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Short Communication

Cellular injuries of spray-dried *Lactobacillus* spp. isolated from kefir and their impact on probiotic properties

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ABSTRACT

The injuries caused by spray drying (SD) of three potential probiotic lactobacilli isolated from kefir grains and the impact on some probiotic properties, were evaluated. Results demonstrated that *Lactobacillus plantarum* 83114 and *L. kefir* 8321 showed a slight reduction of viability (0.11 and 0.29 log CFU/ml respectively) after SD process, and *L. kefir* 8348 was found to be more sensitive to the process with a reduction in viability of 0.70 log CFU/ml. Neither membrane damage, evaluated by increased sensitivity to NaCl, lysozyme, bile salt and penicillin G, nor changes in acidifying activity in MRS and milk by lactobacilli were detected after SD. *L. plantarum* 83114 and *L. kefir* 8321 after SD did not lose their capacity to adhere to intestinal cells. Nevertheless, *L. kefir* 8348 showed a significant loss of adhesion capacity after SD. In addition, rehydrated spray-dried *L. kefir* 8321 retained the ability to protect against *Salmonella* invasion of intestinal cells. This effect was observed when *L. kefir* is co-incubated with *Salmonella* before invasion assay.
This work shows that the membrane integrity evaluated by indirect methods and some probiotic properties of lactobacilli isolated from kefir did not change significantly after SD, and these powders could be used in functional foods applications.

1. Introduction

Probiotic bacteria, mainly specific strains of lactobacilli and bifidobacteria, have been shown to have beneficial effects on immunomodulation and in the alleviation or prevention of diverse intestinal disorders (Servin and Coconnier, 2003; Shah, 2007). Probiotic bacteria are reported to prevent the adherence, establishment and invasion of specific enteropathogens (Servin, 2004). Many criteria have been suggested for the selection of probiotics, among them lack of pathogenicity, tolerance of gastrointestinal conditions (acid and bile), ability to adhere to the gastrointestinal mucosa, and competitive exclusion of pathogens can be mentioned (Ouwehand et al., 1999; Coconnier et al., 2000; Lievin-Le Moal et al., 2002; Servin and Coconnier, 2003).

A large variety of microorganisms with potential use as a probiotic have been isolated from kefir grains. These microorganisms produce organics acids, bacteriocins and have the ability to adhere to intestinal cells and antagonize intestinal pathogens (Yüksekdag et al., 2004; Powell et al., 2007; Golowczyc et al., 2007, 2008). Several lactobacilli strains isolated from kefir grains in our laboratory, were proved to have some characteristics present in a probiotic microorganism, among them can be mentioned: *L. kefir* CIDCA 8321 which adheres to Caco-2 cells and protects epithelial cells against *Salmonella* invasion *in vitro* when they are previously co-incubated (Golowczyc et al., 2007), *L. kefir* CIDCA 8348 that adheres strongly to Caco-2 cells (Golowczyc et al., 2007) and *L. plantarum* CIDCA 83114 inhibits *Salmonella* and *Escherichia coli in vitro,* is able to adhere to Caco-2 cells (Golowczyc et al., 2008) and antagonizes *E. coli* O157:H7 on Hep-2 cells (Hugo et al., 2008).

The preservation of microorganisms by different drying methodologies has been used for decades. Nowadays, workers have been investigating the use of spray drying (SD) as a convenient method for producing large quantities of some bacterial probiotic cultures (Gardiner et al., 2000; Desmond et al., 2001; Silva et al., 2002; Corcoran et al., 2004; Golowczyc et al, 2010). The principal advantages of SD are that it is less expensive and faster for producing large quantities of dried cells, than other techniques used to preserve microorganisms (Teixeira et al. 1995a, 1995b; Gardiner et al., 2000; Silva et al., 2005). The main disadvantage is that this process results in exposing the microorganisms to high temperatures which can be detrimental to the integrity of the cell (Teixeira et al., 1995a, 1995b; Gardiner et al., 2000; Silva et al., 2002, 2005). This process may affect a

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large number of cellular components, including DNA, RNA, cytoplasmic membrane and cell wall (Santivarangkna et al., 2008). Since some probiotic properties are closely related to the structure of the bacterial surface, it is very important to evaluate the cellular damage after SD to determine whether these spray-dried microorganisms maintain their properties. Therefore, the objective of this paper is to study injuries caused by the SD process on selected *Lactobacillus* spp., isolated from kefir grains and their effect on probiotic properties.

2. Materials and methods

2.1. Strains and growth conditions

Lactobacillus plantarum CIDCA 83114, *Lactobacillus kefir* CIDCA 8321 and *Lactobacillus kefir* CIDCA 8348 were previously isolated from kefir grains and identified (Garrote et al., 2001). Stock cultures were stored in 120 g/l non-fat milk solids at −80 °C. Working cultures were cultivated in De Man, Rogosa, Sharpe broth (MRS, Difco, Detroit, USA) at 30 °C for 48 h under static conditions. To prepare the inoculum, strains were inoculated into MRS broth (1% v/v) and incubated at 30 °C for 24 h (*L. plantarum*) or 48 h (*L. kefir*) under static conditions. Cells were harvested by centrifugation at 7000×*g* at 4 °C for 10 min and washed twice by centrifugation with sterile Ringer's solution.

Salmonella enterica serovar Enteritidis CIDCA 101 (Golowczyc et al., 2007) was used for invasion experiments. It was grown in nutrient broth (Biokar Diagnostics, Beauvais, France, ref no. BK003HA) for 18 h at 37 °C.

2.2. Spray drying process

The cell pellets obtained as described above were resuspended to the original volume in reconstituted skim milk powder (11% w/v, Oxoid, Hampshire, UK) 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 °C, inlet air temperature 160 °C and atomizing air pressure 3 Bar. Powder was collected in a single cyclone separator.

2.3. Enumeration of viable bacteria

To calculate the survivors after spray drying, 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 30 min and then, serially diluted. Bacteria were enumerated on MRS agar by the drop count technique.

2.4. Cell damage evaluation

The sensitivity of microorganisms to NaCl, lysozyme, bile salt and penicillin G, before and after drying processes, was determined. To select the adequate concentrations for each agent, the minimum inhibitory concentration (MIC) for each selective agent was determinated, and sub-inhibitory concentration was used (lower than MIC). All chemical additives except NaCl were added to MRS molten agar after filter sterilization. Fresh and dried *L. plantarum* cultures were plated on MRS agar plates supplemented separately with: 5% (w/v) NaCl (Merck), 10 mg/ml lysozyme (Sigma, St. Louis, MO, USA), 0.75 μg/ml penicillin G (Sigma) or 0.25% (w/v) bile salt (Sigma). Fresh and spray-dried *L. kefir* cultures were plated on MRS agar plates supplemented separately with: 2% (w/v) NaCl, 1 mg/ml lysozyme, 0.125 μg/ml penicillin G or 0.12% (w/v) bile salt. The plates were examined after 2–3 days of aerobic incubation and viable numbers were compared with numbers obtained on unsupplemented MRS plates (without these selective agents).

Kinetics of acidification of MRS and milk added with yeast extract (1% w/v) were performed with fresh and rehydrated spray-dried microorganism. One ml of fresh or rehydrated spray-dried microorganism were transferred to 49 ml of MRS or milk previously equilibrated at 30 °C (final concentration was approx. 2×10^6 CFU/ml). At regular intervals, samples were taken and pH values were determined.

2.5. Caco-2/TC-7 cell culture and adhesion assay

Growth conditions of cells culture and adhesion assays were carried out according to Golowczyc et al. (2007). Briefly, a Caco-2/TC-7 monolayer was incubated with 0.5 ml of fresh or rehydrated spray-dried lactobacilli suspension (2×10^8 CFU/ml of PBS) and 0.5 ml of DMEM of adhesion (without antibiotics or supplements) for 1 h at 37 °C in a 5% CO_2-95% air atmospheres. Then, the monolayer was washed and lysed (40–50 min) by adding sterile distilled water. To determine the number of viable lactobacilli adhered to Caco-2/TC-7 cells, appropriate dilutions in 0.1% (w/v) tryptone (Biokar Diagnostics) were plated in MRS and colony counts after 48 h incubation, were performed. Experiments were performed in triplicate on three consecutive cell passages.

2.6. Salmonella invasion assays

The invasion assay was carried out according to Golowczyc et al. (2007). Briefly, Caco-2/TC-7 monolayers at post-confluence were washed twice with sterile PBS (pH 7.2) and then 0.5 ml of *Salmonella* suspension (2×10^8 CFU/ml of PBS) and 0.5 ml of DMEM were added to each well and incubated for 1 h at 37 °C in a 5% CO_2-95% air atmosphere. Then, for counting only bacteria located inside Caco-2/TC-7 cells, 0.5 ml of gentamicin (Sigma, 100 μg/ml PBS) were added to each well and the monolayer was incubated for 1 h at 37 °C. After that, monolayer was washed twice, lysed by adding sterile distilled water and colony counts were performed on nutrient agar (Biokar Diagnostics, ref no. BK021HA).

To study the *L. kefir* protection against *Salmonella* invasion, fresh (washed twice with PBS) or rehydrated spray-dried *L. kefir* 8321 (2×10^8 CFU/ml) and *Salmonella* (2×10^8 CFU/ml) were mixed and co-incubated in PBS (pH 7.2) for 1 h at 37 °C. Then, 0.5 ml of the mixture was added to Caco-2/TC-7 monolayers and *Salmonella* invasion was determined as explained above.

2.7. Statistical analysis

Results were expressed as means ± standard deviation of at least two independent experiments. For statistical comparisons, analysis of variance (ANOVA) was performed using the statistical program InfoStat Software (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). The Student t test was used for mean comparison of survival after SD as well as Caco-2/TC-7 adhesion and invasion assays. All differences were considered statistically significant at a $p<0.05$.

3. Results and discussion

3.1. Survival after spray drying

Table 1 shows the ability of the three investigated lactobacilli to survive the spray drying process (column named “initial count”). No significant differences were obtained among the three samples in terms of survival. Under dehydration conditions used, *L. plantarum* 83114 and *L. kefir* 8321 showed very little reduction of viability (0.11 and 0.29 log CFU/ml respectively) while *L. kefir* 8348 was found to be more sensitive to the process with a reduction in viability of 0.70 log CFU/ml. Previously, we demonstrated that the outlet air temperature of 70 °C was associated with the highest survival rate for microorganisms during drying and subsequent storage (Golowczyc et al., 2010). For this reason, in this work the microorganisms were dried at an outlet air temperature of 70 °C, because