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Inactivation and recovery of *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus* after high hydrostatic pressure treatments up to 900 MPa

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Summary. High hydrostatic pressure (HP) processing is used in the food industry to enhance the safety and extend the shelf-life of food. Although a drastic decrease in microbial viability is achieved immediately after the application of HP treatments, under favorable conditions the injured bacteria can recover. The present study evaluated the inactivation and recovery of five strains of *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus* subjected to pressures of 400, 600, and 900 MPa under stressing and non-stressing conditions in a complex medium. Treatments at 400 and 600 MPa were found to greatly affect the viability of *L. monocytogenes* and *S. enterica*, but only a treatment of 5 min at 900 MPa decreased the levels of the three pathogens to below the detection limit (8–9 log units reduction). After HP treatment, not only the baroresistant *S. aureus* but also several replicates of *L. monocytogenes* and *S. enterica* strains recovered during subsequent incubation under favorable conditions. However, when HP was combined with low pH and nitrite but not with NaCl or lactate, the viability of pressurized *S. aureus* cells progressively decreased. As pathogenic bacteria can recover even after the application of very high pressure levels, the combination of HP with other hurdles for microbial growth, either intrinsically present in the food product or extrinsically applied, may be needed to guarantee the efficacy of technologies aimed at pathogen reduction and shelf-life extension. [**Int Microbiol** 2010; 13(3):105-112]

Keywords: high hydrostatic pressure · food-borne pathogens · bacterial recovery · sublethal injury · hurdle technology

Introduction

High hydrostatic pressure (HP) is an emerging technology that has been shown to enhance the safety and shelf-life of many food products but with minimal influence on the sensory, physical, and nutritional properties of the food, in contrast to thermal processing, especially if low-temperature HP processing is performed [1]. Pressure levels higher than 200–300 MPa have been described to inactivate bacterial cells by causing structural changes in the cell membrane and the inactivation of enzymes. At the protein level, pressure-induced changes are reversible in the range of 100–300 MPa but irreversible at pressures above 400 MPa, due to the cleavage of intermolecular and intramolecular bonds [23]. A number of inactivation studies have demonstrated that not only different bacterial species but also different strains of the same species differ in their resistance to HP [16,18,22]. This observation indicates that the inhibitory effect of HP depends on the combination of a number of factors related to the bacteria itself, such as shape, Gram type, physiological state, or strain particularities. In addition, the nature of the medium (e.g., pH, the presence of salt and/or nutrients) and the pressurization variables (pressure level, pressurization time, and temperature) have also been described to influence HP-mediated inactivation [22].

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The commercial application of HP has steadily increased over the past 10 years. In 2008, 110 facilities existed worldwide, with total annual production volume of more than 150,000 tons [2]. In the food industry, HP processing consists of the application of pressure treatments of up to 600 MPa for short periods of time (1–10 min). Data on the effects of pressure levels higher than 600 MPa are very scarce and come from research studies performed using pilot equipment.

It is well known that pressure can produce sublethal injury [15]; however, the extent of this phenomenon is not well characterized and not always considered in inactivation studies. Microorganisms are said to be sublethally damaged if they survive an inactivation treatment and the damage incurred is eventually, under favorable conditions, repaired. The main characteristics of sublethally injured cells are: their high sensitivity to acid, alkali, bile salts, lytic enzymes, and oxidative and osmotic stresses; alterations in metabolism or nutritional requirements; and increased mutation rates, restriction in the range of growth temperatures, increased sensitivity to secondary stresses, and extension of the lag phase [14].

From the safety point of view, it is important to consider that through an adequate combination of HP, together with other preservation treatments or unfavorable environmental factors ("hurdle technology" [12]), it is possible to prevent the recovery of sublethally injured cells and to increase the severity of their inactivation by hampering damage repair.

The aim of this study was to evaluate the resistance of *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus*, three widely distributed food-borne pathogens, to different HP treatments (400, 600, and 900 MPa) in a complex medium. In addition, the ability of the strains to recover under favorable and unfavorable conditions was evaluated.

Materials and methods

Bacterial strains. Five strains of *L. monocytogenes*, *S. enterica* and *S. aureus* from animal and meat origin were separately assayed (Table 1).

High hydrostatic pressure treatments. Strains were grown overnight at 37ºC in brain-heart infusion (BHI, BD-Diagnostic Systems, Franklin Lakes, NJ, USA), a complex medium. Before treatment, cultures were transferred to sterile plastic Pasteur pipettes, which were then thermosealed, vacuum-packed individually in plastic bags, and pressurized at 400 and 600 MPa for 10 min at 15ºC in a 120-l high-pressure unit (Wave 6000 from NC Hyperbaric, Burgos, Spain). The pressurization liquid was water.

Table 1. Description of the strains of *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus*

Species	Reference[1]	Abbreviation	Serovar	Origin
L. monocytogenes	CTC1011	L1011	1/2c	Meat product
	CTC1034	L1034	4b	Meat product
	CECT937	L937	3b	Human
	CECT940	L940	4d	Sheep
	CECT5873	L5873	1/2a	Human
S. enterica	CTC1003	S1003	London	Meat product
	CTC1015	S1015	Schwarzergrund	Meat product
	CTC1022	S1022	Derby	Meat product
	GN-0003	S3	Enteritidis	Pork entrails
	GN-0006	S6	Typhimurium	Pork gut
S. aureus	CTC1008	A1008		Meat product
	CTC1019	A1019		Meat product
	CTC1021	A1021		Meat product
	CECT976	A976		Meat product
	CECT4466	A4466		Turkey salad

[1]CTC and CECT strains belong to collections from the IRTA and the Spanish Type Culture Collection, respectively. GN strains were kindly provided by Dr. Badiola (CReSA, Bellaterra, Spain).

Table 2. Levels of NaCl, pH, $NaNO_2$, and potassium lactate applied to pressurized *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus*

	NaCl (% w/v)				pH			$NaNO_2$ (mg/ml)			Lactate (% w/v)			
L. monocytogenes	–	4	7	10	7.4	6.3	5.1	5	10	20	–	4.8	7.2	9.6
S. enterica	1	4	7	–	7.4	6.3	5.1	5	10	20	–	4.8	7.2	9.6
S. aureus	–	4	7	10	7.4	6.3	5.1	5	10	20	2.4	4.8	7.2	–
a_w	0.990	0.975	0.956	0.933	0.996	0.996	0.996	0.992	0.990	0.985	0.989	0.982	0.976	0.969

Come-up times were 130 s and 170 s for treatments at 400 and 600 MPa, respectively, and pressure release was almost immediate (<10 s). Pressurization at 900 MPa for 5 min was performed in a 2-l pilot (Thiot Ingenierie, NC Hyperbaric, Bretenoux, France–Burgos, Spain), with di-2-ethylhexyl azelate as the pressurization fluid. The come-up and release times were 240 s and 40 s, respectively. Three independent experiments were carried out for each bacterial strain.

Enumeration by differential plating. Differential plate counting was performed before and after HP treatment in tryptic soy agar plus 0.6% yeast extract (TSAYE, from BD) for enumeration of viable cells, and in TSAYE with NaCl (4% for *S. enterica* and *L. monocytogenes*, and 10 % for *S. aureus*) for enumeration of uninjured cells only. The number of sublethally injured cells was estimated by the difference between counts in TSAYE and TSAYE with NaCl. The limit of detection (LOD) was 10 colony-forming units (CFU)/ml.

Recovery of pressurized cells under non-stressing conditions. Five ml of cultures pressurized at 400, 600, and 900 MPa were mixed with 5 ml of fresh BHI and incubated at 14 and 22ºC for 21 days. After 2, 7, and 21 days, cultures were enumerated in TSAYE plates. The LOD was 10 CFU/ml.

Viability of pressurized cells under stressing conditions. Cultures (5 strains per species) pressurized at 400, 600, and 900 MPa were inoculated (15 ml) in wells of microtiter plates containing 135 ml of BHI together with different concentrations of NaCl (Panreac Quimica, Castellar del Vallès, Spain), $NaNO_2$ (Panreac Quimica), or potassium lactate (Purasal P/Hi Pure 60, Purac Biochem, Gorinchem, Netherlands), and different levels of acidity (pH adjusted with HCl) (Table 2), all previously determined to allow growth of the strains. Plates were incubated at 14 and 22ºC for up to 6 weeks. After 1 day and 1, 3, and 6 weeks of incubation, the presence/absence of viable pathogens was analyzed by spotting 10 ml of culture onto selective medium plates, with brilliant green agar (Difco, Sparks, NV, USA), chromogenic *Listeria* agar (Oxoid, Basingstoke, UK) and Baird Parker (Oxoid) used to detect *L. monocytogenes*, *S. enterica* and *S. aureus*, respectively. The LOD was 100 CFU/ml.

Physicochemical analyses. A Crison Basic 20 pH-meter (Crison Instruments, Alella, Spain) was used to measure pH. Water activity (a_w) was measured at 25ºC using an Aqualab (Decagon Devices, Pullman, WA, USA).

Statistical analysis. Bacterial counts and HP inactivation (expressed as log reduction) were statistically analyzed using analysis of variance (ANOVA) and the post-hoc Tukey test. Viability data of pressurized *S. aureus* under stressing conditions were analyzed with logistic regression. All statistical analyses were carried out using Statistica 7.0 software (Statsoft, Tulsa, OK, USA), with $P < 0.05$ considered significant.

Results

Immediate high hydrostatic pressure-induced inactivation of pathogens. The application of 400, 600, and 900 MPa resulted in important decreases (>8 log reduction) in the counts of *L. monocytogenes* to below the detection limit (1 log CFU/ml) (Table 3). No differences were observed among the five strains assayed ($P > 0.05$), and the CFU of sublethally injured cells was below the LOD. All *S. enterica* strains, by contrast, were drastically inactivated at 400 MPa (7–8 log units reduction) but few sublethally injured cells (1.0–1.6 log CFU/ml) remained viable. After pressurization at 600 MPa, viable cells were detected only in *S. enterica* strain S1003 (1.1 log CFU/ml, including 0.2 log of sublethally injured cells). Exposure to 900 MPa reduced the counts of all *S. enterica* strains to below the LOD. *Staphylococcus aureus* was much more resistant than *L. monocytogenes* and *S. enterica* to HP treatments of 400 and 600 MPa ($P < 0.05$) and the viable counts of the five strains of the pathogen decreased by only 1.5–2.0 log units after treatment at 400 MPa and by 5.8–8.0 log units after treatment at 600 MPa. In both cases, strain A1019 was the most resistant, with fewer sublethally injured cells. Following treatment at 900 MPa, counts of all *S. aureus* strains were under the LOD.

Recovery of pressurized cultures at 14 and 22ºC. Although the CFU of *L. monocytogenes* cells was below the LOD immediately after all HP treatments (Table 4, after HP data), not all cultures were completely inactivated because the pathogen grew during the subsequent recovery period (Table 4). After 2 days of incubation at 22ºC, all *L. monocytogenes* strains recovered in at least one of the three replicates exposed to 400 MPa. During the subsequent incubation, only one additional replicate from strain L937 grew. A similar trend was observed in cells treated with 600 MPa, although the number of recovered replicates was lower and no recovery of strain L940 was observed. After treatment at 900

Table 3. Counts of *Listeria monocytogenes*, *Salmonella enterica*, and *Staphylococcus aureus* (log CFU/ml ± SD) before and after HP treatment at 400, 600, and 900 MPa

		After 400 MPa		After 600 MPa		After 900 MPa	
	Before HP	TSAYE	$TSAYE_{NaCl}$[1]	TSAYE	$TSAYE_{NaCl}$	TSAYE	$TSAYE_{NaCl}$
L. monocytogenes							
L1011	9.21 ± 0.06[a]	< 1	< 1	< 1	< 1	< 1	< 1
L1034	9.31 ± 0.05[a]	< 1	< 1	< 1	< 1	< 1	< 1
L937	9.30 ± 0.10[a]	< 1	< 1	< 1	< 1	< 1	< 1
L940	8.48 ± 0.40[ab]	< 1	< 1	< 1	< 1	< 1	< 1
L5873	9.13 ± 0.58[a]	< 1	< 1	< 1	< 1	< 1	< 1
S. enterica							
S1003	9.17 ± 0.09[ab]	1.16 ± 0.75[a]	< 1	1.13 ± 0.31	0.98 ± 0.05*	< 1	< 1
S1015	9.14 ± 0.13[b]	1.07 ± 0.84[a]	< 1	< 1	< 1	< 1	< 1
S1022	9.16 ± 0.14[b]	1.32 ± 1.25[a]	< 1	< 1	< 1	< 1	< 1
S3	8.62 ± 0.56[ab]	1.58 ± 0.99[a]	< 1	< 1	< 1	< 1	< 1
S6	8.63 ± 0.53[ab]	1.01 ± 0.05[a]	< 1	< 1	< 1	< 1	< 1
S. aureus							
A1008	8.94 ± 0.24[ab]	6.93 ± 0.37[b]	5.67 ± 0.36[a]	0.98 ± 0.05[a]*	< 1	< 1	< 1
A1019	8.92 ± 0.78[a]	7.37 ± 0.88[b]	7.32 ± 0.59[a]	3.14 ± 0.77[b]	2.54 ± 1.02	< 1	< 1
A1021	8.93 ± 0.24[ab]	7.11 ± 0.63[b]	6.48 ± 0.81[a]	1.61 ± 0.62[ab]	< 1	< 1	< 1
A976	8.92 ± 0.11[ab]	7.11 ± 0.54[b]	5.70 ± 1.31[a]	1.13 ± 0.32[a]*	< 1	< 1	< 1
A4466	8.97 ± 0.10[ab]	7.03 ± 0.19[b]	6.46 ± 0.78[a]	1.07 ± 0.21[a]	< 1	< 1	< 1

[1]TSAYE plates were supplemented with 4% NaCl for *L. monocytogenes* and *S. enterica* and 10% for *S. aureus* differential plating. For each species, different letters within a column indicate significantly different reductions among strains ($P < 0.05$).
*Limit of detection was 1 log CFU/ml; replicates with counts below this level were assigned a value of 0.95 for average and SD calculation. Results are the mean of three independent experiments.

MPa, only one replicate of strain L5873 and one of strain L937 were able to grow at 22 and 14ºC, respectively. For both the 600 MPa and the 900 MPa treatments, no differences in recovery were observed from day 2 to 21 at 22ºC. In contrast, at 14ºC, recovery of the replicates was slower but more replicates treated at 400 and 600 MPa had recovered after 21 days.

The five strains of *S. enterica* pressurized at 400 MPa fully recovered after 2 days of incubation at 22 and 14ºC (Table 4). After treatment with 600 MPa, the degree of inhibition was higher and the recovery slower, with some of the replicates unable to grow even after 21 days, at either 14 or 22ºC. Strain S1003, the only strain with counts above the LOD after treatment at 600 MPa, recovered the fastest at 14ºC. After pressurization at 900 MPa, only one replicate of strain S1015 recovered, after 7 days at 14ºC.

Due to the poor inactivation of *S. aureus* following pressurization (Table 3), most of the strains treated at 400 and 600 MPa were detected immediately after HP treatment, and all of them were detected after 2 days at 22ºC and after 7 days at 14ºC (Table 4). Following treatment at 900 MPa, no viable cells from strain A976 (incubated at 14 or 22ºC) or strain A1008 (at 14ºC) were detected after 21 days of incubation. However, the high number of *S. aureus* cultures, 7/14 after 21 days at 22ºC and 5/14 at 14ºC, able to recover after this very HP treatment is noteworthy.

Recovery of pressurized cells under stressing conditions.

Listeria monocytogenes and *S. enterica* were drastically inactivated by HP and no viable cells were found under any of the stressing conditions assayed. In fact, viable cells were only observed in control conditions (pH 7.4) after treatment at 400 MPa (data not shown). The viability of 400-MPa-treated *S. aureus* was only affected by subsequent incubation of the cultures at pH 5.1 and especially in $NaNO_2$ (10

Table 4. Number of viable replicates immediately after pressurization (I) and after 2, 7, and 21 days of incubation at 14 and 22ºC under non-stressing conditions (BHI medium) of *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus*

	Pressure:	400 MPa							600 MPa							900 MPa						
	Temperature:	22ºC				14ºC			22ºC				14ºC			22ºC				14ºC		
	Strains/Day:	I	2	7	21	2	7	21	I	2	7	21	2	7	21	I	2	7	21	2	7	21
L. monocytogenes	L1011	–	2	2	2	1	1	1	–	1	1	1	–	1	1	–	–	–	–	–	–	–
	L1034	–	2	2	2	–	1	3	–	1	1	1	–	1	1	–	–	–	–	–	–	–
	L937	–	1	2	2	–	2	3	–	2	2	2	1	1	2	–	–	–	–	–	–	1
	L940	–	1	1	1	–	1	2	–	–	–	–	–	–	1	–	–	–	–	–	–	–
	L5873	–	2	2	2	2	3	2	–	2	2	2	–	2	2	–	1	1	1	–	–	–
S. enterica	S1003	3	3	3	3	3	3	3	1	2	3	3	1	3	3	–	–	–	–	–	–	–
	S1015	2	3	3	3	3	3	3	–	1	2	2	1	1	2	–	–	–	–	–	1	1
	S1022	2	3	3	3	3	3	3	–	1	3	2	1	2	3	–	–	–	–	–	–	–
	S3	2	3	3	3	3	3	3	–	1	3	3	2	2	2	–	–	–	–	–	–	–
	S6	2	3	3	3	3	3	3	–	2	2	2	2	2	2	–	–	–	–	–	–	–
S. aureus	A1008	3	3	3	3	3	3	3	1	3	3	3	1	3	3	–	–	–	2	–	–	–
	A1019	3	3	3	3	3	3	3	3	3	3	3	3	3	3	–	1	1	2	–	1	2
	A1021	3	3	3	3	3	3	3	2	3	3	3	2	3	3	–	1	1	2	–	1	1
	A976	3	3	3	3	3	3	3	1	3	3	3	3	3	3	–	–	–	–	–	–	–
	A4466	3	3	3	3	3	3	3	1	3	3	3	2	3	3	–	1	1	1	–	1	2

"–" indicates no detected replicates with counts above the detection limit (10 CFU/ml) and "3" indicates the recovery of all the three cultures assayed in three independent experiments. Note that at days 7 and 21 recovery of the cultures was 90–100% compared to the counts before pressurization.

and 20 mg/ml) ($P < 0.001$) (Table 5). In both cases, most of the strains of the pathogen were viable after 1 week but progressively died. Incubation temperature significantly affected the viability of *S. aureus* cells in the presence of $NaNO_2$ ($P < 0.05$) but not at pH 5.1. When incubation was performed at 22ºC in the presence of 20 mg $NaNO_2$/ml, only some of the replicates of strains A1019, A1021, A976 and A4466 were inhibited after 3 weeks while at 14ºC and the same $NaNO_2$ concentration all strains were dead at this time. At 14ºC, an inhibitory effect of $NaNO_2$ was achieved at a concentration of 10 mg/ml. After treatment at 600 MPa, the behavior of strain A1019 stood out, as the replicates were viable in the presence of any of the antimicrobials. This resistance may have been related to the strain's poor immediate inactivation by HP (Table 3). Only the highest levels of acidity, $NaNO_2$, and lactate inhibited the growth of strain A1019, and no significant differences were observed between 14 and 22ºC ($P > 0.05$). *Staphylococcus aureus* strain A1008, by contrast, was the most sensitive strain and viable cells were not detected under any of the assayed conditions. Moreover, no viable *S. aureus* cells pressurized at 900 MPa were observed under any of the recovery conditions.

Discussion

The strains of *L. monocytogenes* and *S. enterica* evaluated in the present study, although belonging to different serotypes and having completely different origins, did not show major strain-specific differences either in barotolerance or in the

Table 5. Number of viable cultures of *Staphylococcus aureus* after treatment at 400 and 600 MPa after 1, 3, and 6 weeks of incubation in BHI medium at 14 and 22ºC in the presence of three different concentrations of NaCl, $NaNO_2$, and potassium lactate (KLac) and at three different levels of acidity

HHP treatment:		400 MPa																											
Recovery temperature:		22ºC															14ºC												
Weeks of recovery:		1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6
Strain:		A1008			A1019			A1021			A976			A4466			A1008			A1019			A1021			A976			A4466		
NaCl (% w/v)	4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	7	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	10	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
pH	7.4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	6.3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	5.1	3	3	3	3	3	3	2	2	2	1	1	1	2	3	3	3	2	2	3	2	2	3	2	2	2	1	1	2	3	3
$NaNO_2$ (mg/ml)	5	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	10	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2	3	3	3	3	3	2	3	1	–	3	1	–
	20	3	3	3	3	2	2	3	2	2	3	2	2	3	1	2	3	–	–	3	1	–	3	–	–	3	–	–	3	–	–
KLac (% w/v)	2.4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	4.8	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	7.2	3	3	–	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

HHP treatment:		600 MPa																											
Recovery temperature:		22ºC															14ºC												
Weeks of recovery:		1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6
Strain:		A1008			A1019			A1021			A976			A4466			A1008			A1019			A1021			A976			A4466		
NaCl (% w/v)	4	–	–	–	3	3	3	–	–	–	–	–	–	–	–	–	–	–	–	3	3	3	–	–	–	–	–	–	–	–	–
	7	–	–	–	3	3	3	–	–	–	–	–	–	–	–	–	–	–	–	3	3	3	–	–	–	–	–	–	–	–	–
	10	–	–	–	3	3	3	–	–	–	–	–	–	–	–	–	–	–	–	3	3	3	–	–	–	–	–	–	–	–	–
pH	7.4	–	–	–	3	3	3	–	–	–	1	1	1	1	1	1	–	–	–	3	3	3	2	2	2	2	2	2	–	–	–
	6.3	–	–	–	3	3	3	–	–	–	–	–	–	–	–	–	–	–	–	3	3	3	2	1	2	–	–	–	–	–	–
	5.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
$NaNO_2$ (mg/ml)	5	–	–	–	3	3	3	–	–	1	–	–	2	–	–	1	–	–	–	3	3	3	1	1	1	–	–	–	–	–	–
	10	–	–	–	3	3	2	–	–	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	20	–	–	–	0	0	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
KLac (% w/v)	2.4	–	–	–	3	3	3	1	1	2	–	–	1	–	–	1	–	–	–	3	3	3	3	3	3	–	–	–	–	–	–
	4.8	–	–	–	3	3	3	–	–	–	–	–	–	–	–	–	–	–	–	2	3	3	–	–	–	–	–	–	–	–	–
	7.2	–	–	–	–	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–

"–" indicates no detected replicates with counts above the detection limit (100 CFU/ml) and "3" indicates recovery of all three cultures assayed in three independent experiments.

levels of sublethal injury after pressurization at 400, 600, and 900 MPa. By contrast, higher barotolerance and variability characterized the *S. aureus* strains, as previously observed for other species, stresses, and conditions [21]. While it is generally accepted that gram-positive bacteria are more resistant than gram-negative to HP, the opposite was observed in this study, consistent with reports for contaminated cooked ham [9] and whole milk [4]. Other studies, performed in buffer systems, have shown either similar [16] or quantitatively different [24] results; but, in general, very important reductions of *L. monocytogenes* and *S. enterica* counts following exposure to HP at 600 MPa have been achieved and differences among studies may be attributed to the use of different strains, the different physiological states of the cells, or different experimental conditions (e.g., food product vs. liquid medium). Until now, however, very few studies have examined the effects of pressure levels higher than 600 MPa. Yuste et al. [24] reported that at least 5 min at 700–800 MPa are necessary to completely inactivate *S. aureus*. In the present study, pressures as high as 900 MPa dramatically decreased the levels of *L. monocytogenes*, *S. enterica* and *S. aureus* (>9 log units) in a complex medium; however, the bacterial populations, and especially *S. aureus*, were not completely inactivated and thus able to recover under favorable growth conditions. Accordingly, to avoid overestimation of the efficacy of HP treatments, it is necessary to consider that immediately after pressurization some cells may be sublethally damaged and thus able to recover depending on the strain, the species, and environmental conditions. For example, while *S. enterica* and *S. aureus* recovered better at 22 than at 14ºC, the opposite was found for *L. monocytogenes*. These results are in line with those of Koseki et al. [10], who reported better recovery of *L. monocytogenes* at 4 and 25ºC than at 37ºC. As membrane fluidity affects pressure resistance [3], the better recovery of *L. monocytogenes* at 14ºC could be related to the psychrotrophic characteristics of this species, reflecting differences in its membrane lipid fatty acyl composition [20].

Treatments of up to 600 MPa are currently being applied in the food industry to enhance food safety and to extend the shelf-life of food products [8]. Nevertheless, if environmental conditions (mainly the physicochemical properties of the food product and the storage temperature) are non-stressing and favorable, pathogens that survive HP treatment could soon recover and reach high levels. However, this eventual recovery can be impaired by further antimicrobial treatments, which the pressurized cells, with alterations in their membranes, enzymatic reactions, etc., could not withstand [22]. This agrees with the widely accepted hurdle theory of Leistner, which proposes the use of sublethal levels of agents, such as salt, acid, or heat, that interact synergistically to inhibit or kill food-borne bacteria [13]. When pressurized cells of *S. aureus* were incubated in acidic medium (pH 5.1) and medium containing $NaNO_2$ (10 and 20 mg/ml), cells that had previously survived HP treatment, i.e., both uninjured and sublethally injured cells, progressively died, probably because over the long term they were unable to cope with the stress conditions generated by the two antimicrobials. Acidity, in contrast to other stress factors such as temperature and a_w, requires a large amount of cellular energy to maintain homeostasis [6,11], and the decreased survival of pressurized cells subsequently exposed to low pH buffer and fruit juices has been reported [7]. The effectiveness of $NaNO_2$, by contrast, is related to the formation of nitrous acid [17]. The high osmotolerance of *S. aureus* could explain, at least partially, the poor inhibitory effects of NaCl and lactate [5,19].

In conclusion, several strains of *L. monocytogenes*, *S. enterica* and *S. aureus* at initial populations of 8–9 log CFU/ml were reduced to undetectable levels by HP processing at 400 MPa, 600 MPa, and 900 MPa, respectively. However, subsequent incubation under favorable conditions showed that even after the application of 900 MPa, a few replicates of the three species, but especially those of *S. aureus*, were able to recover. Recovery rates were lowered by combining HP treatment with subsequent incubation in the presence of antimicrobials.

As physicochemical conditions highly affected both the viability and the ability of pressurized cells to recover, evaluations of the effectiveness of HP treatments must include not only an immediate but also a delayed counting. To guarantee the safety of treated food products, it may well be necessary to combine HP treatments with other inhibitors of microbial growth.

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