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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

(Sigma, Milan, Italy) were added, followed by 17 mL of synthetic duodenum juice, prepared by completely dissolving $NaHCO_3$ (6.4 g/L), KCl (0.239 g/L), and NaCl (1.28 g/L) in distilled water and by adjusting the pH to 7.4 with 5 mol/L HCl before sterilising at 121 °C for 15 min (Mathara et al., 2008). After mixing, the samples were further incubated at 37 °C and viable counts were performed after 60, 120, 180 and 240 min of incubation by plate counting. Results were expressed as the differences in Log cell counts between time zero and the end of the experiment (referred to cell load decreases).

2.4. Safety properties

2.4.1. Antibiotic resistance

Antibiotic susceptibility of control and HPH treated cells in PBS was determined according to Belletti et al. (2009) with some modifications. M.I.C.E valuator™ (M.I.C.E.™) strips, obtained from Oxoid (Basingstoke, United Kingdom), were used. The antibiotic susceptibility of control and treated cells was determined for 11 antibiotics (ampicillin, erythromycin, gentamycin, tetracycline, amoxycillin, penicillin G, levofloxacin, ciprofloxacin, oxacillin, linezolid and vancomycin). Through the application of an M.I.C.E. strip to a pre-inoculated agar plate, the antimicrobial released from the polymer strip, formed a defined concentration gradient in the area surrounding it. After incubation at 37 °C for 3 days, the inhibition halo around the strip allowed the rapid and accurate determination of the Minimally Inhibitory Concentration (MIC) of the target strain to the tested antibiotics.

2.4.2. Biogenic amine production

Screening for biogenic amine production (histamine, tyramine, cadaverine and putrescine) of control and HPH treated cells suspended in PBS was investigated by means of amino acid decarboxylation test. In fact, the presence of decarboxylase activity was evaluated by an agar plate assay using the improved decarboxylase differential growth medium with or without (negative control) the corresponding amino acid precursor (1 g/100 mL) and the procedure described by Bover-Cid and Holzapfel (1999).

2.5. Viability and tolerance to SGA during the refrigerated storage in a dairy medium

To study the tolerance to SGA, the strains were grown in MRS medium for 18 h at 37 °C. After harvesting by centrifugation (8000 g, 10 min, 4 °C), the cells were re-suspended in buttermilk, acidified to pH 4.6 with lactic acid (Sigma, Milan, Italy) and treated at 50 MPa or not (control). This lyophilized medium was purchased in a local dairy industry, previously reconstituted (77 g/L) and sterilized at 115 °C for 30 min.

The viability and the SGA of control and HPH-treated strains were monitored over a refrigerated period of 30 days by plate counts according to the methods previously described. Buttermilk was chosen as a dairy medium able to confer adequate growth and survival during storage of LAB, according to previous studies (Burns, Vinderola, Molinari, & Reinheimer, 2008).

2.6. Data statistical analysis

The results obtained are the mean of six independent replicates (carried out on different days). The data were analysed by 1-way ANOVA using the statistical package Statistica for Windows 6.1 (Statsoft Inc., Tulsa, OK). The differences between mean values were detected by the HSD Tukey's test.

3. Results

3.1. Cell viability followed HPH treatment

The High Pressure Homogenizaton (HPH) treatment performed (50 MPa) did not statistically modify the viability of cells suspended in PBS. In fact, the treatment at 50 MPa reduced the cell loads of the strains under study in a magnitude lower than 0.2 Log CFU/mL (data not shown).

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

The results for cell hydrophobicity test are shown in Fig. 1. The HPH treatment induced a significant reduction of cell hydrophobicity for all strains, except for *L. paracasei* A13 whose hydrophobicity significantly increased ($p < 0.05$) approximately 5 folds (as observed by Tabanelli et al., 2012).

The auto-aggregation test was performed on control and HPH-treated cells suspended in PBS solution. Fig. 2 shows the percentage of auto-aggregation for the strains under study. The HPH treatment caused a reduction of this property for *L. delbrueckii* subsp. *lactis* 200 and 200+, *L. acidophilus* Dru+ and 08. Differently, the auto-aggregation of *L. acidophilus* Dru was not affected by the treatment while *L. paracasei* A13 increased its auto-aggregation capacity (over one third) after the HPH treatment.

Bile resistance was tested for control and HPH-treated cells at increasing bile salt concentrations (0.3, 0.5, 1.0 g/100 mL). The data showed that the HPH treatment did not affect the bile resistance, except for *L. acidophilus* Dru+ which increased of about one third its resistance at a bile salt concentration of 0.3 g/100 mL. Among the strains studied, *L. acidophilus* 08 was the one showing the highest bile salt resistance. In fact, the viability of this strain was not affected even by the highest bile concentration considered. Also *L. paracasei* A13 was very resistant to the bile. In fact, it slightly decreased (25%) the absorbance value recorded after 24 h of exposure to 0.3 g/100 mL of bile growth with respect to control without bile (data not shown).

All *L. acidophilus* strains displayed the capacity to deconjugate bile salts, having the HPH treatment no effect on this capacity. The other strains considered were unable to deconjugate bile salts. The HPH treatment was able to increase the values of cholesterol assimilation for *L. paracasei* A13, *L. delbrueckii* subsp. *lactis* 200+ and *L. acidophilus* Dru ranging between 10 and 15% while decreases

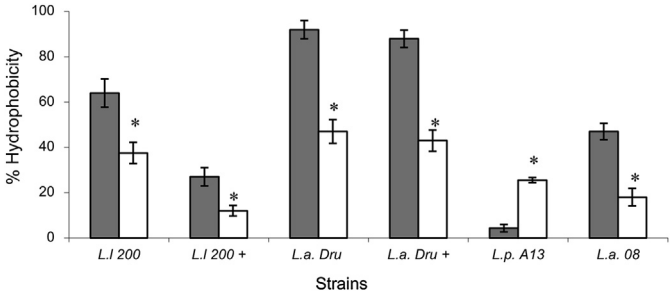

Fig. 1. Cell hydrophobicity of *L. delbrueckii* subsp. *lactis* 200, 200+, *L. acidophilus* Dru, Dru+, *L. paracasei* A13 and *L. acidophilus* 08 treated at 0.1 MPa (grey bar) and 50 MPa (white bar) of HPH. The hydrophobicity percentage was calculate with the formula $[(A0 - At)/A0] \times 100$, where At represented the absorbance at 560 nm after 1 h of incubation at 37 °C and A0 the absorbance at t0. For each strain considered the statistical analysis was performed between control and cells treated at 50 MPa. The results obtained are the mean of six independent replicates (carried out on different days). * Statistically different ($p < 0.05$). +: bile-resistant derivatives.

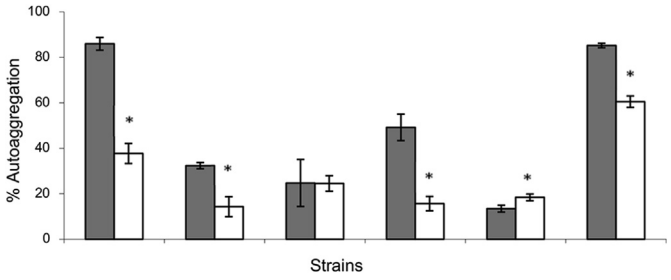

Fig. 2. Cell auto-aggregation of *L. delbrueckii* subsp. *lactis* 200, 200+, *L. acidophilus* Dru, Dru+, *L. paracasei* A13 and *L. acidophilus* 08 treated at 0.1 MPa (grey bar) and 50 MPa (white bar) of HPH. The auto-aggregation percentage was expressed as 1−(At1/A) × 100, where At1 represented the absorbance at 600 nm after 5 h of incubation at 37 °C and A the absorbance at t0. For each strain considered the statistical analysis was performed between control and cells treated at 50 MPa. The results obtained are the mean of six independent replicates (carried out on different days). * Statistically different ($p < 0.05$). +: bile-resistant derivatives.

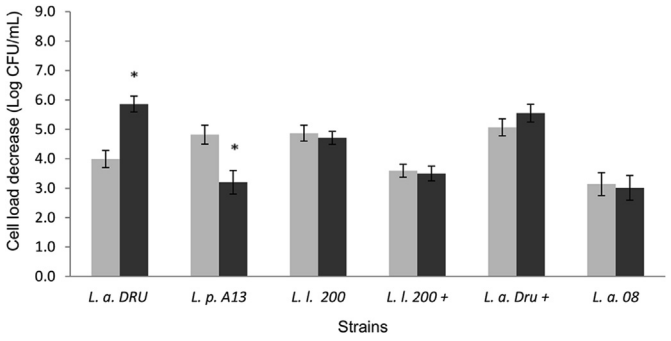

Fig. 4. Cell load reductions (Log CFU/mL) due to exposure to simulated stomach duodenum passage (SSDP) in phosphate buffer solution solution of cells treated at 0.1 MPa or 50 MPa immediately after the sub-lethal HPH treatment. For each strain considered the statistical analysis was performed between control and cells treated at 50 MPa. The results obtained are the mean of six independent replicates (carried out on different days). * Statistically different ($p < 0.05$). +: bile-resistant derivatives.

of 15 and 20% were observed for the strains *L. acidophilus* Dru+ and *L. acidophilus* 08, respectively (data not shown).

3.3. Tolerance to SGA and SSDP followed HPH treatment

The resistance to simulated gastric acidity resulted strain-dependent. Bile-resistant derivatives *L. acidophilus* Dru+ and *L. delbrueckii* subsp. *lactis* 200+ showed a rapid decrease of viability independently of the treatment. In fact, they attained levels under the detection limit (1.0 Log CFU/mL) in 90 min of exposure (Fig. 3). Higher resistance was observed for by *L. paracasei* A13 *L. acidophilus* Dru and 08 and *L. delbrueckii* subsp. *lactis* 200. HPH-treated cells of these strains showed a higher resistance with respect to their controls.

Generally the SSDP assay showed high rates of survival and indicated higher tolerance to acid and bile environment for the strains taken into consideration (Fig. 4). The HPH treatment reduced the resistance to simulated stomach duodenum-passage of the strains *L. acidophilus* Dru while it increased that of *L. paracasei* A13 in ca. 1.5 Log CFU/mL. The resistance to SSDP of the remaining strains was not affected by the HPH treatment.

3.4. Antibiotic resistance and biogenic amine production

Regarding the antibiotic resistance, among the tested strains, *L. acidophilus* Dru, Dru+, 08 and *L. delbrueckii* subsp. *lactis* 200 were resistant to ciprofloxacin at concentrations ranging between 0.002 and 32 μg/mL. Moreover, treated and control cells of *L. paracasei* A13 exhibited resistance to vancomycin between 0.015 and 256 μg/mL while *L. acidophilus* strains were resistant to levofloxacin (0.015–256 μg/mL) (data not shown). The sub-lethal HPH treatment did not modify the antibiotic resistance of the strains.

None of the strains showed decarboxilase activity on the amino acids under study (tyrosine, histidine, ornithine and lysine). The application of HPH treatment did not modify the decarboxilase activity of the strains.

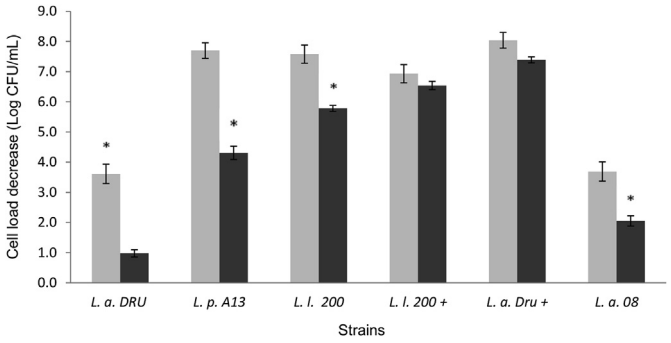

Fig. 3. Cell load reductions (Log CFU/mL) due to exposure to simulated gastric acidity (90 min) in phosphate buffer solution of cells treated at 0.1 MPa (grey bar) or 50 MPa (black bar) immediately after the sub-lethal HPH treatment. For each strain considered the statistical analysis was performed between control and cells treated at 50 MPa. The results obtained are the mean of six independent replicates (carried out on different days). * Statistically different ($p < 0.05$). +: bile-resistant derivatives.

3.5. Viability and tolerance to SGA during the refrigerated storage in a dairy matrix

Considering buttermilk acidified at pH 4.6, no significant differences in cell counts between control and HPH-treated cells were observed over the 30 d storage period except for *L. acidophilus* Dru+ (Table 1). This strain when subjected to HPH treatment maintained a higher cell load (ca. 1.0 Log CFU/mL) compared to its control. After 30 d of storage at 4 °C, the higher lost in cell viability was observed for *L. acidophilus* Dru which attained levels of about 3.0 Log cycles, independently of the application of the HPH treatment. On the contrary, *L. paracasei* A13 maintained levels higher than 7.0 Log CFU/mL also after 30 days of storage. *L. delbrueckii* subsp. *lactis* 200 and 200+ reduced their viability in about 1.5 Log CFU/mL by the end of the refrigerated storage period.

Finally, the control and treated cells stored at 4 °C in acidified buttermilk were exposed to a simulated gastric digestion (Table 1). It is showed that, immediately after the treatment, *L. acidophilus* Dru and 08 were the most resistant ones regardless the treatment applied while the other strains were very sensitive. The HPH negatively affected the resistance of *L. acidophilus* Dru and 08 and *L. paracasei* A13. However, after 30 days of storage, the resistance of HPH-treated *L. paracasei* A13 to SGA significantly increased.

4. Discussion

The data obtained showed the capacity of a sub-lethal treatment with HPH to enhance some *in vitro* probiotic features and related properties (i.e. hydrophobicity and tolerance to simulated gastric acidity) in a strain-dependant way. This technology has already demonstrated to possess a great potential to improve some LAB properties when applied at sub-lethal levels. In fact, it has been reported that HPH did not affect LAB cell viability but increased their technological properties such as fermentation time and

Table 1
Viability and cell load reductions (Log CFU/mL) due to exposure (90 min) to simulated gastric acidity of cells stored at 4 °C in acidified buttermilk (pH 4.6) in relation to the pressure applied.

Strain	Pressure (MPa)	Cell counts (Log CFU/mL) over storage			Cell load reduction (Log CFU/mL) after simulated gastric acidity over storage		
		0 d	15 d	30 d	0 d	15 d	30 d
L. paracasei A13	0.1	8.0 (0.2)[a]	8.2 (0.3)	7.4 (0.3)	−4.8 (0.2)[a]	−8.2 (0.3)	−5.8 (0.2)^{A}
	50	8.0 (0.3)	8.1 (0.2)	7.5 (0.3)	−8.0 (0.3)	−8.1 (0.2)	−4.4 (0.3)^{B}
L. acidophilus Dru	0.1	7.6 (0.3)	7.6 (0.4)	3.3 (0.3)	−1.3 (0.2)	−3.7 (0.1)	−1.9 (0.1)^{C}
	50	7.7 (0.4)	7.6 (0.3)	3.3 (0.3)	−3.4 (0.2)	−3.8 (0.2)	−3.3 (0.2)^{D}
L. acidophilus Dru+	0.1	8.1 (0.3)	7.6 (0.3)	6.2 (0.3)^{A}	−8.1 (0.3)	−7.6 (0.3)	−6.2 (0.2)^{E}
	50	7.8 (0.2)	7.3 (0.3)	7.3 (0.3)^{B}	−7.8 (0.2)	−7.3 (0.2)	−7.3 (0.3)^{F}
L. acidophilus 08	0.1	8.9 (0.4)	8.7 (0.3)	8.2 (0.2)^{C}	−1.3 (0.1)	−3.5 (0.1)	−2.0 (0.1)^{G}
	50	9.0 (0.3)	8.5 (0.3)	7.9 (0.2)^{D}	−3.0 (0.2)	−3.6 (0.2)	−3.4 (0.1)^{H}
L. delbrueckii lactis 200	0.1	7.1 (0.2)	7.2 (0.3)	5.8 (0.3)	−6.1 (0.2)	−6.5 (0.3)	−5.8 (0.2)
	50	6.9 (0.4)	6.7 (0.3)	5.9 (0.2)	−6.9 (0.2)	−5.7 (0.2)	−5.9 (0.3)
L. delbrueckii lactis 200+	0.1	8.0 (0.3)	7.9 (0.3)	6.6 (0.2)	−8.0 (0.3)	−7.9 (0.3)	−6.6 (0.3)
	50	7.7 (0.2)	7.3 (0.2)	6.6 (0.3)	−7.6 (0.2)	−7.3 (0.3)	−6.6 (0.2)

+: bile-resistant derivatives.
For each strain considered the statistical analysis was performed between cell treated at 0.1 and at 50 MPa. Values, for the same strain, with different superscript are statistically different ($p < 0.05$). Results are the mean of six independent replicates (carried out on different days). The detection limit for the method employed is 1 CFU/mL.
[a] Standard deviation.

hydrolytic properties, among other. (Lanciotti et al., 2007; Patrignani, Lanciotti, Mathara, Guerzoni, & Holzapfel, 2006). The HPH treatment severity was chosen to not affect cell viability because it is an indicator of probiotic capacity (Granato, Branco, Cruz, Faria, & Shah, 2010; Vinderola & Reinheimer, 2003). In fact, reduction in cell counts after a 50 MPa HPH treatment did not exceed 0.20 Log CFU/mL and was not significant. The data obtained in this work about cell viability after HPH treatment are in agreement with previously reports (Lanciotti et al., 2007). Vannini, Lanciotti, Baldi, and Guerzoni (2004) also observed a scarce viability reduction after HPH of some *Lactobacillus helveticus* and *Lactobacillus plantarum* strains using pressure levels up to 130 MPa, confirming the tolerance to moderate pressure of LAB.

Only *L. paracasei* A13 increased its hydrophobicity and autoaggregation capacity after HPH treatment at 50 MPa. On the contrary, the HPH treatment decreased these features in the other strains considered. Highest values of hydrophobicity were found for *L. acidophilus* Dru and its bile-resistant derivative *L. acidophilus* Dru+, while lower values were obtained for *L. paracasei* strain in agreement with previously reported values (Mathara et al., 2008). Although several mechanisms are involved in the adhesion of microorganisms to intestinal epithelial cells, hydrophobicity and autoaggregation are important because they correlate with the microbial capacity to adhere and, at least temporarily, to colonize the intestinal epithelium (Del Re, Sgorbati, Miglioli, & Palenzona, 2000; Kiely & Olson, 2000; Schillinger, Guigas, & Holzapfel, 2005; Zareba, Pascu, Hryniewicz, & Waldström, 1997). In particular, hydrophobicity is reported to confer a competitive advantage, important for bacterial maintenance in the human gastrointestinal tract (Schillinger et al., 2005). The literature data concerning the effects of pressure on cell hydrophobicity is scarce. The different hydrophobicity characteristic of the strains in relation to HPH treatment can be attributed to the compositional and structural differences in the cellular outer structures. Lanciotti, Gardini, Sinigaglia, and Guerzoni (1996) showed the ability of sub-lethal hydrostatic pressure treatments to modify the hydrophobicity in yeasts and this feature resulted strain dependent. This increase of hydrophobicity has been attributed to the formation of a complex between polar groups of cell wall and ions of the system. Iucci, Patrignani, Vallicelli, Guerzoni & Lanciotti (2006) attributed the enhanced activity of HPH-treated lactoferrin and lysozyme against *Listeria monocytogenes* to the increase of molecular hydrophobicity induced by the HPH treatment. HPH treatment is reported to increase the exposure of hydrophobic regions of proteins (Guerzoni et al., 1999). The well-documented HPH effects on macromolecules can explain the modifications of the strain hydrophobicities suggesting also a key role in the interactions with the gut immune cells.

Concerning the bile resistance, the HPH treatment did not significantly affect strain tolerance to bile, except for *L. acidophilus* Dru+ which increased of about one third its resistance at a bile concentration of 0.3 g/100 mL. Also Muramalla and Aryana (2011) showed, studying the effect of low pressure homogenization on yogurt bacteria and *L. acidophilus* LA-K, that the changes of this property in relation to the pressure applied was strain-dependent. No decrease in bile resistance was observed after the HPH treatment both in sensitive and resistant strains. Among the resistant strains, *L. acidophilus* 08 showed the highest tolerance and it is interesting to note that it was able to grow even in presence of 1 g/100 mL bile, exceeding the physiological human bile level (Mathara et al., 2008), also when subjected to a sub lethal treatment of 50 MPa. The capacity of deconjugating bile salts was neither affected by HPH. The treatment did not induce the acquirement of this ability in strains such as *L. paracasei* A13 which was not endowed with this feature.

As far as the tolerance to simulated gastric acidity (SGA), HPH can influence this property even if it resulted strain-dependent. *L. paracasei* and *L. acidophilus* (and in particular HPH-treated cells) were in general more resistent than *L. delbrueckii* subsp. *lactis*. Also Muramalla and Aryana (2011) demonstrated that sublethal HPH treatment can improve the acid tolerance of specific strains with respect to untreated controls. As far as the SSDP assay, cells showed high rates of survival, independently of the HPH treatment, probably because this test was performed at a higher pH value (pH 3) than the SGA assay. In addition, the different cell response mechanisms could be induced by combined stress application (acidity and bile). It is interesting to note that HPH-treated *L. paracasei* A13 showed an enhanced resistance to simulated gastric digestion in both assays performed (resistance to SGA and to SSDP).

The tolerance to simulated SGA was also affected by the storage period. Homogenized cells were less acid resistant than untreated cells immediately after the HPH-treatment for all the strains under study. After 30 days, instead, HPH-treated *L. paracasei* A13 cells maintained higher acid resistance than control cells. This could be explained by the mechanisms of stress response to the initial conditions (i.e. pressure, low pH). It is well-known that these stress responses enable survival under more severe conditions, enhancing

resistance to subsequent processing conditions (Chung, Chen, Yet, Layne, & Perrella, 2006).

The applied HPH treatment did not modify the antibiotic resistance and the decarboxilase activity of the strains. These features had been considered due to the importance they have in the selection criteria of potential probiotic bacteria (Saarela, Mogensen, Fonde'n, Mättö, & Mattila-Sandholm, 2000) and to exclude a potential negative effect of HPH treatment on the transfer of gene involved in antibiotic resistance and on the activation of the enzymes involved in the production of biogenic amines.

The data obtained showed that HPH treatment at 50 MPa can favour the maintenance of cell viability during a refrigerated storage in buttermilk, a suitable medium to maintain the cell viability during refrigeration (Burns, Vinderola, Molinari, et al., 2008). The high cell loads recorded after 30 d of storage at 4 °C for the HPH treated cells are in agreement with the findings of Burns, Patrignani, et al. (2008) and Patrignani et al. (2009). According to these authors, this increased viability can be attributed to the increased precocious availability of low molecular weight peptides and free fatty acids such as oleic acid.

5. Conclusions

This study showed that the HPH treatment could increase some important functional characteristics in a strain dependant way. The response to the treatment varied according to the strain as well as the time of storage and the suspension media. Since probiotic strains have to maintain not only a good viability but also a good functionality during manufacture, storage and consumption, the results of the present study, enlarge the knowledge of the effect of HPH on the strain properties and could be exploited to improve the quality of functional foods. In particular, *L. paracasei* A13 enhanced its *in vitro* probiotic features by HPH treatment. The application of a treatment able to enhance the raw material quality in terms of safety (microbial inactivation) and nutrient availability to milk inoculated with probiotic strains could result in safer products with enhanced functional properties. Further investigations are necessary to elucidate the *in vivo* effects of HPH-treatment on functional properties.

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