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Effect of high hydrostatic pressure treatment on the viability and acidification ability of lactic acid bacteria

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ABSTRACT

The effect of high pressures of 100–450 MPa combined with temperatures of 20–40 °C on *Lactobacillus delbrueckii* subsp. *bulgaricus* ACA-DC0105, *Streptococcus thermophilus* ACA-DC0022 and *Lactococcus lactis* ACA-DC0049 cell viability and acid production ability was studied. The rates of decrease in pH and cell viability were estimated for all the process combinations studied. The viability and acidification ability of the cells depended on the process conditions. More intense process conditions resulted in a lower number of viable cells and simultaneous reduction of lactic acid production correlated to lower rates of decrease of pH. *Lb. bulgaricus* appeared to be the microorganism most resistant to pressure, while *Lc. lactis* the most sensitive. Similar behaviour was observed for the acidification ability of these microorganisms, with *Lc. lactis* being least able to decrease the pH value of cheese. The HP-treated strains could be used as adjunct starters for cheese production.

1. Introduction

Cheeses in brine are one of the oldest type of cheeses, manufactured according to a specific technology and procedure that involves ripening and storage in brine (Moatsou & Govaris, 2011). Optimisation of cheese ripening, in terms of duration and development of sensory characteristics, is of continued interest in the dairy field.

Lactic acid bacteria (LAB), used in cheese manufacture, have dual functionality. The main functionality is the production of lactic acid through the fermentation of lactose that in turn causes the decrease of the pH of cheese curd. Thus, the growth of undesirable microorganisms is inhibited by deprivation of nutrients while the particular textural, sensorial and physicochemical characteristics of the cheese mass are developed. LAB have also a number of specific intracellular peptidases that can be active in the cheese environment after cell lysis; these hydrolyse low molecular weight peptides to form free amino acids that contribute either directly or after further metabolism to the organoleptic characteristics of mature cheese (Christensen, Dudley, Pedersen, & Steele, 1999).

High hydrostatic pressure (HP) could affect the acidification potential as well as the activity of intracellular peptidases of LAB. It has been established that controlled HP treatment can increase the activity of intracellular peptidases of certain starter cultures, boosting desirable enzymatic reactions. Previous studies have focused on the effect of HP on the activity of LAB intracellular enzymes used in cheese production (Giannoglou et al., 2018; Giannoglou, Katsaros, & Taoukis, 2016a; Katsaros, Giannoglou, & Taoukis, 2009) and, by extension, on the potential of cheese ripening acceleration and more specifically on the relevant proteolytic reactions (Giannoglou et al., 2016b; Messens, Arevalo, Dewettinck, & Huyghebaert, 1999; O'Reilly, Kelly, Murphy, & Beresford, 2001; Saldo, Sendra, & Guamis, 2000; Trujillo, Guamis, & Carretero, 2000). On the other hand, it has been observed that HP processing of LAB decreases lactic acid production depending on the strain studied and the processing conditions, due to the effect of HP on the activity of glycolytic enzymes (Casal & Gomez, 1999; Daryaei, Coventry, Versteeg, & Sherkat, 2010; Malone, Wick, Shellhammer, & Courtney, 2003; Miyakawa, Anjitsu, Ishibashi, & Shimamura, 1994).

The present research work was undertaken from the perspective that specific LAB strains with negligible acidification ability could be used as adjunct starters that fortify cheese with additional

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enzymatic activities. The aim was to investigate the impact of HP treatment on the acidification ability of *Lactobacillus delbrueckii* subsp. *bulgaricus* ACA-DC 0105, *Streptococcus thermophilus* ACA-DC 0022 and *Lactococcus lactis* ACA-DC 0049. The effect of pressures from 0.1 to 450 MPa combined with temperatures from 20 to 40 °C on cell counts and the ability of the strains to produce acid was studied.

2. Materials and methods

2.1. Sample preparation

Bacterial strains *Lb. delbrueckii* subsp. *bulgaricus* ACA-DC 0105 (*Lb. bulgaricus*), *S. thermophilus* ACA-DC 0022 (*S. thermophilus*) and *Lc. lactis* ACA-DC 0049 (*Lc. lactis*), isolated from Feta cheese, were kindly provided by the Library of Agricultural University of Athens, Greece. The *Lb. bulgaricus* strain was grown under anaerobic conditions at 45 °C in MRS-broth (Merck, Darmstadt, Germany); strains of *S. thermophilus* and *Lc. lactis* were grown aerobically at 37 °C in M17-broth (Merck). Bacterial growth was monitored by estimating the absorbance at 600 nm (A_{600nm}). The bacterial cultures used in the experiments were at the late exponential phase (approximately 10^8 cfu g^{-1}, with a corresponding absorbance $A_{600nm} = 4.2$). A volume of 10 mL of each culture was centrifuged (1125×*g*, 10 min). The pellet was washed twice with 20 mM phosphate buffer, pH 7.0, then reconstituted in 10 mL sterile reconstituted skimmed milk [10% total solids (TS) and 1.5% fat] and finally packed in UV sterile multi-layer (film: PP-aluminium-PE) laminated pouches.

2.2. High hydrostatic pressure processing

HP experiments were conducted in triplicate at all the combinations of pressure at 100 and 200 MPa for processing times 0–40 min, 300 MPa for 0–30 min and 450 MPa for 20 min and temperatures at 20, 30 and 40 °C.

Bacterial strains cells without any treatment were considered as control samples. The employed HP unit (Food Pressure Unit FPU 1.01, Resato International BV, Roden, The Netherlands), comprised a pressure intensifier and a multi-vessel system consisting of six vessels of 45 mL capacity each; a maximum operating pressure and temperature of 1000 MPa and 90 °C was used. The pressure transmitting fluid used was polyglycol ISO viscosity class VG 15 (Resato International BV). Process temperature in the vessels was achieved by liquid circulation in the outer jacket controlled by a heating-cooling system. The desired pressure value was set and after pressure build up (20 MPa s^{-1}), the pressure vessels were isolated. This point defined the time zero of this process. Pressure of each vessel was released ($t_{release} < 3$ s) after a pre-set time interval (according to the experimental design) by opening the pressure valve. The initial adiabatic temperature increase during pressure build up was taken into consideration to achieve the desired operating temperature during pressurisation. Pressure and temperature in the chamber were constantly monitored and recorded (in 1 s intervals) during the process.

2.3. Viability of high pressure treated cells

The counts of the viable HP-treated bacteria were determined. The pour plate method was used for the enumeration of *Lb. bulgaricus* on MRS Agar (Merck) after anaerobic incubation for 72 h at 42 °C. The spread plate method was used for the enumeration of *S. thermophilus* and *Lc. lactis* on M-17 agar (Merck) after aerobic incubation for 72 h at 42 and 30 °C, respectively.

2.4. Acidification ability of the high pressure treated cells

The ability of the pressure-treated cells to produce acid was evaluated by measuring the rate of decrease of pH over time (0–5 h) in inoculated (4%, v/v) sterile reconstituted skimmed milk samples (1.5% total fat, 10% TS). The inoculated samples with the strain of *Lb. bulgaricus* were incubated at 45 °C under anaerobic conditions while the inoculated samples with the strains of *S. thermophilus* and *Lc. lactis* were incubated aerobically at 37 °C.

2.5. Determination of the cell free PepX aminopeptidase activity of *S. thermophilus* high pressure treated cells

To study the effect of HP on *S. thermophilus* cell lysis, the activity of PepX aminopeptidase was determined in all the treated samples.

To measure the activity of the cell-free PepX, the treated samples were centrifuged (1125×*g*, 10 min) and the supernatants were collected. Aminopeptidase PepX was evaluated by using two substrates, Gly-Pro-p-NA and Ala-Pro-p-NA (Christensen et al., 1999; Malone et al., 2003) supplied by Bachem Ltd. (St. Helens, UK). PepX activity was measured as described by Mierau et al. (1996) and Vesanto, Savijoki, Rantanen, Steele, and Palva (1995), as modified by Malone et al. (2003) as follows: 0.2 mL cell extract was added to 0.8 mL 20 mM Tris-HCl (pH 7) containing 0.2 mM substrate and measured spectrophotometrically at 30 °C, at 410 nm (SPECTRAmax 250 Microplate Spectrophotometer, Molecular devices, San Jose, CA, USA). Hydrolysis of p-nitroaniline (p-NA) for each substrate was measured through time for a total of 5 min.

2.6. Data analysis

The microbial load (average of three estimations) was plotted versus the processing time for different process conditions. The decrease of bacterial counts (log cfu g^{-1}/log cfu_o g^{-1}) was modelled using the Baranyi equation (Baranyi & Roberts, 1994) and the constants rates (k) of the viability of the HP-treated cells were estimated at all pressure-temperature conditions tested The temperature dependence of the inactivation rate constants of the bacterial strains, was expressed through the activation energy value (E_a), which was calculated using Arrhenius equation (Eq. (1)):

$$k_T = k_{Tref} \times \exp\left[-E_a/R \times \left(1/T - 1/T_{ref}\right)\right] \quad (1)$$

where k_T is the inactivation constant rate of the bacterial cells at a process temperature T (K), k_{Tref} the inactivation constant rate of the bacterial cells at a reference process temperature $T_{ref} = 293.15$ K (20 °C), E_a the activation energy (J mol^{-1}) and R the universal gas constant (8.314 J mol^{-1} K^{-1}).

The effect of the pressure on the activation energy, was expressed through an exponential equation (Eq. (2)):

$$E_a = E_{aPref} \times \exp\left[B1 \times \left(P - P_{ref}\right)\right] \quad (2)$$

where E_a is the activation energy at a pressure P (MPa), $P_{ref} = 200$ MPa and E_{aPref} the E_a value at P_{ref}. The parameters of the linearised form of Eq. (2), were estimated by linear regression.

The pressure dependence of the inactivation rate constants of the bacterial strains at each temperature was expressed through the activation volume V_a, calculated using the Eyring equation (Eq. (3)):

$$k_P = k_{Pref} \times \exp\left[(-Va/RT) \times \left(P - P_{ref}\right)\right] \quad (3)$$

where k_P is the inactivation rate constant at a pressure P (MPa), k_{Pref} the inactivation rate constant at a reference pressure (P_{ref} = 200 MPa), V_a the activation volume (mL mol^{-1}), R the gas constant (8.314 J mol^{-1} K^{-1}) and T (K) the process temperature.

The effect of the temperature on the activation volume was expressed by a linear equation (Eq. (4)):

$$V_a = V_{aTref} - A_1 \times \left(T - T_{ref}\right) \quad (4)$$

where V_a is the activation volume at a temperature T, V_{aTref} the activation volume at a reference temperature (T_{ref} = 20 °C) and A_1 a constant.

The pH (average of three estimations) decrease of the inoculated reconstituted skimmed milk samples with the treated cells was plotted against the incubation time. The decrease of the pH was calculated using linear equation and the constant rates (k) were estimated at all pressure-temperature conditions tested.

3. Results and discussion

3.1. Effect of high pressure on cell viability

Fig. 1 shows the effect of treatment at 100–450 MPa on the reduction of cell counts (log cfu g^{-1}) of *S. thermophilus* at 20, 30 and 40 °C. It was evident that the intensification of HP-treatment conditions in terms of pressure or temperature enhanced cell count reduction. A similar effect was observed for the *Lb. bulgaricus* and *Lc. lactis* strains (data not shown). The inactivation rate constants (k) of the HP-treated cells for each one of the three strains are presented in Table 1. The effect of the temperature (E_a) and pressure (V_a) on the inactivation rate constants is also shown (T_{ref} = 20 °C, P_{ref} = 200 MPa). E_a values decreased with increased process pressure for all three microorganisms studied, depicting a dependence of the inactivation from temperature at higher pressures. The same trend was observed for the pressure dependence of the inactivation rate constants for all three microorganisms; higher process temperatures lead to decreased absolute values of V_a, indicating less pressure dependence for the inactivation.

The *Lb. delbrueckii* strain was the most resistant under the HP-temperature combinations tested, while the *Lc. lactis* strain was the most sensitive. In contrast, Shah, Tsangalis, Donkor, and Versteeg (2008), after treatment of *Lb. delbrueckii* subsp. *bulgaricus*, *S. thermophilus* and *Lactobacillus acidophilus* in reconstituted milk at 480 MPa, observed higher pressure sensitivity for *Lb. delbrueckii* subsp. *bulgaricus*, compared with *S. thermophilus*. Similar work was conducted by Malone, Shellhammer, and Courtney (2002) and they reported complete inactivation of *Lc. lactis* subsp. *cremoris* MG1363 and SK11 in phosphate-buffered pH 7.0, for any pressure higher than 400 MPa combined with 25 °C, for 5 min. Processing at lower pressures (300 MPa, 25 °C, 5 min), resulted in a reduction by 7.3 and 2.5 log of the pre-mentioned strains, respectively. O'Reilly, O'Connor, Murphy, Kelly, and Beresford (2002), studied the effect of HP on the viability of *Lc. lactis* 303, 223, 227 and AM2 at pH 5.3 and depending on strain, a reduction of 6–8 log cycles in starter numbers was observed after treatment in 0.1 M citrate buffer (pH 5.3) at 300 MPa. No viable cells remained after treatment at 400 MPa. Daryaei et al. (2010), studied the effect of HP processing on *Lc. lactis* subsp. *lactis* C10 at 300 and 600 MPa (≤20 °C, 5 min). They observed that processing at 600 MPa resulted in reduction of 11.1 and 10.7 logs at pH 5.5 and 6.5, respectively, while processing at 300 MPa caused no significant reduction of bacterial log counts.

Fig. 2 shows the effect of HP on E_a and the effect of the process temperature on V_a. Increase of pressure decreased E_a, indicating higher temperature resistance for the strains at higher pressures. The effect of HP on the E_a was expressed by Eq. (2). The parameters of the equation were determined using linear regression, i.e., E_{aPref} = 50.1 kJ mol^{-1}, B_1 = 0.0050 MPa^{-1} (R^2 = 0.968) for *S. thermophilus*, E_{aPref} = 73.4 kJ mol^{-1}, B_1 = 0.0043 MPa^{-1} (R^2 = 0.972) for *Lb. delbrueckii* and E_{aPref} = 61.3 kJ mol^{-1}, B_1 = 0.0048 MPa^{-1} (R^2 = 0.958) for *Lc. lactis*.

Temperature increase led to an increase of the V_a values, reflecting the growing impact of the HP on the inactivation rate of the bacterial cells by increasing temperature. The effect of temperature on the V_a was described by eq. (4). The parameters of the equation were determined using linear regression, i.e., V_{aTref} = −3.8 mL mol^{-1}, A_1 = 0.669 mL mol^{-1} K^{-1} (R^2 = 0.991) for *S. thermophilus*, V_{aTref} = −30.1 mL mol^{-1}, A_1 = 0.837 mL mol^{-1} K^{-1} (R^2 = 0.962) for *Lb. delbrueckii* and V_{aTref} = −27.9 mL mol^{-1}, A_1 = 0.804 mL mol^{-1} K^{-1} (R^2 = 0.970) for *Lc. lactis*.

3.2. Effect of high pressure on acidification ability

The study of the ability of the bacterial cells to produce acid was based on pH changes of inoculated reconstituted skimmed milk (1.5% total fat and 10% TS). The values of pH and pH/pH$_o$ were plotted versus incubation time. Fig. 3 presents the change of the pH and pH/pH$_o$ of milk inoculated with *S. thermophilus* treated at 200 MPa for 0–30 min and 450 MPa for 0–20 min, at processing temperatures 20 and 40 °C.

The HP treatment impaired the rate of acid production depending on the process parameters. All the results obtained were kinetically modelled. More intense process conditions resulted in higher decrease of the strain acidification ability. For the *Lb. bulgaricus* and *Lc. lactis* strains, similar trends were observed (results not shown). The exponential rate constants of the change of pH/pH$_o$ of the milk samples inoculated with HP treated strains were calculated at all conditions tested (Table 2). In general, *Lc. lactis* appeared to be less effective in decreasing pH than the other

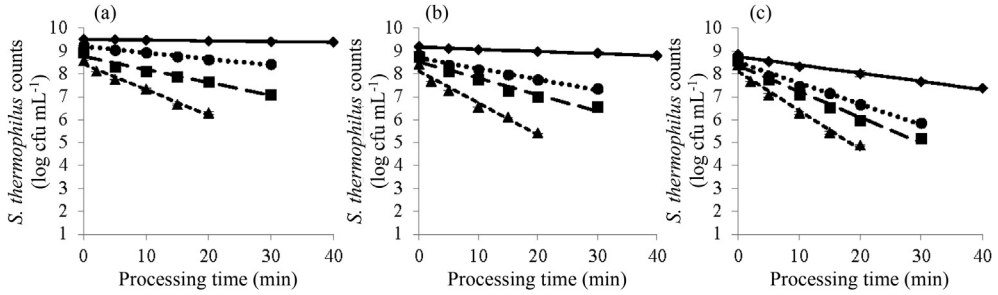

Fig. 1. Effect of high pressure treatments (—◆—, 100 MPa; ····●····, 200 MPa; – ■ -, 300 MPa; --▲--, 450 MPa) on *S. thermophilus* ACA-DC 0022 counts (log cfu g^{-1}) at processing temperatures of: a, 20 °C; b, 30 °C; c, 40 °C.

Table 1
Inactivation rate constants of the high pressure treated bacterial cells of *Lb. bulgaricus* ACA-DC 0105, *S. thermophilus* ACA-DC 0022 and *Lc. lactis* ACA-DC 0049, estimated under the tested pressure-temperature conditions.[a]

Temperature (°C)	Pressure (MPa)				V_a (mL mol^{-1})
	100	200	300	450	
Lb. bulgaricus ACA-DC 0105					
20	0.001 ± 0.001	0.009 ± 0.001	0.031 ± 0.001	0.064 ± 0.003	−31.4 ± 4.1
30	0.006 ± 0.001	0.031 ± 0.001	0.063 ± 0.003	0.105 ± 0.006	−18.9 ± 2.3
40	0.014 ± 0.001	0.058 ± 0.003	0.092 ± 0.003	0.131 ± 0.019	−14.7 ± 2.2
E_a (kJ mol^{-1})	122.5 ± 12.5	70.8 ± 4.5	41.8 ± 2.7	27.4 ± 3.5	
S. thermophilus ACA-DC 0022					
20	0.003 ± 0.001	0.027 ± 0.002	0.056 ± 0.005	0.112 ± 0.008	−23.4 ± 3.0
30	0.009 ± 0.001	0.047 ± 0.003	0.090 ± 0.008	0.139 ± 0.009	−17.9 ± 2.4
40	0.036 ± 0.002	0.091 ± 0.006	0.113 ± 0.008	0.170 ± 0.013	−10.1 ± 1.2
E_a (kJ mol^{-1})	93.1 ± 3.5	45.9 ± 2.3	26.6 ± 3.0	16.1 ± 2.0	
Lc. lactis ACA-DC 0049					
20	0.002 ± 0.001	0.033 ± 0.002	0.070 ± 0.006	0.200 ± 0.023	−28.7 ± 3.4
30	0.016 ± 0.001	0.087 ± 0.005	0.127 ± 0.005	0.274 ± 0.013	−18.3 ± 2.1
40	0.050 ± 0.004	0.128 ± 0.004	0.175 ± 0.009	0.341 ± 0.010	−12.7 ± 1.0
E_a (kJ mol^{-1})	115.7 ± 6.1	51.9 ± 4.7	34.9 ± 3.4	20.5 ± 2.2	

[a] Values are means ± standard error.

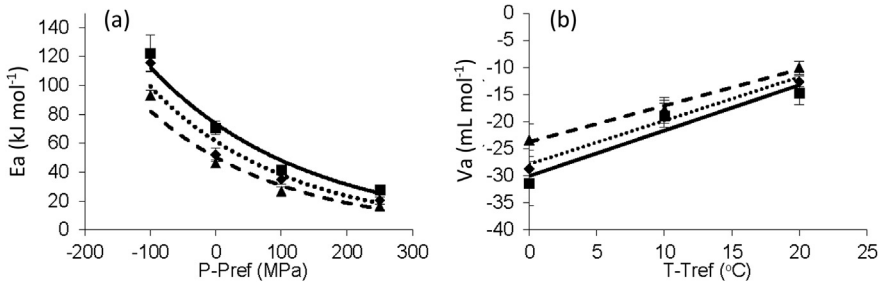

Fig. 2. The effect of (a) high pressure on the activation energy (E_a) (P_{ref} = 200 MPa) and of (b) temperature on the activation volume (V_a) (T_{ref} = 20 °C) for *S. thermophilus* ACA-DC 0022 (– ▲ -), *Lb. bulgaricus* AC. A-DC 0105 (—■—) and *Lc. lactis* ACA-DC 0049 (····◆····).

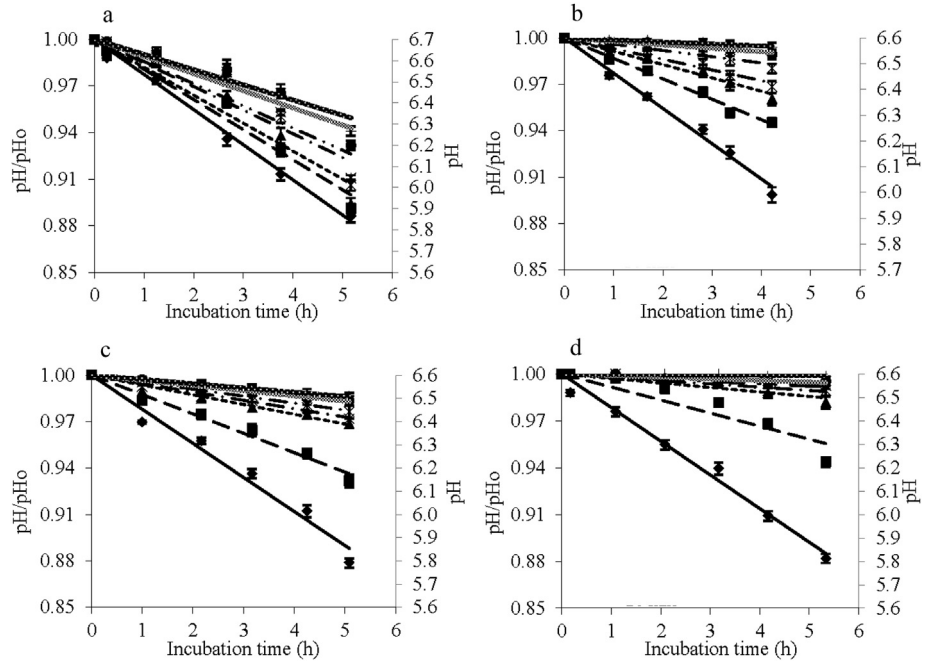

Fig. 3. Decrease of the pH value (secondary axis) and pH/pH$_0$ (pH$_0$ is pH value at zero time) (primary axis) of milk inoculated with *S. thermophilus* ACA-DC 0022 cells, treated at (a and b) 200 MPa (at 20 °C and 40 °C, respectively), for (– – –) 0 min, (-----) 5 min, (– · –) 10 min, (— · · ·) 15 min, (▪▪▪▪) 20 min, (▬▬) 30 min and at (c and d) 450 Pa (at 20 °C and 40 °C), respectively for (– – –) 0 min, (-----) 2 min, (– · –) 5 min, (— · · ·) 10 min, (▪▪▪▪) 15 min, (▬▬) 20 min; (——) control.

Table 2
Rate constants of the decrease of pH/pH$_o$ (pH$_o$ is pH value at zero time) of the milk samples inoculated with High Pressure treated *Lb. bulgaricus ACA-DC 0105*, *S. thermophilus ACA-DC 0022 and Lc. lactis ACA-DC 0049*.[a]

Pressure (MPa)/ Temperature (°C)	Control	Processing time (min)							
		0	2	5	10	15	20	30	40
Lb. bulgaricus ACA-DC 0105									
100/20	0.170 ± 0.006	0.166 ± 0.006		0.165 ± 0.006	0.163 ± 0.005		0.162 ± 0.004	0.159 ± 0.004	0.158 ± 0.004
100/30	0.169 ± 0.006	0.165 ± 0.005		0.161 ± 0.005	0.152 ± 0.003		0.149 ± 0.004	0.147 ± 0.002	0.145 ± 0.003
100/40	0.173 ± 0.007	0.161 ± 0.004		0.150 ± 0.004	0.140 ± 0.009		0.127 ± 0.006	0.116 ± 0.004	0.104 ± 0.002
200/20	0.173 ± 0.003	0.161 ± 0.005		0.148 ± 0.006	0.136 ± 0.005		0.128 ± 0.004	0.119 ± 0.002	0.110 ± 0.004
200/30	0.174 ± 0.010	0.148 ± 0.008		0.133 ± 0.005	0.121 ± 0.004		0.109 ± 0.006	0.093 ± 0.003	0.084 ± 0.005
200/40	0.172 ± 0.003	0.140 ± 0.004		0.126 ± 0.005	0.115 ± 0.005		0.103 ± 0.004	0.089 ± 0.005	0.077 ± 0.005
300/20	0.172 ± 0.009	0.140 ± 0.005		0.124 ± 0.008	0.109 ± 0.007	0.095 ± 0.007	0.081 ± 0.004	0.071 ± 0.007	
300/30	0.170 ± 0.008	0.122 ± 0.003		0.106 ± 0.005	0.090 ± 0.006	0.077 ± 0.005	0.063 ± 0.003	0.048 ± 0.002	
300/40	0.168 ± 0.006	0.113 ± 0.003		0.095 ± 0.004	0.079 ± 0.004	0.055 ± 0.002	0.035 ± 0.002	0.025 ± 0.002	
450/20	0.169 ± 0.009	0.107 ± 0.005	0.090 ± 0.003	0.075 ± 0.004	0.051 ± 0.003	0.041 ± 0.002	0.029 ± 0.002		
450/30	0.173 ± 0.003	0.081 ± 0.003	0.066 ± 0.004	0.053 ± 0.004	0.044 ± 0.002	0.034 ± 0.001	0.028 ± 0.002		
450/40	0.169 ± 0.005	0.068 ± 0.003	0.058 ± 0.003	0.046 ± 0.003	0.031 ± 0.003	0.019 ± 0.001	0.015 ± 0.001		
S. thermophilus ACA-DC 0022									
100/20	0.147 ± 0.004	0.137 ± 0.003		0.130 ± 0.002	0.128 ± 0.003		0.125 ± 0.004	0.123 ± 0.003	0.118 ± 0.003
100/30	0.145 ± 0.008	0.131 ± 0.007		0.121 ± 0.011	0.115 ± 0.011		0.108 ± 0.010	0.098 ± 0.009	0.091 ± 0.009
100/40	0.147 ± 0.011	0.124 ± 0.005		0.110 ± 0.006	0.102 ± 0.007		0.089 ± 0.008	0.077 ± 0.010	0.065 ± 0.009
200/20	0.147 ± 0.006	0.135 ± 0.012		0.130 ± 0.015	0.113 ± 0.017		0.107 ± 0.017	0.081 ± 0.012	0.071 ± 0.009
200/30	0.150 ± 0.008	0.110 ± 0.010		0.101 ± 0.005	0.086 ± 0.005		0.079 ± 0.011	0.065 ± 0.006	0.053 ± 0.007
200/40	0.151 ± 0.009	0.087 ± 0.005		0.059 ± 0.005	0.049 ± 0.005		0.030 ± 0.004	0.015 ± 0.003	0.009 ± 0.001
300/20	0.148 ± 0.007	0.115 ± 0.011		0.080 ± 0.006	0.066 ± 0.004	0.055 ± 0.003	0.040 ± 0.004	0.038 ± 0.004	
300/30	0.144 ± 0.006	0.082 ± 0.006		0.052 ± 0.004	0.045 ± 0.005	0.037 ± 0.004	0.025 ± 0.002	0.019 ± 0.001	
300/40	0.149 ± 0.006	0.066 ± 0.004		0.029 ± 0.002	0.018 ± 0.002	0.014 ± 0.001	0.010 ± 0.001	0.008 ± 0.001	
450/20	0.145 ± 0.097	0.084 ± 0.007	0.038 ± 0.003	0.034 ± 0.003	0.031 ± 0.001	0.023 ± 0.001	0.018 ± 0.001		
450/30	0.150 ± 0.009	0.078 ± 0.004	0.038 ± 0.004	0.028 ± 0.004	0.022 ± 0.003	0.015 ± 0.002	0.013 ± 0.002		
450/40	0.149 ± 0.007	0.064 ± 0.010	0.022 ± 0.003	0.015 ± 0.002	0.010 ± 0.001	0.007 ± 0.001	0.002 ± 0.001		
Lc. lactis ACA-DC 0049									
100/20	0.137 ± 0.005	0.130 ± 0.004		0.127 ± 0.005	0.126 ± 0.006		0.125 ± 0.008	0.124 ± 0.008	0.120 ± 0.010
100/30	0.139 ± 0.008	0.121 ± 0.008		0.111 ± 0.010	0.100 ± 0.010		0.093 ± 0.009	0.082 ± 0.007	0.071 ± 0.005
100/40	0.135 ± 0.003	0.115 ± 0.004		0.100 ± 0.005	0.088 ± 0.005		0.079 ± 0.004	0.067 ± 0.006	0.056 ± 0.003
200/20	0.137 ± 0.002	0.117 ± 0.011		0.112 ± 0.010	0.099 ± 0.009		0.093 ± 0.008	0.075 ± 0.003	0.063 ± 0.002
200/30	0.136 ± 0.006	0.102 ± 0.004		0.086 ± 0.002	0.073 ± 0.002		0.060 ± 0.005	0.048 ± 0.001	0.041 ± 0.003
200/40	0.139 ± 0.008	0.076 ± 0.002		0.052 ± 0.003	0.038 ± 0.003		0.024 ± 0.002	0.011 ± 0.001	0.008 ± 0.001
300/20	0.140 ± 0.004	0.095 ± 0.003		0.062 ± 0.002	0.055 ± 0.005	0.043 ± 0.004	0.031 ± 0.002	0.029 ± 0.003	
300/30	0.136 ± 0.003	0.072 ± 0.003		0.051 ± 0.004	0.039 ± 0.005	0.029 ± 0.003	0.020 ± 0.001	0.014 ± 0.002	
300/40	0.139 ± 0.003	0.059 ± 0.002		0.027 ± 0.002	0.018 ± 0.001	0.014 ± 0.001	0.010 ± 0.001	0.005 ± 0.011	
450/20	0.137 ± 0.008	0.080 ± 0.004	0.034 ± 0.001	0.030 ± 0.002	0.027 ± 0.002	0.020 ± 0.002	0.016 ± 0.001		
450/30	0.138 ± 0.006	0.063 ± 0.004	0.031 ± 0.004	0.022 ± 0.002	0.017 ± 0.002	0.013 ± 0.002	0.009 ± 0.001		
450/40	0.137 ± 0.004	0.043 ± 0.004	0.015 ± 0.001	0.011 ± 0.001	0.008 ± 0.001	0.006 ± 0.001	0.001 ± 0.000		

[a] Values are means ± standard error.

microorganisms under the same process conditions, while *Lb. bulgaricus* was the microorganism least affected by the processing.

Miyakawa et al. (1994) studied the ability of *Lactobacillus helveticus* LHE-511 to produce acid after processing at 400 MPa, sonication and incubation of the cell extract (2%, w/v, in 100 mM phosphate buffer, pH 7.0) with 1.1% (w/v) glucose for 1 h at 37 °C. They observed complete inhibition of the acidification ability of the treated strain in contrast to the untreated one. Similar results were reported by Malone et al. (2003) for *Lc. lactis* subsp. *cremoris* MG1363, since they measured decreased lactic acid production ability after processing at 300–800 MPa, mainly attributed to loss of activity of glycolytic enzymes. Upadhyay, Huppertz, Kelly, and McSweeney (2007) studied the acidification ability of *Lc. lactis* subsp. *cremoris* HP and 303, treated at 100, 200 or 300 MPa for 1 s, 10 or 20 min by measuring the change of pH of reconstituted skimmed milk. According to their results and in agreement with the results of the present study, intense processing conditions led to increasing loss of the acidification ability of the two strains. After treatment at 200 or 300 MPa for 10 or 20 min, complete inhibition of the acidification ability of both strains was observed.

Daryaei et al. (2010) studied the effect of HP on the ability of *Lc. lactis* subsp. *lactis* C10, *S. thermophilus* TS1 and *Lb. acidophilus* 2400 to produce acid and on the activity of the glycolytic enzymes phospho-β-galactosidase and β-galactosidase. Acidification ability was studied after processing at 300 and 600 MPa at 22 °C for 5 min in 100 mM phosphate buffer pH 6.5 and incubation with 5% (w/v) lactose at 30 °C, determining the titratable acidity. According to their results, processing at 600 MPa inhibited the acid production for the strain of *Lc. lactis*, which was attributed to the 94% reduction in the activity of the phospho-β-galactosidase that has a significant role in the metabolism of lactose. The activity of β-galactosidase decreased by 97 and 27% in the strains of *S. thermophilus* and *Lb. acidophilus*, respectively (Daryaei et al., 2010).

In Fig. 4 the effect of the HP processing at 200 MPa, 20 °C on both the viability and the acidification ability of the three strains is depicted. After 20 min treatment at these conditions, inactivation of the strains of *Lb. bulgaricus*, *S. thermophilus* and *Lc. lactis* of about 2.2, 11.1 and 12.7% was observed, while a 30.3, 44.8 and 45.4% reduction of acidification ability was measured, respectively.

Treatment of the lactic acid bacteria of the present study with different combinations of HP and temperature resulted in reduction of their counts and acidification ability. Representatively, at 200 MPa, 20 °C and 20 min the counts of *Lb. bulgaricus* strain decreased by 2.2% with a simultaneous reduction in acidification ability by 30.3% (Fig. 4a). The respective percentages for the strain of *S. thermophilus* were 11.1% and 44.8% (Fig. 4b) and for the strain of *Lc. lactis* were 12.7% and 45.4% (Fig. 4c).

These results could be combined with those of Giannoglou et al. (2016a) and Katsaros et al. (2009) that reported increase of intracellular aminopeptidase activity (necessary for cheese ripening) of

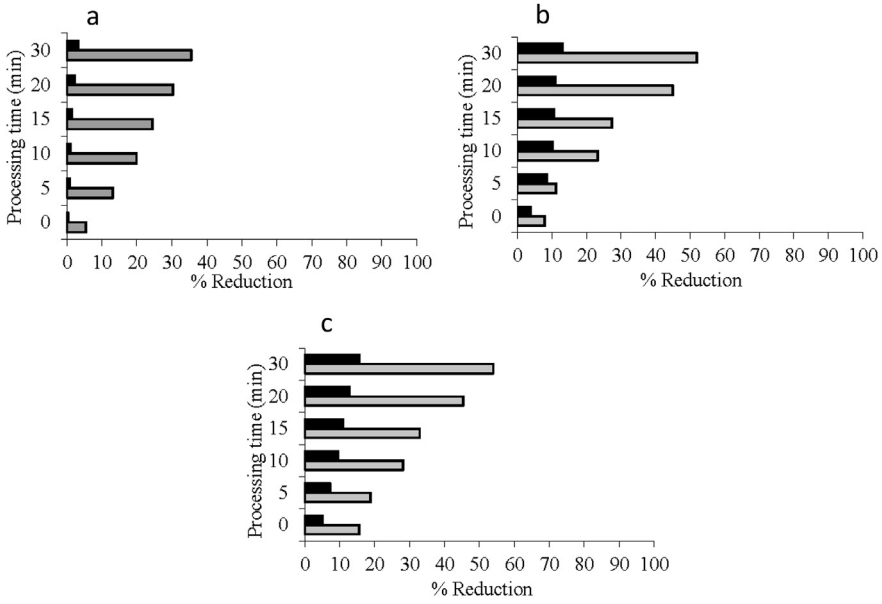

Fig. 4. Effect of high pressure processing at 200 MPa, 20 °C on the inactivation rate decrease (black bars) and the acidification ability (grey bars) of a) *Lb. delbrueckii* subsp. *bulgaricus* ACA-DC 0105, b) *S. thermophilus* ACA-DC 0022 and c) *Lc. lactis* ACA-DC 0049.

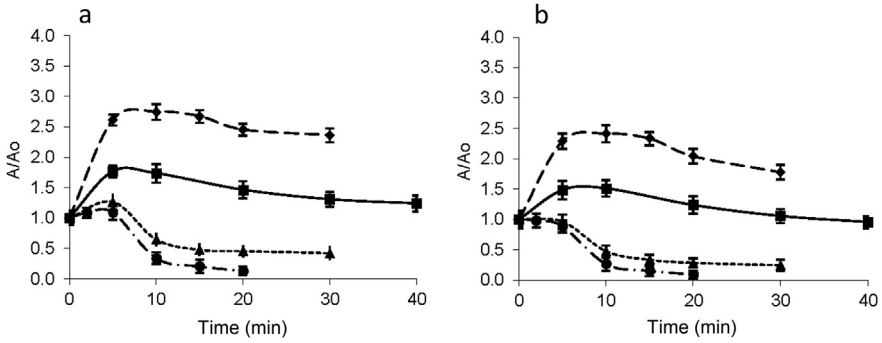

Fig. 5. The activity of the cell-free PepX aminopeptidase of *S. thermophilus* ACA-DC 0022 for processing at (■) 100, (◆) 200, (▲), 300 and (●) 450 MPa and at (a) 20 °C and (b) 40 °C, where A is PepX activity after processing for a time period t (min), A_0 the initial activity of PepX (at a time zero of processing).

the same strains studied within this manuscript, after HP treatment. For example, processing at conditions 200 MPa, 20 °C for more than 10 min, a reduction of the acidification ability for more than 30% along with minimum cell count decrease (<13%) was observed, while at the same time a 3-fold increase of intracellular peptidases was reported (Giannoglou et al., 2016a; Katsaros et al., 2009). This allows the estimation of a possible overcompensation of the amount of HP treated starter culture that can be used to obtain the benefits of the positive effect on the aminopeptidase action to achieve overall an acceleration in the ripening process, while maintaining adequate acidification action (Giannoglou et al., 2016a,b).

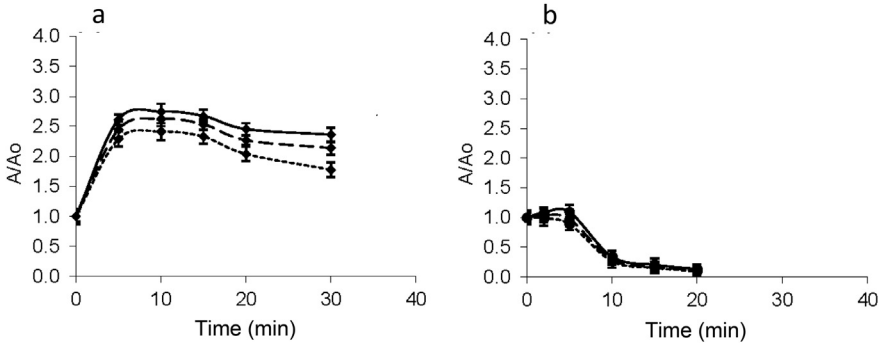

Fig. 6. The activity of the cell-free PepX aminopeptidase of *S. thermophilus* ACA-DC 0022 for processing at (———) 20, (– – – -) 30 and (------) 40 °C and at (a) 200 MPa and (b) 450 MPa, where A is PepX activity after processing for a time period (min), A_0 the initial activity of PepX (at time zero of processing).

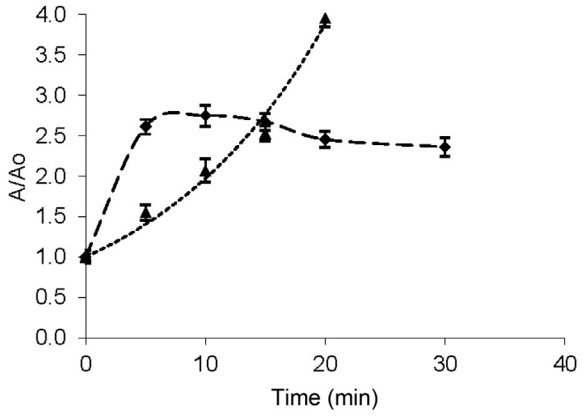

Fig. 7. Comparison between the cell-free (– – – -) and intracellular (------) activity (Giannoglou et al., 2016a) of the PepX aminopeptidase of *S. thermophilus* ACA-DC 0022 for processing at 200 MPa and 20 °C, where A is PepX activity after processing for a time period t (min), A_0 the initial activity of PepX (at a time zero of processing).

3.3. The effect of high pressure on *S. thermophilus* cell lysis: determination of cell-free PepX aminopeptidase activity

The effect of pressures 100, 200, 300 and 450 MPa combined with temperatures 20, 30 and 40 °C and for processing times 0–40 min, on the cell lysis of *Streptococcus themophilus*, was investigated through measuring PepX aminopeptidase activity of the HP-treated cell extracts. Similar work was conducted for *Lb. bulgaricus* and *Lc. lactis* (data not shown).

A double effect of HP processing was observed as HP led to cell lysis and release of PepX aminopeptidase in the medium and also led to an increase/decrease of PepX aminopeptidase activity, depending on the pressure-temperature conditions applied, suggesting interspecific variation in its pressure sensitivity.

Fig. 5 shows the effect of pressure (100–450 MPa) on the release and/or on the activity of the cell-free PepX aminopeptidase of *S. thermophilus*, pressurised at 20 and 40 °C. Increased activity of the PepX aminopeptidase was observed until 10 min processing at 100 and 200 MPa at 20–40 °C and slight increase until 5 min for processing at 300 and 400 MPa at 20 °C. Stabilisation or slight decrease of PepX activity for higher processing time was observed at 100 and 200 MPa. Processing at more intense pressure conditions led to decreased enzyme activity. PepX activity was found to be significant higher after treatment at 200 MPa at all temperatures studied. The higher activation of PepX was observed after treatment at 200 MPa and 20 °C as observed in the investigation of Giannoglou et al. (2016a) for *S. thermophilus* intracellular PepX.

Fig. 6 shows the effect of temperature (20–40 °C) on the release and/or on the activity of the cell-free *S. thermophilus* PepX aminopeptidase, treated at 200 and 450 MPa. Increase in temperature led to decrease of activity increase for processing at 200 MPa. An increase of PepX activity reduction at pressures higher than 200 MPa was observed.

Comparing the results of the effect of HP on the cell free and intracellular PepX aminopeptidase (Giannoglou et al., 2016a), the differences in the behaviour for processing at 200 MPa and 20 °C are depicted in Fig. 7. Cell free PepX aminopeptidase activity increased till 10 min processing time and then was stabilised for higher processing times, in contrast to intracellular PepX activity that was increased.

4. Conclusions

The evaluation of the effect of HP processing on the viability and acidification ability of the strains *Lb. delbrueckii* subsp. *bulgaricus* ACA-DC 0105, *S. thermophilus* ACA-DC 0022 and *Lc. lactis* ACA-DC 0049 is related to their use as adjunct starters. The viability depended on the processing conditions with lower counts for more intense processing conditions. *Lc. lactis* appeared to be the more sensitive microorganism to pressure treatment, while *Lb. bulgaricus* was the more resistant. Similarly, the acidification ability was impaired due to HP treatment. In general, *Lc. lactis* was less effective as far as acidification ability was concerned, compared with *Lb. bulgaricus* and *S. thermophilus*, after HP processing under the same conditions.

Combining these results with others cited in the literature describing a significant increase in intracellular peptidases activity after pressure treatment, it is evident that *Lb. bulgaricus*, *S. thermophilus and Lc. lactis* could be used as an adjunct starter.

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We thank Professor E. Tsakalidou in the Department of Food Science and Technology of the Agricultural University of Athens for kindly providing the strains of the lactic acid bacteria needed for conducting the research. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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