and the storage period itself (Matto *et al.* 2006; Vinderola *et al.* 2012) have been indicated as possible sources of variation in the functionality of probiotic cultures, compared with the one displayed by cells as fresh cultures obtained in the laboratory. To the best of our knowledge, there are no reports indicating the effect of spray-drying on the immune-stimulating capacity of probiotic bacteria *in vivo*. In this context, the aim of the present work was to characterise the functionality of three probiotic cultures submitted to spray-drying in skim milk.

MATERIALS AND METHODS

Strains and culture conditions

Three strains of probiotic lactobacilli, whose commercial names were changed to avoid any conflict of interest, were used in this study. The strains are kept in the culture collection of the INLAIN (UNL-CONICET, Santa Fe, Argentina) and were identified as *Lactobacillus acidophilus* A9, *Lactobacillus paracasei* A13 and *Lactobacillus casei* Nad. When needed, overnight cultures (16 h, 37 °C) were obtained in MRS (deMan, Rogosa and Sharp) broth (Biokar, Beauvais, France), after three transfers from frozen (−70 °C) stocks maintained in MRS added with 18% (w/v) glycerol (Ciccarelli, Santa Fe, Argentina).

Spray-drying in skim milk

Overnight cultures of the three strains in MRS broth were harvested (6000 × *g*, 15 min, 5 °C), washed twice with PBS (phosphate-buffered saline solution, pH 7.4) and resuspended in 20% (w/v) skim milk. Cell suspensions were spray-dried in a laboratory scale spray dryer (Buchi mini spray dryer model B290, Flawil, Switzerland). A constant inlet air temperature of 170 °C, an outlet temperature of 85 °C and a flux of 600 L/h were used. Cell suspensions were atomised and sprayed into the drying chamber using a two-fluid nozzle. The product dried almost instantaneously, and the residence time was negligible. Three independent replicates were performed for each strain. Spray-dried powders were vacuum sealed in individual samples of 10 g. Residual moisture (% w/w) was determined in triplicate at 101 ± 1 °C (FIL-IDF 26 A: 1993). Cell counts of lactobacilli were performed before and after spray-drying on MRS agar (37 °C, 48 h aerobic incubation).

Electron microscopy studies

The spray-dried powders obtained were characterised using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Overnight fresh cultures were obtained as previously described, resuspended in PBS solution (pH 7.4) and fixed with glutaraldehyde (according to the instructions of the laboratory that carried out the SEM and TEM studies). Spray-dried powders and fresh cultures were submitted (at 5 °C) to the Electron Microscopy Service of the Superior Institute for Biological Researches (INSIBIO, UNT-CONICET, Tucumán, Argentina) for the SEM and TEM studies. The time between harvesting and fixation of cells and further processing of samples at INSIBIO was less than 18 h.

Resistance to simulated gastrointestinal digestion

Overnight cultures obtained as previously described were suspended in 20% (w/v) skim milk. Spray-dried powders were resuspended in distilled water at a concentration of 20% (w/v) of total solids. For studying the resistance to simulated gastrointestinal digestions, cell suspensions (20 mL) were mixed with the same volume of a simulated 'saliva-gastric' solution containing $CaCl_2$ (0.22 g/L), NaCl (16.2 g/L), KCl (2.2 g/L), $NaHCO_3$ (1.2 g/L) and 0.3% (w/v) porcine pepsin (Merck, Darmstadt, Germany) and adjusted to pH 2.50 with 5 M and 1 M HCl (Vinderola *et al.* 2011). One millilitre samples were removed immediately after mixture (before pH adjustment) for cell counts (MRS agar, 37 °C, aerobic incubation, 48 h). Cell suspension was incubated at 37 °C in a water bath for 90 min (simulated gastric digestion), and cell counts were performed after incubation. A sample was removed after 90 min, centrifuged (6000 × *g*, 15 min, 5 °C) and re-suspended in MRS broth containing 0.5% (w/v) bovine bile salts (Sigma, St Louis, MO, USA) at pH 7.4. Cell suspension was incubated at 37 °C for 60 min (bile exposure), and counts were performed after incubation (MRS, 37 °C, aerobic incubation, 48 h).

In vivo trial

Animals

A total of 52 6-week-old BALB/c female mice weighing 18–20 g were obtained from the random bred colony of the Centro de Experimentaciones Biológicas y Bioterio, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe, Argentina). Animals were allowed to stand at the INLAIN animal facility for a week before starting the assays. Each experimental group consisted of four mice housed together in plastic cages kept in a controlled atmosphere (temperature, 21 ± 1 °C; humidity 55% ± 2%) with a 12-h light/dark cycle. All animals received simultaneously and *ad libitum*, tap water and a sterile conventional balanced diet (Cooperación, Buenos Aires, Argentina) containing proteins, 230 g/kg; raw fibre, 60 g/kg; total minerals, 100 g/kg; Ca, 13 g/kg; P, 8 g/kg; water, 120 g/kg and vitamins. Mice were maintained and treated according to the guidelines of the National Institute of Health (NIH, USA). The animal assays were approved by the Ethical Committee for Animal Experimentation of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza). The 3 R's principle was taken into account when using animals.

Strains administration

Mice in treated groups received daily (by gavage) 0.2 mL of a cell suspension containing *ca.* 9.3 log colony-forming-units (cfu)/mL of the strain under study for 5 or 10 consecutive days. Strains were administered as overnight fresh cultures, washed and resuspended in 20% (w/v) skim milk, or as spray-dried cultures in skim milk reconstituted in distilled water to a 20% (w/v) of total solids. In the control group, mice received by gavage 0.2 mL of 20% (w/v) skim milk for 10 consecutive days.

Translocation assay

At the end of each feeding period, mice were anaesthetised and sacrificed by cervical dislocation. Liver was removed and homogenised in sterile PBS. Serial dilutions were plated in MacConkey (Britania, Buenos Aires, Argentina) for detection of enterobacteria. Plates were incubated at 37 °C for 24 h under aerobic conditions. Results were expressed as positive or negative translocation, as liver is a sterile organ under normal conditions.

Immunofluorescence assays for detection of Immunoglobulin A (IgA)-producing cells in the small intestine

Small intestine was removed and processed for routine histological preparation for paraffin inclusion. The number of IgA+ cells was measured on histological slices from the gut by a direct immunofluorescence test according to Vinderola *et al.* 2005. Briefly, the test was performed using the anti-IgA mono-specific antibody (a-chain specific) conjugated with FITC (Sigma). Deparaffinised histological samples were incubated with a 1/100 dilution of the antibody in 0.01 M Na PBS solution (pH 7.4) for 30 min at 37 °C. Then, samples were washed three times with PBS solution and examined using a fluorescence light microscope with Hg lamp. Results were expressed as the mean number of positive (fluorescent) cells per 10 fields (magnification 100 ×) after counting at least 30 fields in duplicate for each animal by blinded operators.

Statistical analysis

The results of cell counts were transformed to log10 cfu/mL and expressed as mean ± standard deviation or log difference of at least three independent experiments in each trial. The treatments were processed according to a 3 × 2 × 2 factorial arrangement (3 strains, 2 feeding periods and 2 forms of delivery: fresh or spray-dried) in a randomised complete design plus a control (without treatment). The statistical model included the main effects of and their interactions. Differences between factors were determined using analysis of variance and means separated using the least square means. Differences were deemed significant at $P < 0.05$. Comparisons were made using the statistical program InfoStat Software (developed by Grupo InfoStat, Facultad de Ciencias Agrarias, Universidad Nacional de Córdoba, Córdoba, Argentina).

RESULTS

Overnight cultures of three strains of commercial probiotic lactobacilli, identified in this work as *L. acidophilus* A9, *L. paracasei* A13 and *L. casei* Nad, were washed and resuspended in 20% skim milk and spray-dried. The powders obtained presented a residual moisture content of less than 4% (w/v) and were very easy to disperse and to solubilise in distilled water at room temperature. No significant differences ($P = 0.067$) in cell counts were observed between the cell suspensions before spray-drying and the reconstituted powders obtained after the application of this technological process (data not shown).

Fresh cultures and spray-dried powders were studied by scanning and transmission electron microscopy (Figure 1). No lactobacilli cells were left outside bubbles formed by skim milk after spray-drying (Figure 1a–d). Additionally, transmission electron microscopy images (Figure 1e,f) obtained directly on powders (avoiding sample rehydration) allowed the observation of individual lactobacilli cells inside skim milk bubbles, surrounded by particles of milk solids. The same behaviour was observed for the three strains studied.

Fresh cultures and spray-dried powders were submitted to a simulated gastrointestinal digestion that comprised two successive steps: gastric digestion for 90 min and bile exposure for 60 min. Reductions in cell counts were significant, compared with initial values, during simulated gastric digestion, but not during bile exposure (Table 1). Diminution in cell counts due to exposure to low pH ranged from 5.31 to 6.49 log cycles for fresh cultures and from 4.54 to 5.34 log cycles for spray-dried cultures. Spray-drying significantly enhanced the resistance of *L. casei* A13 and *L. paracasei* Nad in approximately 1 and 2 log cycles, respectively.

Finally, an *in vivo* trial was performed to determine the effect of spray-drying on the immune-stimulating capacity of the strain under study. The oral administration of the strain induced no translocation of intestinal flora to the liver; thus, the dose used in this study was regarded as safe in this animal model. Figure 2 shows the effects of the oral administration of the strains under study for 5 or 10 consecutive days on the number of IgA-producing cells in the small intestine lamina propria of mice. All strains used were effective for significantly enhancing ($P < 0.05$) this parameter of functionality for both feeding periods assessed, either administered as fresh or spray-dried cultures, when compared with the control group. Regarding the technological treatment applied (spray-drying), a significantly higher number of IgA-producing cells were induced by spray-dried cultures compared with fresh cultures, for the three strains when administered for 5 days.

DISCUSSION

Freezing and freeze-drying are the most commonly used methods to produce probiotic cultures at an industrial level.

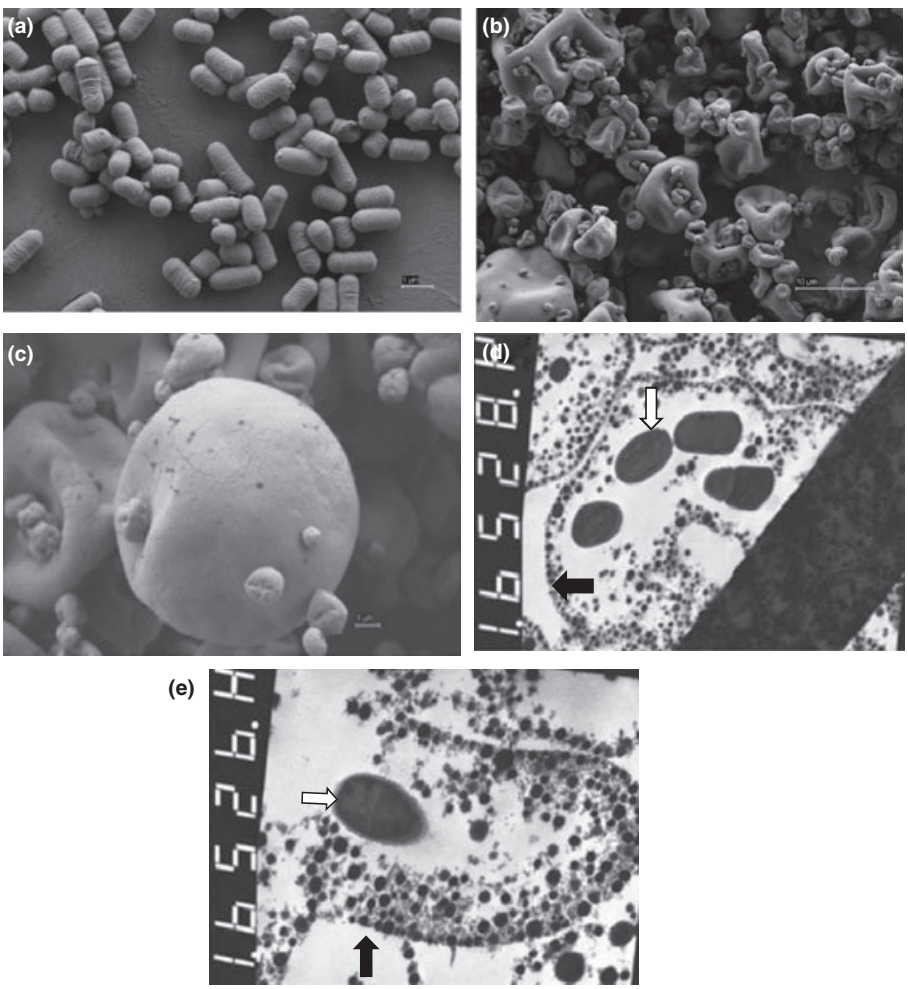

Figure 1 Scanning (a, b, c) and transmission (d, e) electron micrographs of *Lactobacillus casei* A13 before (a) and after (b, c, d, e) spray-drying in skim milk. Black arrows in pictures d and e indicate the external surface of the bubbles observed in pictures b and c. White arrows in pictures d and e indicate cells of lactobacilli.

Table 1 Resistance of fresh or spray-dried lactobacilli in skim milk to simulated gastric digestion and bile exposure

		Cell count (log cfu/mL) at time (min)			*Mean reduction in log cycles due to gastric digestion*[3]	*Enhancement of cell viability due to spray-drying*[4]
Strain	*Condition*	*0*	*90 (gastric digestion)*	*150 (60 min of bile exposure)*		
Lactobacillus paracasei Nad	Fresh	$8.58 \pm 0.57^{a,1}$	$2.09 \pm 0.17^{a,2}$	$2.00 \pm 0.11^{a,2}$	6.49	1.95
	Spray-dried	$8.42 \pm 0.58^{a,1}$	$3.88 \pm 0.14^{b,2}$	$3.84 \pm 0.35^{b,2}$	4.54	
Lactobacillus acidophilus A9	Fresh	$8.69 \pm 0.14^{a,1}$	$3.38 \pm 0.65^{a,2}$	$2.86 \pm 0.68^{a,2}$	5.31	0.03
	Spray-dried	$8.90 \pm 0.17^{a,1}$	$3.56 \pm 0.49^{a,2}$	$3.66 \pm 0.10^{a,2}$	5.34	
Lactobacillus casei A13	Fresh	$8.60 \pm 0.53^{a,1}$	$2.45 \pm 0.78^{a,2}$	$2.36 \pm 0.32^{a,2}$	6.15	0.96
	Spray-dried	$9.19 \pm 0.15^{a,1}$	$4.05 \pm 0.25^{b,2}$	$3.99 \pm 0.16^{b,2}$	5.19	

[a,b]Values in columns, for the same strain, with different letter as superscript are significantly different ($P < 0.05$).

[1,2]Values in rows with different number as superscript are significantly different ($P < 0.05$).

[3]Difference between Mean cell counts at time zero and Mean cell counts after simulated gastric digestion (90 min), performed in each row.

[4]Difference between Mean reduction in cell counts as fresh culture and Mean reduction in cell counts as spray-dried culture, after simulated gastric digestion.

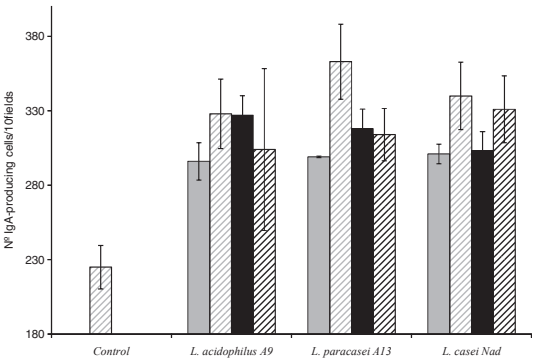

Figure 2 Effects of the oral administration of *L. acidophilus* A9, *L. paracasei* A13 and *L. casei* Nad for 5 (grey bars) or 10 (black bars) consecutive days as fresh (full bars) or spray-dried (stripped bars) cultures, in the number of IgA-producing cells in the small intestine lamina propria of mice, compared with control animals (white bar).

Spray-drying, as an alternative technology for this purpose, may have as advantage its rapidity and relatively lower cost compared with freeze-drying. Additionally, spray-drying is already available for industrial applications, compared with other micro-encapsulation techniques (Burgain *et al.* 2011). However, one of the disadvantages of spray-drying is the risk of cell-death associated with the use of high temperatures, which are not always compatible with survival of all bacterial strains (Burgain *et al.* 2011). However, spray-drying has been successfully used for manufacture of powders from skim milk–based media containing high viable bacterial numbers of different species (Gardiner *et al.* 2000; O'Riordan *et al.* 2001; Lian *et al.* 2002; Simpson *et al.* 2005; Paéz *et al.* 2012). In this study, three strains of probiotic bacteria used currently in fermented dairy products were spray-dried in skim milk and the obtained powders were characterised by electron microscopy. The resistance to simulated gastrointestinal digestion was studied along with strains capacity to enhance the number of IgA-producing cells in mice's small intestine lamina propria as an indicator of functionality at an animal level.

Electron microscopy studies showed that lactobacilli cells were successfully entrapped inside milk solids, leaving no cells outside them. The microparticles obtained were spherical and varied in size. The skim milk surface microparticles appeared grainy with visible cracks. Lian *et al.* 2002 suggested that these cracks may facilitate heat escape from inside the particle after drying, causing less heat injury to the entrapped micro-organisms and contributing to survival after spray-drying. Gardiner *et al.* (2002) revealed the presence of cells of *Lactobacillus paracasei* NFBC 338 inside spray-dried skim milk powder by using confocal scanning laser microscopy. No cells of this strain outside skim milk powder particles (Gardiner *et al.* 2002) or skim milk-gum acacia powder (Desmond *et al.* 2002b) were observed, as in SEM micrographs. Additionally, TEM studies with *L. paracasei* NFBC 338 (Meng *et al.* 2008) showed that cells were surrounded by milk solid particles, in agreement with observations of the present study. Micrographs of *Bifidobacterium animalis* subsp. *lactis* BB-12 microcapsules produced with skim milk or skim milk combined with inulin or oligofructose also showed absence of cells outside particles obtained by spray-drying (Fritzen-Freire *et al.* 2012). Lian *et al.* (2002) showed by SEM that *B. longum* B6 was successfully spray-dried inside microparticles of gum arabic, gelatin, soluble starch and skim milk. O'Riordan *et al.* (2001) entrapped cells of *Bifidobacterium* PL1 in starch by spray-drying, leaving no cells outside particles. Finally, Semyonov *et al.* (2011) reported the encapsulation of *L. casei* subsp. *paracasei* LMG P-21380 in a maltodextrin–trehalose matrix by ultrasonic vacuum spray drier, a traditional spray-drying modification that uses less harsh conditions that enhance cell viability after drying. SEM images of microcapsules produced also showed a successful incorporation of cell insides particles. These visual evidences put together suggest that spray-drying is effective for entrapping viable cells isolating them from the surrounding environment.

In this study, spray-drying in skim milk did not affect cell viability. However, differences in resistance to simulated gastrointestinal digestion were detected when comparing fresh cultures with spray-dried cultures. The resistance to simulated gastric digestion was enhanced by spray-drying for *L. casei* Nad and *L. paracasei* A13. Our results agree with those of Fávaro-Trindade and Grosso (2002) who showed an enhanced survival of *L. acidophilus* La-05 and *B. lactis* Bb-12 spray-dried in cellulose acetate phthalate when exposed to low pH and bile. However, this fact might be strain and conditions dependant, as O'Riordan *et al.* (2001) reported no differences in resistance to acid conditions between free or spray-dried starch-coated bifidobacteria. An increased resistance to a stress factor (low pH) might be accomplished by a previous exposure to low levels of another stress factor (spray-drying), a phenomenon called crossed adaptation that has been previously reported for lactobacilli strains exposed to different stress factors (Lou and Yousef 1996; O'Driscoll *et al.* 1996).

Finally, an *in vivo* trial in mice was conducted to assess the immune-stimulating capacity of the strains when administered as fresh or spray-dried cultures. IgA is the most abundant immunoglobulin at the mucosal surfaces whose main function in the gut is to exert immune exclusion by intimate cooperation with the innate nonspecific defence mechanisms (Macpherson *et al.* 2001). Numerous reports indicate proliferation of IgA in the gut, on animal models (Vinderola *et al.* 2007; Chiang and Pan 2012; Souza *et al.* 2012) or humans (Kabeerdoss *et al.* 2011; Holscher *et al.* 2012), as a desirable indicator of functionality. In this study, spray-drying did not negatively modify strains capacity to enhance the number of IgA-producing cells. Interestingly, for the 5-day period of administration, the capacity of spray-dried

cultures to promote IgA proliferation was higher compared with fresh cultures. In a previous study, we observed that spray-dried peptides induced a higher number of IgA-producing cells in mice (Burns *et al.* 2010), compared to untreated peptide suspension. It may be hypothesised that exposure to heat of the outer shell of proteins in lactobacilli cells may have somewhat induced protein aggregates that could have behaved, *in vivo*, more as particulate rather than as soluble antigens, enhancing their interaction capacity with the immune cells associated with the gut (Perdigón *et al.* 2002). Another technological treatment consisting in the application of sublethal levels of high pressure (named High Pressure of Homogenisation) also significantly enhanced the capacity of *L. paracasei* A13 to induce the proliferation of IgA-producing cells compared with nontreated cells (Tabanelli *et al.* 2012).

CONCLUSIONS

At the laboratory level, spray-drying is a promising technological tool for the development of probiotic cultures in skim milk when the strains are able to overcome the thermal treatment involved in dehydration. Resistance to simulated gastrointestinal digestion and the immune-stimulating capacity may be enhanced by spray-drying in a strain-specific manner. Scaling-up of drying of probiotic strains is still needed to verify whether high yields of viable cells with enhanced *in vitro* and *in vivo* properties are maintained at the industrial level.

ACKNOWLEDGEMENTS

Luisina Lavari is a doctoral fellow of CONICET (Argentina). This work was funded by the projects PNLEC Nº 071021 and AETA Nº 281721, supported by INTA.

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