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Effect of sub-lethal high pressure homogenization treatments on the *in vitro* functional and biological properties of lactic acid bacteria

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ABSTRACT

The aim of this work was to assess if a sub-lethal high pressure homogenization (HPH) treatment could modulate *in vitro* functional and biological properties of probiotic bacteria. *Lactobacillus paracasei* A13, *Lactobacillus acidophilus* 08 and Dru, *Lactobacillus delbrueckii* subsp. *lactis* 200 and bile-resistant derivatives *L. acidophilus* Dru+ and *L. delbrueckii* subsp. *lactis* 200+ were suspended in phosphate buffered saline solution and treated at 50 MPa. Data obtained showed that HPH can modulate hydrophobicity and auto-aggregation without modification of viability and decarboxylase activity. Resistance to simulated gastric conditions resulted strain-dependent. High resistance was observed for treated *L. paracasei* A13, *L. acidophilus* Dru and 08 and *L. delbrueckii* subsp. *lactis* 200. The HPH-treatment reduced the resistance to simulated stomach duodenum-passage of *L. acidophilus* Dru while increased it for *L. paracasei* A13.

Strain viability and resistance to simulated gastric conditions were evaluated treating at 50 MPa cells suspended in acidified buttermilk (pH 4.6) and stored at 4 °C for 30 days. The highest cell viability loss, after 30 d of refrigerated storage, was observed for *L. acidophilus* Dru, independently of the application of HPH. However, after 30 days of storage, the resistance of *L. paracasei* A13 to simulated gastric digestion significantly increased in HPH treated cells.

1. Introduction

Functional foods represent one of the fastest growing areas in the global food industry because it is considered as a dietary strategy to reduce the incidence of illness in the humankind (Li, Zhang, Lee, & Pham, 2003). Among functional foods, certain food products have received great attention due to their importance as suitable vehicles for probiotic bacteria, defined as 'live microorganisms which when consumed in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Scientific evidence indicates that conditions such as diarrhoea, gastroenteritis, irritable bowel syndrome, inflammatory bowel disease, depressed immune function, cancer and genitourinary tract infections have all been reported to benefit from the ingestion of specific probiotic strains (Gupta & Garg, 2009; Hong et al., 2011).

Recently, a growing interest has raised toward some technologies, such as Pulsed Electric Field (PEF), High Hydrostatic Pressure (HHP), High Pressure Homogenization (HPH), that are able to enhance the survival of probiotic strains or to enhance their overall functionality (Cueva, 2009; Patrignani, Lanciotti, & Guerzoni, 2010). Although the majority of these technologies have been firstly studied as an alternative to thermal treatment for microbial inactivation (Knorr, 1999; Knorr, Zenker, Heinz, & Lee, 2004; Lado & Yousef, 2002; Shah, 2007; Wan, Conventry, Sanguansri, & Versteeg, 2009), several evidences proved their exploitation in the functional field. For example, Cueva (2009) investigated the effect of PEF treatment on viability, bile and acid tolerance and protease activity of *Lactobacillus acidophilus* LA-K, demonstrating that specific PEF conditions allowed the modulation of the functional characteristics of this strain.

Among the processes involving the pressure, the High Hydrostatic Pressure field (HHP) is one of the most studied (Kelly & Zeece, 2009; Senorans, Ibanez, & Cifuentes, 2003; Wan et al., 2005) and its efficacy in inactivating different microbial species is well documented (Ananta & Knorr, 2003; Desmond, Stanton, Fitzgerald, Collins, & Ross, 2001; Knorr & Heinz, 2001). As far as the contribution of high pressure processing for functional food formulation, the majority of the literature available deals with the effect of HHP on bioactive milk proteins. Also HPH has recently showed good potential for the formulation of functional foods, mainly probiotic products. In fact, in the functional dairy sector, HPH has been

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proposed to manufacture probiotic fermented milks and cheeses with improved sensorial or functional properties i.e. improved strain viability over refrigerated storage and accelerated fermentation kinetics (Burns, Patrignani, et al., 2008; Patrignani et al., 2009). Also Lanciotti, Patrignani, Iucci, Saracino, and Guerzoni (2007) showed that HPH is able to modify, in relation to the strain and to the treatment applied, both the fermentation kinetics and the enzymatic activities of starter and non starter lactic acid bacteria (LAB) without detrimental effects on cell viability. Regarding *in vitro* functional properties, to the best of our knowledge, only Muramalla and Aryana (2011) published on the use of some low homogenization pressures (up to 13.8 MPa for 5 passes) to improve certain probiotic characteristics of yogurt bacteria and *L. acidophilus* LA-K. These authors demonstrated that treatments at 13.80 and 6.90 MPa, repeated for 5 times, improved acid tolerance and bile tolerance, respectively, of *L. acidophilus* LA-K but had no effect on protease activity and its growth, recommending this technique for improvement of certain probiotic characteristics.

Thus, the aim of this work was to test if the application of a sublethal HPH treatment can positively modify the functional and biological properties of some strains of lactobacilli, already tested for their *in vitro* probiotic properties. In particular, the effects of a treatment of 50 MPa on the cell viability, cell hydrophobicity, cell auto-aggregation, bile resistance, bile salt deconjugation capacity, cholesterol assimilation, acid tolerance and the response to simulated stomach duodenum-passage were studied. The effects of storage temperature and suspension medium on cell viability and resistance to gastric conditions were also evaluated.

2. Materials and methods

2.1. Strains

Lactobacillus paracasei A13, *L. acidophilus* 08 and Dru, *Lactobacillus delbrueckii* subsp. *lactis* 200 and their bile-resistant derivatives *L. acidophilus* Dru+ and *L. delbrueckii* subsp. *lactis* 200+ were used. These strains had been previously tested for their functional properties and are commonly used in commercial dairy products (Burns, Reinheimer, & Vinderola, 2011; Burns, Vinderola, Binetti, et al., 2008; Vinderola, Prosello, Ghiberto, & Reinheimer, 2000).

Bile-resistant derivatives were obtained in a previous study by progressive adaptation to increased concentrations of bile salts (Burns, Vinderola, et al., 2008). The strains were maintained in de Man, Rogosa and Sharpe (MRS) broth (Biokar, Beauvais, France) with sterile glycerol (20 mL/100 mL) at −70 °C in the collection of the Instituto de Lactologia Industrial (INLAIN, UNL-CONICET, Santa Fe, Argentina). Fresh cultures of each strain were obtained by two consecutive daily transfers in MRS broth (Biokar, Beauvais, France) using a 1 mL/100 mL inoculum, incubated at 37 °C in aerobic conditions for 18 h.

2.2. High pressure homogenization treatment

Cell cultures grown in MRS broth for 18 h at 37 °C were harvested by centrifugation (8000 *g*, 10 min, 4 °C). Pellets were washed twice with 9 g/L NaCl solution and re-suspended in sterile phosphate buffered saline (PBS) solution (pH 7.4). The strains were subjected to a high pressure homogenization (HPH) treatment at 50 MPa with a high pressure homogenizer PANDA (Niro Soavi, Parma, Italy). The machine was supplied with a homogenizing PS type valve; the valve assembly includes a ball type impact head made of ceramics, a stainless steel large inner diameter impact ring and a tungsten carbide passage head. The inlet temperature of samples was 20 °C and the increase rate of temperature was 3 °C/10 MPa. As control samples, cell suspensions were treated at a level of 0.1 MPa in the homogenizer. Immediately after the treatments, the samples were rapidly cooled at 10 °C in a water bath.

2.3. Viability and in vitro probiotic properties

2.3.1. Cell viability

Cell viability was assessed before and immediately after the HPH treatment by cell counts on MRS agar (Biokar, Beauvais, France). Plates were incubated at 37 °C for 48 h in aerobic conditions.

2.3.2. Hydrophobicity, auto-aggregation capacity, cholesterol assimilation, bile resistance and bile salt deconjugation

The influence of HPH treatment on these parameters was assessed for cells treated with HPH suspended in PBS solution. The hydrophobicity, as the ability to adhere to hydrocarbons, was assessed according to Vinderola and Reinheimer (2003). The hydrophobicity percentage was calculated with the formula $[(A0 - At)/A0] \times 100$, where At represented the absorbance at 560 nm after 1 h of incubation at 37 °C and A0 the absorbance at t0.

Auto-aggregation assay and cholesterol assimilation test were performed according to Mathara et al. (2008). As far as auto-aggregation, HPH-treated or control strains were suspended in PBS solution at a level of approximately 10^8 CFU/mL and 4 mL were mixed by vortexing for 10 s. Auto-aggregation was determined during 5 h of incubation at 37 °C. Every hour 0.1 mL of the upper suspension was transferred to another tube with 0.9 mL of PBS and the absorbance (A) was measured at 600 nm. The auto-aggregation percentage was expressed as $1-(At1/A) \times 100$, where At1 represented the absorbance at 600 nm after 5 h of incubation at 37 °C and A the absorbance at t0 (immediately after HPH treatment). The ability of HPH-treated or control cells to grow in the presence of bovine bile was determined according to Vinderola and Reinheimer (2003). Cultures were incubated at 37 °C for 24 h in the presence of 0.3, 0.5 and 1 g/100 mL of bovine bile (Sigma, Milan, Italy) and the results were expressed as the percentage of growth (A560 nm) in the presence of bile compared to a control culture (without bile salts).

The strain capacity to deconjugate bile salts (BSH activity) was determined according to Dashkevicz and Feighner (1989) with some modifications suggested by Mathara et al. (2008). Cultures were grown onto MRS agar plates supplemented with 0.5 g/100 mL sodium salt of taurodeoxycholic acid (Sigma, Milan, Italy) and 0.37 g/L of $CaCl_2$ at 37 °C for 48 h. The BSH activity was detected by the presence of precipitation zones on the plates.

2.3.3. Tolerance to simulated gastric acidity (SGA) and simulated stomach duodenum-passage (SSDP) immediately after HPH treatment

Two different stresses were applied to control or HPH-treated cells: exposure to SGA and to SSDP. To determine the tolerance of cells to SGA, a solution of $CaCl_2$ (0.022 g/100 mL), NaCl (1.62 g/100 mL), KCl (0.22 g/100 mL) and $NaHCO_3$ (0.12 g/100 mL) was used according to Vinderola and Reinheimer (2003). One volume of this solution was added to one volume of the different samples and the pH was adjusted to pH 2.5 with HCl 8 mol equi/L. Viable counts were performed, as detailed above, before and after an incubation period of 90 min at 37 °C. Results were expressed as the differences in Log cell counts between time zero and the end of the experiment (referred to cell load decreases).

The resistance to SSDP was performed immediately after HPH treatment on cell suspensions in PBS having a pH value of 3 adjusted with HCl 8 mol equi/L according to Mathara et al. (2008). For each sample (200 mL), 10 mL were withdrawn after 1 h of incubation at 37 °C at pH 3 and 4 mL of 10 g/100 mL oxgall solution