Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 102 (2007) 542–550
Food Chemistry
www.elsevier.com/locate/foodchem

Potential of high pressure homogenization in the control and enhancement of proteolytic and fermentative activities of some *Lactobacillus* species

Rosalba Lanciotti, Francesca Patrignani, Luciana Iucci, Pasquale Saracino, M. Elisabetta Guerzoni *

Dipartimento di Scienze degli Alimenti, University of Bologna, via Fanin 46, 40127 Bologna, Italy

Abstract

Different species of *Lactobacillus* involved in dairy product fermentation and ripening were considered in order to study the effect of high pressure homogenization (HPH) on: (i) fermentation kinetics of HPH treated cells inoculated in milk; (ii) metabolic profiles; (iii) release of intracellular proteolytic enzymes; and (iv) enhance of the activity of extracellular or cellular wall located proteolytic enzymes. The HPH treatments applied were 50, 100, 150 MPa, 2 cycles at 50 and at 100 MPa. The viability loss did not exceed 1.3 log cfu/ml after the higher treatments applied. The electrophoretic profiles of α- or β-casein incubated with the different cell free filtrates shown that HPH positively affected the proteolytic activity of some strains. Moreover, HPH affected the acidification rates of the milk inoculated with the processed cells and the primary metabolism of some strains. Regarding volatile compounds, ethanol, acetoin and 2-methyl butyric acid were subjected to the major changes when the inoculum had been processed.

Keywords: High pressure homogenization; Proteolytic activity; *Lactobacillus* species; Fermentative kinetics

1. Introduction

Semi-continuous high pressure homogenization (HPH) is a technology which has been proposed for the non-thermal fluid food microbial decontamination. Its effectiveness in the inactivation of pathogenic and spoilage microorganisms in model and real systems is well documented (Diels, Wuytack, & Michiels, 2003; Kheadr, Vachon, Paquin, & Fliss, 2002; Lanciotti, Gardini, Sinigaglia, & Guerzoni, 1996; Lanciotti, Sinigaglia, Angelici, & Guerzoni, 1994; Thiebaud, Dumay, Picart, Guiraud, & Cheftel, 2003; Vachon, Kheadr, Giasson, Paquin, & Fliss, 2002; Wuytack, Diels, & Michiels, 2002). The application of this process, alternative to heat treatment, to improve safety and microbiological quality of milk and whole liquid eggs has been proposed (Guerzoni, Lanciotti, Westall, & Pittia, 1997; Guerzoni, Vannini, Lanciotti, & Gardini, 2002). Cavitation and viscous shear have been identified as the primary mechanisms of microbial cell disruption during HPH (Kleinig & Middelberg, 1998; Middelberg, 1995). In addition to the effects on microbial cells, HPH treatment is active on food constituents, especially proteins, leading to changes in their functional properties and activities (Kheadr et al., 2002; Vannini, Lanciotti, Baldi, & Guerzoni, 2004). More specifically, HPH treatment of skim and whole milk has been reported to modify the ratio of the nitrogen fractions and the soluble forms of calcium and phosphorous, improve the coagulation characteristics of milk as well as increase the cheese yields (Guerzoni et al., 1999; Hayes & Kelly, 2003a; Hayes, Fox, & Kelly, 2005; Humbert, Driou, Guerin, & Alais, 1980; Kheadr et al., 2002; Lanciotti et al., 2004). Moreover, the HPH treatment of milk was associated with an enhancement and an acceleration of both proteolytic and slytic activities of goat cheese during ripening (Guerzoni et al., 1999). Accelerated

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[*] Corresponding author. Tel.: +39 0 51 2096573; fax: +39 0 51 2096574.
E-mail address: guerzoni@foodsci.unibo.it (M.E. Guerzoni).

0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.06.043

lipolysis has also been observed in Crescenza, a traditional Italian soft cheese, when produced using milk HPH-treated at 100 MPa (Lanciotti et al., 2004). In addition, the principal biotechnological applications regarded large-scale cell disruption also for the recovery of intracellular metabolites or enzymes (Clarkson, Lefevre, & Titchenerhooker, 1993; Geciova, Bury, & Jelen, 2002; Keshavarz-Moore, Hoare, & Dunnill, 1990) and activation or deactivation of enzymes (Fantin et al., 1996; Hayes & Kelly, 2003b; Vannini et al., 2004). In particular, Vannini et al. (2004) reported that high pressure homogenized antimicrobial enzymes such as lysozyme and lactoperoxisase presented an enhanced activity against several spoilage and pathogenic species. These Authors attributed this activation to an increased exposure of hydrophobic regions of proteins. The hydrophobicity seems to be a key factor for the enhanced antimicrobial action of chemically modified or heat treated lysozyme (Bernkop-Schnurch, Krist, Vehabovic, & Valenta, 1998; Ibrahim, Kato, & Kobayashi, 1993; Ohno & Morrison, 1989). It has been reported that the large supramolecular protein structure is disrupted under hydrostatic pressure, allowing the components to move freely and become independent of the original structure. However, interactions can reform when the pressure instantaneously decreases but the original structure is not reformed because of the independent movements of the components (Payens & Heremans, 1969).

The temperature increase during the process is a key process parameter for enzyme activity modification and microbial inactivation (Hayes et al., 2005). According to Grandi, Rainieri, Guerzoni, and Pagliarini (2005) the temperature increase derives during the pressure drop from the pressure energy transformation into thermal energy and corresponds to about 12 °C each 50 MPa. In a previous work, when the inlet temperature was 5–7 °C and the outlet temperature did not exceed 30 ± 2 °C, activation of endogenous and microbial proteolytic enzymes was observed in cheeses obtained from caw and goat milks treated at 100 MPa (Lanciotti et al., 2006). The Authors suggested that the treatment, as depending on pressure and temperature, increased the activity of milk and extracellular enzymes and of the enzyme located on the cell envelope.

The main components of the proteolytic system of the LAB are the cell envelope associated proteinases, although intracellular proteinases have been reported (Upadhyay, McSweeney, Magboul, & Fox, 2004). Moreover, amino acid and peptide transport systems, and a range of intracellular peptidases have been described. The LAB proteolytic enzymes and their release are the determinant factors in cheese ripening and in the flavour development (Chepot-Chartier, Deniel, Rousseau, Vassal, & Gripon, 1994; Fox, 1998; Gatti, Fornasari, Lazzi, Muccheti, & Neviani, 2004). The various LAB species are characterized by different protease activities and complex systems of endo- and exo-peptidase, differing in nature, specificity and cell location (Mommet & Gripon, 1994). According to Gatti et al. (2004), while a few peptidases were not dependant on their confinement to particular cell compartments, the major part of aminopeptidase activities were partially inhibited or affected by the compartimentalization.

In this work different strains belonging to *Lactobacillus* species, involved in dairy product fermentation and ripening, were considered in order to study the effect of HPH treatment on their proteolytic and metabolic activities. In particular, the aim was to evaluate the potential use of HPH as a tool to:

(i) control the fermentation kinetics of processed cells of LAB to be used as starter;
(ii) modify the metabolic profiles of processed cells;
(iii) increase the release of proteolytic enzymes located in the cytoplasm;
(iv) enhance the activity of extracellular or cellular wall located proteolytic enzymes.

2. Materials and methods

2.1. Strains

The strains employed in this experimental work, belonging to the collection of Dipartimento di Scienze degli Alimeti of Bologna University, were *Lactobacillus arizonensis* 143, 21, *Lactobacillus casei* 28, 80, *Lactobacillus pentosus* 83, 57, 37 and *Lactobacillus plantarum* 75, 42, 8, 186, 147, 63, 58. All the strains were isolated from Caciotta, a typical italian cheese.

Cells were initially grown on MRS Medium (Oxoid, Basingstoke, UK) incubated at 37 °C for 48 h under anaerobic condition. After growth, for each strain, a colony was taken from solid medium and inoculated, under sterile conditions, in flask containing 1000 ml of MRS broth (Oxoid, Basingstoke, UK). The culture broths were incubated an overnight at 37 °C.

2.2. High pressure homogenization treatment

The cells, grown in MRS medium, were refrigerated at 10 °C and subjected to different high pressure homogenization (HPH) treatments. In particular, the suspensions were treated for one cycle at 50, 100 and 150 MPa or for two cycles at 50 and 100 MPa. A continuous high pressure homogenizer PANDA (Niro Soavi, Parma, Italy) was used for all homogenizing treatments. 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 10 °C and the increase rate of temperature was 3 °C/10 MPa. As control samples, for each strain tested, untreated cell suspensions were used. Before and immediately after the HPH treatment, the cell loads were determined by plate counting onto MRS medium (Oxoid, Basingstoke, UK), at the same conditions previously described.

2.3. Proteolytic activity on α- and β-casein

Aliquots of 2 ml of each sample (controls and HPH treated samples) were centrifuged at 7000*g* for 15 min. About 0.5 ml of the different cell free supernatants were incubated at 30 °C for 24 h with 4 ml of α- or β-casein (having a final concentration of 7.6 g/l), 0.8 ml of 0.25 M phosphate Buffer, pH 7.00, and 0.12 ml of $NaNO_3$ (having a final concentration of 0.2 g/l) The reactions were stopped by putting the samples at −18 °C. SDS-polyacrylamide gel electrophoresis was performed according to the method proposed by Andrews (1983) with the following modification: the separating and the stacking gel contained 15% and 5% acrylamide, respectively, in Tris–glycine buffer pH 8.3. The samples were mixed (1:1 v/v) with Leammli Sample Buffer (Bio-Rad, Milan, Italy). Electrophoresis was performed with an SE600 Vertical Slab Gel Unit (Hoefer Scientific, San Francisco, CA) whose power supply (Power Pac 3000, Bio-Rad Laboratories, UK) was set at 70–80 V for the stacking gel and then increased to 250 V for the separating gel for approximately 45 min. Gels were fixed and stained with Coomassie Blue G250 for 2 h and de-stained in a 50 ml/l methanol and 70 ml/l acid acetic solution for 2–4 h.

2.4. Fermentation kinetics in milk

About 100 ml of fresh whole milk were previously heat treated at 105 °C for 7 min and inoculated with 2 ml of HPH treated or control cell cultures obtained as previously described. For every strain and for each pressure level applied, samples were incubated at 37 °C. After reaching pH 4.6, the samples were stored at 4 °C for 12 h. Acidification kinetics were followed using a pH-meter Hanna Instruments 8519 (Incofar, Modena, Italy). The data collected are the mean of three independent repetitions. After the refrigerated storage, the cell loads of each strain in fermented milk, in relation to the HPH treatment applied, were evaluated by plate counting on MRS agar medium (Oxoid, Basingstoke, UK).

2.5. Aromatic profiles

The volatile compounds of coagula obtained were monitored immediately after 12 h of refrigerated storage by using a gas-chromatographic-mass spectrometry coupled with solid phase micro extraction (GC-MS-SPME) technique. For each coagula, 5 g sample were sealed in sterilized vials. Samples were heated at 40 °C for 15 min and volatiles adsorbed for 60 min on a fused silica fibre covered by Carboxen Polydimethyl Siloxane (CAR-PDMS), 75 μm (Supelco, Stheiheim, Germany). Adsorbed molecules were desorbed in the gas-chromatograph for 5 min. For peak detection, an Agilent Hewlett-Packard 6890 GC gas-chromatograph equipped with a MS detector (Hewlett-Packard 5970 MSD) and a 50 m × 0.32 i.d. fused silica capillary column coated with a 1.2 μm polyethylenglycole film (Chrompack CP-Wax 52 CB) as stationary phase were used. The condition were as follows: injection temperature, 250 °C; detector temperature, 220 °C; carrier gas (He) flow rate, 1 ml/min; splitting ratio, 1:20 (v/v). The oven temperature was programmed as follows: 50 °C for 2 min; from 50 °C to 65 °C, with 1 °C/min rate of increase; from 65 °C to 220 °C, with a 5 °C/min increase, then holding for 22 min. The identification of the individual peaks obtained was based on comparison of the retention times of the unknown molecules with those obtained from the known standards (Sigma, Stheiheim, Germany). Moreover, the identification was carried out by computer matching of their mass spectral data with those of the pure compounds contained in the Agilent Hewlett-Pakard NIST 98 and Wiley version 6 mass spectral database.

2.6. Organic acids

The organic acids of coagula obtained were monitored immediately after 12 h of refrigerated storage. 3 g of sample was added to 15 ml of water–acetonitrile (1:4) mixture, shacked and centrifuged at 4000*g* for 15 min. The supernatant was filtered through nylon filters 0.22 μm as described by Kristo, Biliaderis, and Tzanetakis (2003). Organic acids analysis was performed at room temperature by HPLC system consisting in a Jasco PU-1580 intelligent HPLC pump, a manual injector (Rheodyne, Cotati, Ca, USA) equipped with a 20-μl loop, a Jasco MD-1510 multiwavelength detector DAD (Diod Array Detector) and a column Aminex HPX-87H, 300 × 7.8 mm i.d. (Bio-Rad, Richmond, CA, USA) packed with styrene copolymerized with divinilbenzene (9-μm particle diameter). The mobile phase was an aqueous sulphuric acid solution 0.08 M. The flow-rate was 0.6 ml/min and detection was performed by UV absorption measurement at 210 nm.

3. Results

3.1. Effect of pressure severity on cell viability of different strains

Cell suspensions of 14 strains belonging to the species *Lb. plantarum*, *Lb. arizonensis*, *Lb. pentosus* and *Lb. casei*, were subjected to one or two cycles of high pressure homogenisation (HPH) at pressure ranging between 50 MPa and 150 MPa. The inlet temperature was 10 ± 1 °C and the outlet temperature did not exceed 35 °C. When two cycle were applied the samples were cooled at 10 °C immediately after the first cycle. The viability loss under the different conditions (Table 1) confirmed the pressure tolerance of lactic acid bacteria previously reported by Vannini et al. (2004). In fact, the viability loss did not exceed 1.3 log cfu/ml after the more severe treatment applied (one cycle at 150 MPa or two cycles at

Table 1
Cell loads of *Lactobacillus* species (log cfu/ml) recorded after the different homogenization treatment applied to culture suspensions

Strain	Treatment					
	Control[a]	50 MPa	100 MPa	150 MPa	Two cycles at 50 MPa	Two cycles at 100 MPa
Lactobacillus arizonensis 143	9.3 ± 0.2	9.2 ± 0.3	9.1 ± 0.2	8.7 ± 0.2	9.3 ± 0.2	8.6 ± 0.2
Lactobacillus arizonensis 21	6.0 ± 0.1	6.0 ± 0.1	5.2 ± 0.1	4.5 ± 0.1	5.0 ± 0.2	4.7 ± 0.3
Lactobacillus casei 80	9.3 ± 0.4	9.3 ± 0.3	8.9 ± 0.4	9.1 ± 0.3	8.9 ± 0.2	8.7 ± 0.2
Lactobacillus casei 28	9.7 ± 0.2	9.7 ± 0.3	9.3 ± 0.3	9.1 ± 0.2	9.4 ± 0.4	9.2 ± 0.3
Lactobacillus pentosus 57	6.8 ± 0.2	6.6 ± 0.2	6.3 ± 0.2	5.8 ± 0.2	6.5 ± 0.3	5.8 ± 0.2
Lactobacillus pentosus 37	6.2 ± 0.2	6.1 ± 0.2	6.0 ± 0.1	5.8 ± 0.2	6.1 ± 0.1	6.0 ± 0.1
Lactobacillus pentosus 83	9.3 ± 0.2	9.3 ± 0.3	9.4 ± 0.3	8.9 ± 0.3	9.3 ± 0.3	8.9 ± 0.2
Lactobacillus plantarum 75	9.2 ± 0.4	9.1 ± 0.3	9.1 ± 0.2	9.3 ± 0.2	8.5 ± 0.2	8.5 ± 0.2
Lactobacillus plantarum 42	9.2 ± 0.4	9.2 ± 0.2	9.0 ± 0.3	8.7 ± 0.3	9.2 ± 0.3	8.8 ± 0.2
Lactobacillus plantarum 8	6.5 ± 0.2	6.3 ± 0.1	6.1 ± 0.2	5.7 ± 0.3	6.1 ± 0.2	5.7 ± 0.2
Lactobacillus plantarum 186	5.4 ± 0.1	5.4 ± 0.1	5.4 ± 0.3	5.4 ± 0.1	5.4 ± 0.1	5.4 ± 0.2
Lactobacillus plantarum 147	6.8 ± 0.3	6.8 ± 0.2	6.8 ± 0.2	6.7 ± 0.3	6.6 ± 0.3	6.6 ± 0.1
Lactobacillus plantarum 63	8.0 ± 0.3	8.1 ± 0.3	8.0 ± 0.2	7.5 ± 0.2	8.0 ± 0.4	7.8 ± 0.2
Lactobacillus plantarum 58	7.0 ± 0.2	6.8 ± 0.2	6.7 ± 0.1	6.2 ± 0.1	6.8 ± 0.2	6.4 ± 0.3

[a] Cells not subjected to HPH treatment.

100 MPa). The different processed cell suspensions were able to attain the same levels of the untreated controls (9.0 ± 0.5 log cfu/ml) when inoculated in milk.

3.2. Effect of the high pressure homogenization (HPH) on the proteolytic enzymes

Cell free supernatants of overnight cultures of the 14 strains were subjected to the above reported different treatments. The cell free fluids were incubated at 30 °C for 24 h with α- or β-casein buffered solutions. The electrophoretic profiles of the α- or β-caseins incubated with the cell free filtrates of the different strains treated with different pressures were compared. As expected different hydrolysis patterns in relation to strains were observed also when the cells had not been subjected to any HPH treatment. In particular, among the 14 strains tested, *Lb. plantarum* 42 and 147 were active exclusively on α-casein, while *Lb. plantarum* 75, 58 and 63 exhibited their proteolytic activity only on β-casein. *Lb. arizonensis* 143, *Lb. casei* 28, *Lb. pentosus* 57 and 37 and *Lb. plantarum* 186 were able to hydrolyze both α- and β-caseins. *Lb. arizonensis* 21, *Lb. plantarum* 8, *Lb. casei* 80 and *Lb. pentosus* 83 did not show proteolytic activity. The HPH treatment positively affected the proteolytic activity of six of above reported strains. In fact, as the pressure level increased, new or more intense bands appeared or the substrate band intensity decreased in the electrophoretic patterns of *Lb. plantarum* 63, 186, *Lb. arizonensis* 143, *Lb. casei* 28 and *Lb. pentosus* 37, 57 (Fig. 1). The filtrates of *Lb. casei* 28 (Fig. 1a) displayed activity both on α-casein and β-casein under all the adopted conditions. However, the lytic activity was stimulated by HPH treatment. In fact, when the pressure was at level higher than 50 MPa enhancements of the intensity of the bands corresponding to 21,500 kDa were observed in the lysis patterns of both α-casein and β-casein. Moreover, in the proteolytic profile of β-casein bands having molecular weights of about 14,500 kDa and 22,000 kDa appeared at pressure levels higher than 50 MPa.

The filtrates of *Lb. arizonensis* 143 (Fig. 1b) were endowed with proteolytic activity on α- and β-casein. Also for this strain the stimulating effect of HPH treatment on the proteolytic activity was observed. In particular, when the pressure was applied at level of 100 MPa (one or two cycles) two bands at 31,000 kDa and 29,000 kDa were detected. On the contrary, *Lb. pentosus* 37 (Fig. 1c) was active only on β-casein and its activity was enhanced by the HPH treatments. In particular, pressures ranging between 50 MPa and 100 MPa caused the attenuation of the substrate band and the appearance of two bands at about 21,500 kDa and 24,000 kDa. However, two cycles at 100 MPa resulted only in the reduction of β-casein substrate band intensity. Also the activity of *Lb. plantarum* 58 on β-casein was enhanced by pressure levels higher than 50 MPa. Two cycles at 50 MPa and 100 MPa incresead the ability to hydrolyse α-casein as evidenced by the appearance of two bands at 21,500 kDa and 22,000 kDa. The filtrates of *Lb. plantarum* 63 (Fig. 1d) were no active on α-casein while a significant proteolytic activity on β-casein was observed when the cells had been treated twice at 50 MPa. Two bands at about 21,500 kDa and 24,000 kDa, similar to those observed in *Lb. pentosus* 37, were present. Regarding the proteolytic activity of *Lb. plantarum* 186, one cycle at 100 MPa stimulated the activities on β-casein while two cycles at 50 kDa and 100 MPa reduced it. On the contrary the activity of this strain on α-casein was quite unaffected by HPH treatment.

For the others strains no difference in relation to the pressure level applied was observed

3.3. Fermentation kinetics

The milk acidification times, evaluated on the basis of the time necessary to reach pH 4.6, of the processed and not processed cells inoculated are reported in Table 2. On

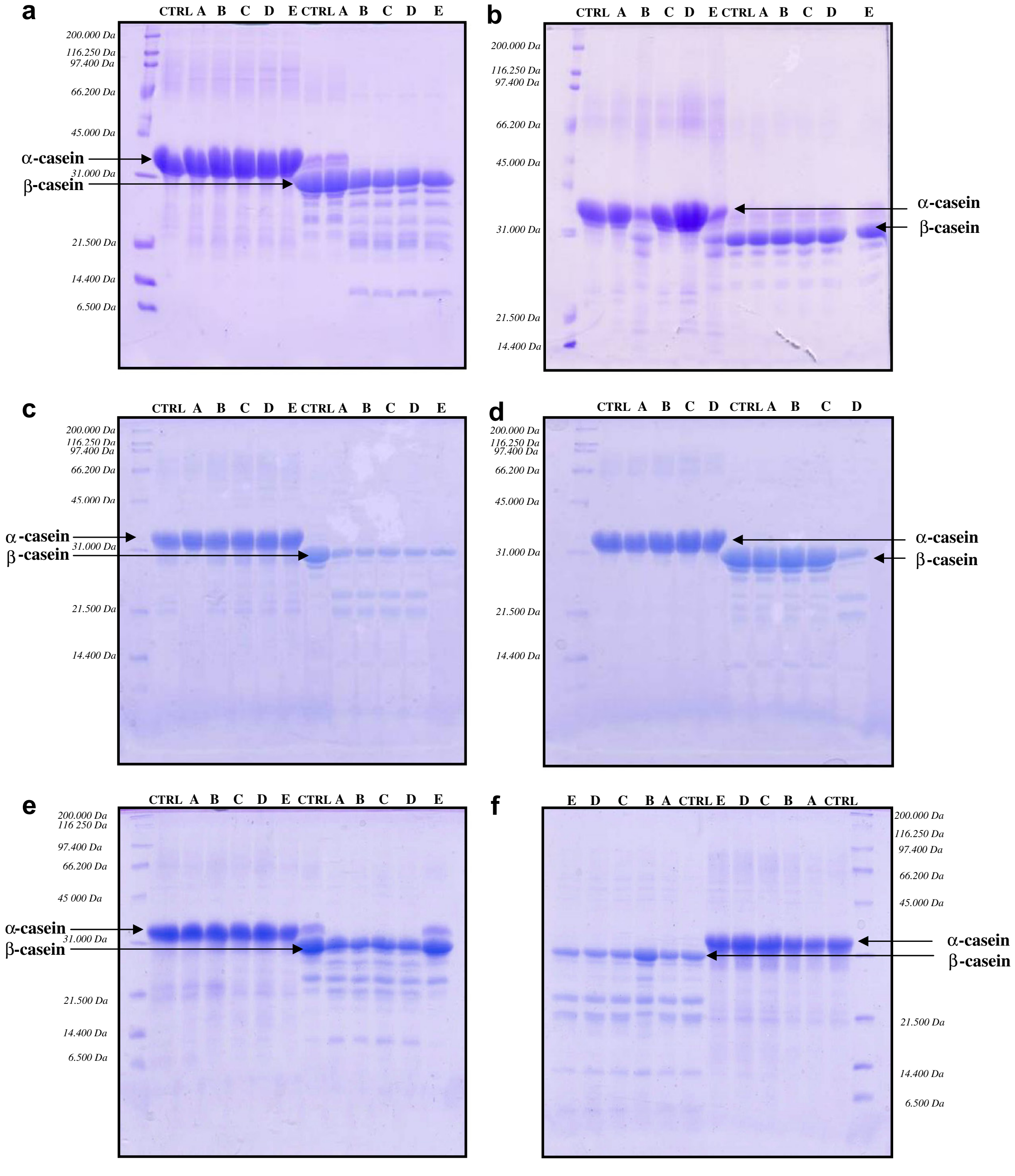

Fig. 1. SDS-polyacrylammide gel electrophoresis of α- and β-casein hydrolysis by cell free filtrates of (a) *Lactobacillus casei* 28, (b) *Lactobacillus arizonensis* 143, (c) *Lactobacillus pentosus* 37, (d) *Lactobacillus plantarum* 63, (e) *Lactobacillus pentosus* 57 and (f) *Lactobacillus plantarum* 186. Lines CTRL, A, B, C, D, and E are cell free filtrates non-HPH treated, 50 MPa, 100 MPa, 150 MPa, 2 cycles at 50 MPa and 2 cycles at 100 MPa, respectively.

the basis of the data obtained three response patterns can be identified. In particular the acidification time of *Lb. pentosus* 83, 57 and 37, *Lb. plantarum* 75, 8, 186, 147, 63 and 58 significantly decreased with the severity of treatment at least up to 150 MPa or two cycles at 50 MPa. On the contrary, the acidification time of *Lb. plantarum* 42 and *Lb. casei* 28, were increased by the increase of pressure levels. Finally the fermentation kinetics of *Lb. arizonensis* 143 and 21 and *Lb. casei* 80 were not affected by the HPH treatment.

3.4. Effect of the inoculum treatment on the fermentation products

The detection of lactic, acetic and citric acids suggested that the primary metabolism of the major part of the

Table 2
Acidification times (expressed as hours necessary to reach pH 4.6) at 30 °C for the tested strains inoculated into whole milk

Strain	Treatment					
	Control[a]	50 MPa	100 MPa	150 MPa	Two cycles at 50 MPa	Two cycles at 100 MPa
Lactobacillus arizonensis 143	30.0	30.5	30.5	30.5	30.0	30.0
Lactobacillus arizonensis 21	28.0	28.5	28.5	28.5	28.5	28.5
Lactobacillus casei 80	18.0	18.0	18.0	18.0	18.0	18.0
*Lactobacillus cas*ei 28	16.0	17.0	18.0	20.0	20.0	20.0
Lactobacillus pentosus 57	31.0	28.0	22.0	21.0	28.0	28.0
Lactobacillus pentosus 37	31.0	26.0	24.0	26.0	26.0	26.0
Lactobacillus pentosus 83	20.5	18.0	18.0	20.5	17.0	18.0
Lactobacillus plantarum 75	26.0	24.0	24.0	24.0	24.0	25.0
Lactobacillus plantarum 42	27.0	29.0	29.0	29.0	29.0	29.5
Lactobacillus plantarum 8	28.0	26.0	25.5	25.5	20.0	25.5
Lactobacillus plantarum 186	28.5	26.0	26.5	26.0	26.0	26.0
Lactobacillus plantarum 147	30.0	28.0	28.0	19.0	19.5	28.0
Lactobacillus plantarum 63	25.0	21.0	23.5	22.0	22.0	22.0
Lactobacillus plantarum 58	27.0	23.0	25.0	22.0	19.0	23.0

The data are the mean of three repetitions. Variability coefficient ranged between 5% and 7% and p was <0.05.

[a] Cells not subjected to HPH treatment.

Table 3
Organic acids (expressed in ppm) detected in coagula obtained by the fermentation of *Lactobacillus* species, previously treated at different pressure level, after 12 h of refrigerated storage

Strain	Organic acid	Treatment					
		Control[a]	50 MPa	100 MPa	150 MPa	Two cycles 50 MPa	Two cycles 100 MPa
Lactobacillus plantarum 75	Citric	207	115	138	154	148	126
	Lactic	5544	5854	5512	6493	6435	6109
	Acetic	376	427	355	514	508	457
Lactobacillus plantarum 58	Citric	331	229	256	230	220	220
	Lactic	3649	4506	4422	4523	4986	4216
	Acetic	445	726	799	802	852	667

The data are the mean of three repetitions. Variability coefficient ranged between 5% and 7% and p was <0.05.

[a] Cells not subjected to HPH treatment.

strains was affected by the previous treatment of the inoculum. In Table 3, as example, the data relative to the strains *Lb. plantarum* 58 and 75 are reported.

The acetic acid and lactic acid significantly increased with the severity of the previous treatment. No important differences were observed in *Lb. pentosus* 37 and 57, *Lb. plantarum* 63 and 42 and *Lb. casei* 80 (data not shown). The other strains showed the same behaviour of *Lb. plantarum* 58 and 75.

3.5. Volatile profiles

In order to asses whether the HPH pre-treatment of the inoculum affected the strain metabolic profile, the coagula obtained were analysed with the SPME-GC. In Table 4 the GC profiles of the coagula obtained with *Lb. casei* 28, *Lb. pentosus* 83, *Lb. plantarum* 75, 42, 8, 186 and 147 under the different pressure conditions were compared. Although with relevant quantitative differences, all the coagula were characterized by the presence of acetaldehyde, 2-propanone, ethanol, 2,3-buthandiol, diacetyl, 2-heptanol, acetoin, butyric acid and 2-methyl butyric acid. Moreover, traces of isoamylic alcohol, isobutanol, 2-eptanone, acetic acid and hexanal were detected.

Ethanol, acetoin and 2-methyl butyric acid, as shown in Table 4 and Fig. 2, were the molecules subjected to the major changes when the inoculum cells had been processed. In general the ethanol and acetoin peak areas increased two or three times with the treatment severity or showed maximum values at 100 MPa or 150 MPa depending on the strain. In particular, the ethanol increased up to 500-fold in the strain *Lb. plantarum* 186. Also the 2-methyl butyric acid increased with pressure level applied.

4. Discussion

The response to the high pressure homogenization (HPH) varied according to the species and the characteristics of the individual strains. In general, the HPH treatment did not significant affect cell viability but display an important influence on (i) extracellular protease activity, (ii) metabolism of the pre-treated cells when inoculated in milk and (iii) chemical features of coagula. Concerning the former effect, the data obtained evidenced that the HPH treatment positively affected the proteolytic activity of some of the strains tested. The increased activity on α- and/or β-casein could be attributed to the enhancement of the release of the cell wall or intra-cellular proteinases or/and

Table 4
Volatile compounds (expressed as area) detected in coagula obtained by the fermentation of *Lactobacillus* species, not treated (control) or previously treated at different pressure level, after 12 h of refrigerated storage

Strain	Volatile compound	Treatment					
		Control	50 MPa	100 MPa	150 MPa	Two cycles at 50 MPa	Two cycles at 100 MPa
Lactobacillus casei 28	Ethanol	17,904	16,625	209,288	13,008	15,710	38,240
	Acetoin	53,164	118,809	116,051	53,678	73,987	33,492
	2-Methyl butyric acid	84,947	158,300	57,381	139,635	251,373	260,687
Lactobacillus pentosus 83	Ethanol	n.d.[a]	n.d.	n.d.	30,555	29,502	98,286
	Acetoin	111,397	453,805	270,595	190,050	179,800	393,210
	2-Methyl butyric acid	249,230	285,397	392,491	358,188	341,805	298,628
Lactobacillus plantarum 75	Ethanol	49,416	10,739	150,356	232,077	283,877	210,951
	Acetoin	132,616	21,504	n.d.	12,484	117,243	24,488
	2-Methyl butyric acid	427,724	n.d.	301,132	506,941	173,139	502,443
Lactobacillus plantarum 42	Ethanol	15,817	107,056	179,975	380,111	233,888	427,376
	Acetoin	34,594	15,784	16,936	19,859	13,982	229,559
	2-Methyl butyric acid	294,465	470,506	449,684	539,517	n.d.	487,630
Lactobacillus plantarum 8	Ethanol	31,144	611,373	512,513	642,661	409,869	510,620
	Acetoin	56,585	376,132	214,818	241,339	206,794	183,533
	2-Methyl butyric acid	392,812	441,476	308,140	379,600	129,956	354,878
Lactobacillus plantarum 186	Ethanol	n.d.	565,543	589,221	586,034	604,974	628,539
	Acetoin	86,300	175,592	369,129	191,448	106,618	218,330
	2-Methyl butyric acid	162,682	350,396	425,001	359,178	389,975	413,247
Lactobacillus plantarum 147	Ethanol	451,752	857,631	691,653	745,337	636,995	662,290
	Acetoin	58,394	437,680	481,173	329,200	358,896	317,933
	2-Methyl butyric acid	126,622	356,071	252,066	565,691	510,523	23,914

The data are the mean of three repetitions. Variability coefficient ranged between 5% and 7% and p was <0.05.
In table only the most significant molecules are reported.
[a] Compound not detected.

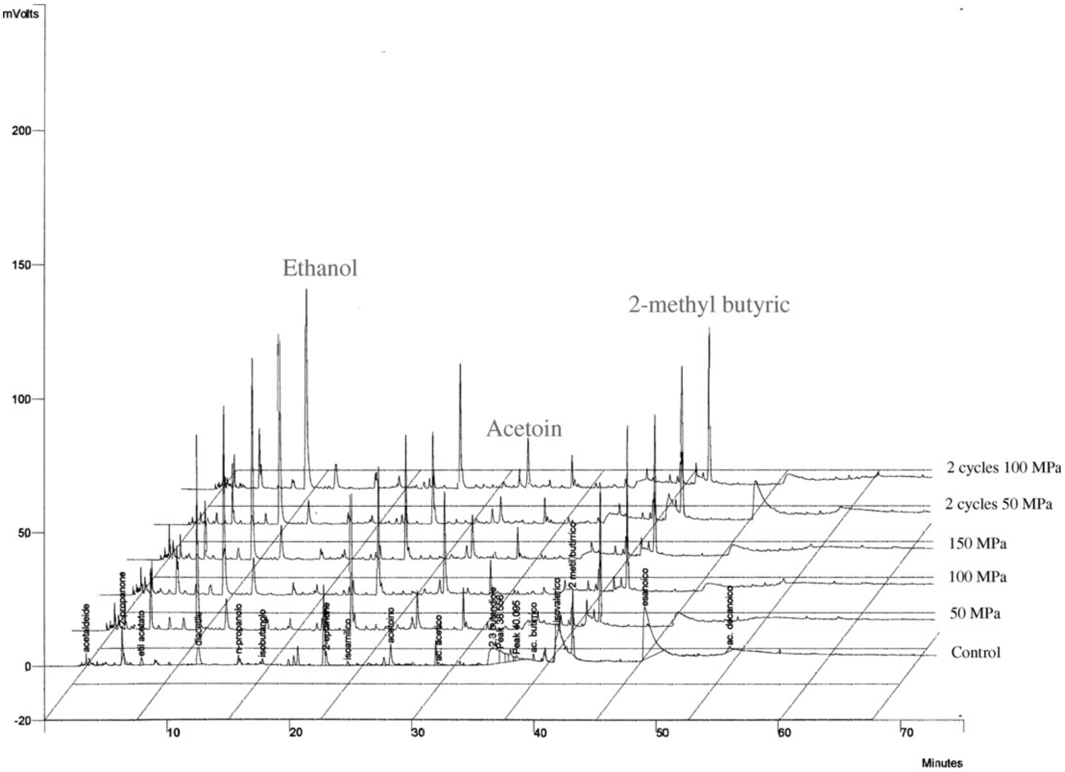

Fig. 2. Effect of the HPH treatment of the inoculum cell on the coagula flavour obtained by *Lactobacillus plantarum* 186 fermentation.

to the enhancement of heir activities by HPH. In fact, an increased release of cell wall proteinases in *Lactobacillus* has been observed also as a consequence of other sublethal stress, i.e. osmotic shock (Piuri, Sanchez-Rivas, & Ruzal, 2003, 2005). In particular, Piuri et al. (2003) evidenced that, in high osmolarity medium, *Lactobacillus casei* cell envelope-associated proteinases increased activity and lost repression by peptides. In addition the HPH treatment has been reported to modify the activity of enzymes and peptides such as lysozyme, lactoperoxidase, lactoferrine and plasmin (Hayes and Kelly, 2003; Iucci et al., in press; Vannini et al., 2004). Taking into consideration that the activity of enzymes is due to their configuration, it can be suggested that also small changes regarding the active sites can increase and, at pressure exceeding a certain threshold, decrease their activities and possibly change their specificity. Preliminary research using Fourier transform Infrared Spectroscopy confirmed that HPH treatments between 50 MPa and 150 MPa induce modifications of the secondary structure and water relationships of the proteins (Unpublished data). Moreover, as reported by Fantin et al. (1996) a HPH processing of a cell suspension of *Yarrowia lipolytica* increased the yields and inverted the enantioselectivity of the reductions of several prochiral ketones.

The activation and the quantitative and qualitative changes of the metabolic activity appears to be the most promising results. In fact, for the major part of the strains, a pre-treatment at different pressure was able to induce relevant changes in term of fermentation dynamics and metabolism with respect to the untreated cells. The levels of the principal fermentation products like lactic or acetic acid and ethanol, and flavour molecules such as acetoin and 2-methyl butyric acid where significantly increased. In particular, a metabolic shift toward ethanol and acetic acid in combination with a higher level of 2-methyl butyric acid and acetoin was observed. The significant changes of the metabolism products, whose release was enhanced when the inoculum cells had been pre-treated, could be the consequence of the response mechanisms activated in the cells exposed to HPH stress. Although, these stimulating aspects have to be deeply investigated also by molecular tools in order to understand the relationships between genomics and metabolic profiles, the HPH seems to be a versatile approach for several biotechnological applications including modulation of the fermentation kinetics, planning of specific sensorial characteristics of dairy products, enhancement of enzyme activities and change of their specificity. A deeper knowledge of the process and its effects on microbial cells, enzymes and food matrices can also result in controlled and predictable use of attenuated starter cultures.

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