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Sensitivity of *Listeria innocua* to high hydrostatic pressure at low temperature in Ringer's solution and milk

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Abstract: The research was performed to investigate the effect of high hydrostatic pressure low temperature (HHPLT) on *Listeria innocua* in Ringer's solution (RS) and raw milk (RM). The physicochemical properties of RM were studied at 300 MPa and 10 °C. Compared with the control, protein and lipase activities were reduced at HHPLT in RM. The *L*-values (luminance) of LHPLT applied RM were significantly ($P < 0.05$) decreased for colour Pressure application at 300 MPa for 90 min totally inactivated *L. innocua* at –20 °C in both RS and RM. *L. innocua* showed resistance to low pressure (200 MPa) in RM. Overall, increasing the pressure with applications at –20 and –30 °C resulted in a higher ($P < 0.05$) inactivation of *L. innocua* than at 10 and 20 °C. HHPLT applications to frozen RM resulted in the retention of textural characteristics.

Keywords: *Listeria innocua*; hydrostatic pressure; low temperature; physicochemical property

High hydrostatic pressure (HHP) is a non-thermal alternative technique to heat treatment for food preservation and produces high-quality products (Erkmen and Bozoglu 2016). Different research has been carried out on the inactivation of spoilage and pathogenic microorganisms by HHP from 100 to over 1 000 MPa and from 0 to 100 °C with various holding times (Dogan and Erkmen 2003; Erkmen 2011; Li et al. 2020; Liu et al. 2021). They showed that HHP could produce microbiologically safe products (such as milk and fruit juices with fresh-like characteristics). Multiple factor combinations can enhance the HHP in the inactivation of microorganisms (Li et al. 2020; Liu et al. 2021).

HHP application could reduce many types of important foodborne pathogens (Erkmen and Bozoglu 2016) and foodborne spoilage microorganisms (Gervilla et al. 2000). HHP application at a near-freezing temperature (0 °C) caused greater inactivation of *Listeria innocua*, *P. fluorescens* and *L. helveticus* than at 25 °C, whereas, for *E. coli* and *S. aureus,* the results were opposite (Gervilla et al. 2000). The effects of HHP on enzymes such as lipase, pectinase, chymotrypsin, protease (Uranga-Soto et al. 2022) and proteins (Liu et al. 2021) were also studied. Little information is available on the inactivation of *L. innocua* and improving food quality and safety at a low temperature on the frozen and unfrozen Ringer's solution (RS) and raw milk (RM). RM is a low-acidity food and contains high organic compounds matrices. The objective of this study was to evaluate the survival of *L. innocua* under different low temperatures (from –30 to 20 °C) and HHP (from 200 to 300 MPa) in the inoculated frozen and unfrozen Ringer's solution (RS) and RM. The physicochemical properties of pressure-processed RM at 300 MPa and 10 °C were also investigated.

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MATERIAL AND METHODS

Microorganisms and chemicals. Three *Listeria innocua* strains (ATCC BAA-680, ATCC 51742, and ATCC 33090) were obtained from the American Type Culture Collection (ATCC, USA). They were cultivated in individual trypticase soy broth supplemented with 0.6% yeast extract (TSB-YE; Difco, USA) at 35 °C for 18 h and transferred into the fresh broth three times after every 18 h incubation. The strains were maintained in individual trypticase soy agar supplemented with 0.6% yeast extract (TSA-YE; Difco, USA) slant at 4 °C.

Preparation of microbial culture. Three strains from stock cultures were inoculated into individual 100 mL TSB-YE, and incubated at 35 °C for 24 h. Each song of 100 mL of 24 h *L. innocua* TSB-YE culture was centrifuged at 4 000 xg for 30 min under aseptic conditions. The cells were resuspended twice in 10 mL of sterile RS (0.85% NaCl solution; pH 6.80), centrifuged again, and finally suspended in 10 mL of skim milk or RS. Three strains were mixed by adding 1% (*v*/*v*) of suspended culture from each strain in 100 mL of RM (3% fat and pH 6.67) or RS. The final average number of *L. innocua* ranged from 7.12 to 7.21 log units in both RS and RM. RS and RM without *L. innocua* inoculation were used as a control.

Hydrostatic pressure application. A hydrostatic pressure vessel (internal diameter 4 cm; length 12 cm; maximum pressure tolerance of 1 500 MPa, with an internal volume of 150 cm^3) and a hydraulic unit (HU014; Kon-hidroliksan, Türkiye) were used for hydrostatic pressurisation. The pressure vessel was made of steel type 45WCRV7 which was processed into the required sizes at the Mechanical Engineering Department, Faculty of Engineering, University of Gaziantep, Gaziantep, Türkiye. Ten millilitres from RS or RM experimental culture were placed into each sterile polyethylene bag (5.5 × 4.0 cm; sterilised by 0.1% H_2O_2). The bags were sealed after eliminating the air inside. For freezing temperature (–20 and –30 °C) applications, the bags containing the culture were frozen in a freezer for 12 h. For low temperatures (10 and 20 °C), the bags were cooled in a cold room for 2 h. The frozen sample in the bag was placed into the hydrostatic pressure (HP) vessel. a hydrostatic piston applied HP using deionised water (Figure 1). The sample in the bag was pressurised individually from 200 to 300 MPa at 20, 10, –20, and –30 °C for different treatment times. The pressure vessel was connected to water circulation from a thermostat to maintain the experimental temperature. The pressure increase and release time rates were about 100 and 100 MPa·s^{-1}, respectively. With water circulation around the vessel, the temperature was held at 20 °C for 20 °C applications and at 10 °C for other applications. All control and treated samples were stored at 4 °C for one hour to stabilise the temperature and for recovery of injured cells before microbial counts.

Microbial counts. *Listeria* selective agar (LSA; Difco, USA) was used to count viable *L. innocua* and confirmed as indicated by Erkmen (2022).

Physicochemical analysis of RM. The physicochemical properties (enzyme activities, pH, thiobarbituric acid, colour, and viscosity) of the RM samples without *L. innocua* inoculation were investigated after HHP applications at 300 MPa and 10 °C. The control RM samples were also analysed. The pH values of RM were measured using a pH meter (Model EM78X; Fisher, USA). The protease and li-

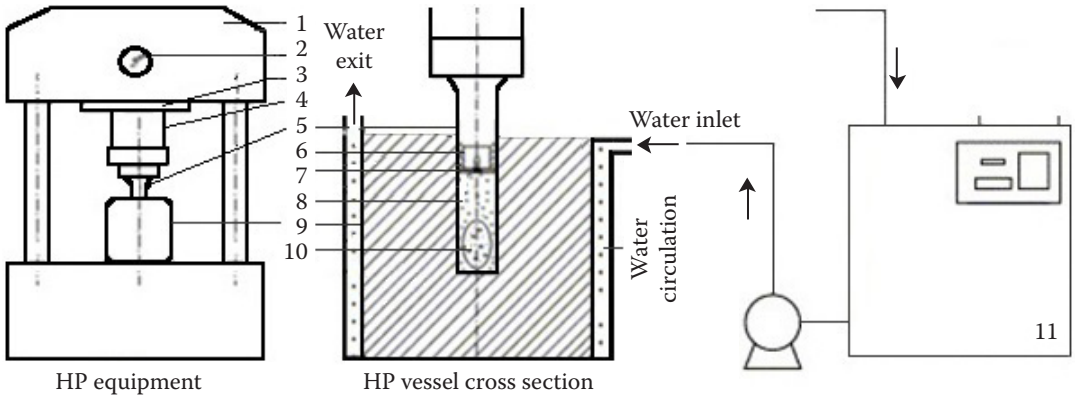

Figure 1. Hydrostatic pressure (HP) units used in pressure treatment

1 – hydraulic pressure instrument; 2 – data logger (pressure); 3 – cylinder; 4 – hydraulic piston; 5 – vessel piston; 6 – V-ring; 7 – water; 8 – vessel; 9 – screw; 10 – medium in bag; 11 – cooler; HP – hydrostatic pressure

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pase activities of samples were measured (Chae 2002). Thiobarbituric acid (TBA) was used to indicate the formation of oxidative products of fatty acids in RM using a spectrophotometric method (Kim et al. 2008). The colour of the samples was measured with a colourimeter (60-1010-615 Model; Color Meter/Hunter-Lab Colorflex, USA), as indicated by Kim et al. (2008) and Chudy et al. (2020). The results for the color measurements of the samples taken before and after the process are given in the Color Solutions International (CSI) system as L^* [luminance; 0 (darkness), 100 (clearness)], a^* [colour; – (green), + (redness)], and b^* [colour, – (blueness), + (yellow)] values. The viscosity value of the sample (5 mL) was measured at 5 °C using a viscometer with a single spindle at 100 rpm. All samples were measured in duplicate.

Statistical analysis. Each experiment, with duplicate bags, was performed in triplicate on separate days, and the results are the average of those values. Analyses of variance were performed on data obtained at different stages of HHP applications using SPSS v22 software (IBM SPSS Statistics v22). One-way ANOVA was performed by comparing sample means using Duncan's multiple-range test. Evaluations were based on a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Physicochemical analysis of milk. Protease activity was 6.94 units·mL^{-1} in RM, and 5.93, 4.59, and 1.99 units·mL^{-1} in pressure applied RM for 6, 12, and 24 min, respectively, at 300 MPa (Table 1). About 14.6, 33.9, and 71.3% reduction of protease activity was obtained after 6, 12, and 24 min of pressure application, respectively. Lipase activity was 0.74 units·mL^{-1} in RM, and 0.62, 0.51, and 0.22 units·mL^{-1} in pressure-applied RM for 6, 12, and 24 min, respectively. About 16.2, 31.1, and 70.3% of lipase activity were reduced after 24 min pressure application. Protease and lipase activities were significantly ($P < 0.05$) reduced after each application time. The results indicated that HHP applications from 6 to 24 min highly reduced RM's protease and lipase activities. The pH values of pressure applied RM were 6.66, 6.66, and 6.65 for 6, 12, and 24 min, respectively (Table 1). High hydrostatic pressure low temperature (HHPLT) application did not significantly ($P > 0.05$) affect the pH value of RM.

TBA slightly increased following 12 and 24-min pressure application; however, 6-min treatment did not affect TBA values (Table 1). TBA value in control RM was 0.046, compared with 0.056 in 24 min pressure application. TBA value in pressure applied RM was significantly ($P < 0.05$) greater for 24 min than for the other application times. TBA values from HHPLT applications were relatively low in comparison with the other methods of milk processing, such as pasteurisation or freezing of milk (Dias et al. 2020). Results indicated that the HHPLT applications did not induce any dramatic lipid oxidation in RM.

The viscosity values of HHPLT applied RM for 6, 12, and 24 min were 0.74, 0.75, and 0.76 MPa, respectively, and that of RM was 0.73 MPa (Table 1), which was not significantly ($P > 0.05$) different from each other.

The L-values of pressure-treated RM were decreased with increasing pressure application (Table 2). L-values in HHPLT applied RM for 12 and 24 min were significantly ($P < 0.05$) decreased but not for 6 min. HHPLT application reduced the L-values of RM, presumably due to the disruption of casein micelles into small fragments that increase the translucence of milk (Kim et al. 2008). The a- and b-values were significantly ($P < 0.05$) decreased and increased in all HHPLT applied RM, respectively (Table 2). The total colour change, presented as ΔE, was significantly ($P < 0.05$) reduced after 6, 12, and 24 min HHPLT applications (Table 2). The colour change occurred with the increasing HHPLT application time.

Antimicrobial effects of pressure on *L. innocua*. The responses of *L. innocua* to pressures from 200 to 300 MPa under different exposure time at 10 and

Table 1. Enzymes activity, pH, thiobarbituric acid (TBA) and viscosity changes in raw milk (RM) at 300 MPa and 10 °C (mean ± SD; $n = 3$)

Application time	Protease	Lipase	pH	TBA	Viscosity (MPa)
Control RM	6.91 ± 0.05^a	0.74 ± 0.02^a	6.67 ± 0.03^a	0.046 ± 0.002^a	0.73 ± 0.1^a
6 min	5.43 ± 0.02^b	0.62 ± 0.01^b	6.66 ± 0.02^a	0.047 ± 0.003^a	0.74 ± 0.1^a
12 min	4.59 ± 00.4^c	0.51 ± 0.02^c	6.66 ± 0.02^a	0.049 ± 0.001^a	0.75 ± 0.2^a
24 min	1.99 ± 0.03^d	0.22 ± 0.0^d	6.65 ± 0.03^a	0.056 ± 0.003^b	0.76 ± 0.3^a

$^{a–d}$ significant survival differences at the same pressure processes determined by the least significant difference test at $P < 0.05$

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Table 2. Changes of colour (*L-*, *a-*, *b-*, and Δ*E* values) in raw milk (RM) at 300 MPa and 10 °C (mean ± SD; *n* = 3)

Application time	*L*-value	*a*-value	*b*-value	Δ*E*
Control RM	75.69 ± 2.28^{a}	–3.27 ± 0.21^{a}	3.57 ± 0.05^{a}	4.16 ± 0.03^{a}
6 min	73.11 ± 1.73^{a}	–3.12 ± 0.10^{b}	3.82 ± 0.10^{b}	4.53 ± 0.03^{b}
12 min	68.16 ± 0.65^{b}	–2.68 ± 0.10^{c}	4.19 ± 0.20^{c}	5.63 ± 0.05^{c}
24 min	61.86 ± 0.62^{c}	–2.07 ± 0.20^{d}	4.59 ± 0.08^{d}	7.79 ± 0.07^{d}

$^{a–d}$ significant survival differences at the same pressure processes determined by the least significant difference test at $P < 0.05$; *L* – luminance parameter; *a*, *b* – colour parameters; Δ*E* – total colour change

Table 3. *Listeria innocua* survival at high hydrostatic pressure (HHP) treatment in Ringer's solution (RS) at 10 and 20 °C (mean ± SD; *n* = 3)

Time (min)	10 °C			20 °C		
	200 MPa	250 MPa	300 MPa	200 MPa	250 MPa	300 MPa
0	7.16 ± 0.03^{aA}	7.19 ± 0.03^{aA}	7.12 ± 0.02^{aA}	7.16 ± 0.05^{aA}	7.15 ± 0.02^{aA}	7.22 ± 0.03^{aA}
5	7.15 ± 0.02^{aA}	7.15 ± 0.04^{aA}	6.95 ± 0.06^{bB}	7.16 ± 0.03^{aA}	7.13 ± 0.03^{aA}	6.93 ± 0.02^{bB}
10	7.14 ± 0.04^{bA}	7.04 ± 0.03^{bA}	6.37 ± 0.08^{cB}	7.15 ± 0.06^{aA}	7.09 ± 0.05^{aA}	6.58 ± 0.04^{cC}
15	7.01 ± 0.06^{cA}	6.58 ± 0.03^{cB}	5.62 ± 0.05^{dC}	7.08 ± 0.02^{aA}	6.71 ± 0.02^{bD}	6.16 ± 0.03^{dE}
30	6.87 ± 0.07^{dA}	6.09 ± 0.06^{dB}	4.85 ± 0.05^{eC}	6.98 ± 0.08^{bD}	6.26 ± 0.06^{cE}	5.33 ± 0.03^{eF}
60	6.58 ± 0.05^{eA}	5.43 ± 0.07^{eB}	3.39 ± 0.09^{fC}	6.71 ± 0.09^{cD}	5.59 ± 0.06^{dE}	4.11 ± 0.09^{fF}
90	6.30 ± 0.06^{fA}	5.08 ± 0.07^{fB}	2.30 ± 0.08^{gC}	6.48 ± 0.07^{dD}	5.29 ± 0.04^{eE}	3.07 ± 0.10^{gF}

$^{a–g}$ significant survival differences at the same pressure processes; $^{A–F}$ significant survival differences among pressure processes, determined by the least significant difference test at $P < 0.05$

20 °C in RS are given in Table 3. The sensitivity of *L. innocua* was not noticeable within 10 min at 200 MPa and 20 °C and 10 °C. *L. innocua* showed higher survival at 200 MPa at 10 °C than at 250 and 300 MPa. At 300 MPa, 4.86 log units of *L. innocua* were inactivated after 90 min while 4.09 log units were inactivated at 20 °C. The increase of temperature from chilling (10 °C) to room temperature (20 °C) at 300 MPa showed a significant ($P < 0.05$) reduction in *L. innocua*. After 10 min pressure application by 200 and 250 MPa at 10 and 20 °C, only less than 0.12 log units of cell inactivation were found, while 0.79 and 0.58 log unit reductions were observed at 10 and 20 °C, respectively, at 300 MPa.

There were significant ($P < 0.05$) differences between –30 and –20 °C concerning *L. innocua* inactivation at both 250 and 300 MPa (Table 4). Pressure application at 200, 250, and 300 MPa in RS at –30 °C showed

Table 4. *Listeria innocua* survival at high hydrostatic pressure (HHP) treatment in Ringer's solution (RS) at –20 and –30 °C (mean ± SD; *n* = 3)

Time (min)	–20 °C			–30 °C		
	200 MPa	250 MPa	300 MPa	200 MPa	250 MPa	300 MPa
0	7.12 ± 0.03^{aA}	7.17 ± 0.03^{aA}	7.16 ± 0.04^{aA}	7.23 ± 0.03^{aA}	7.13 ± 0.01^{aA}	7.15 ± 0.02^{aA}
5	6.87 ± 0.02^{bA}	6.40 ± 0.04^{bB}	5.31 ± 0.07^{bC}	7.02 ± 0.07^{aA}	6.14 ± 0.03^{bD}	5.61 ± 0.08^{bE}
10	6.18 ± 0.05^{cA}	5.08 ± 0.03^{cB}	4.04 ± 0.02^{cC}	6.65 ± 0.05^{bD}	5.45 ± 0.04^{cE}	4.52 ± 0.04^{cF}
15	5.51 ± 0.10^{dA}	3.95 ± 0.04^{dB}	2.87 ± 0.02^{dC}	5.94 ± 0.05^{cD}	4.34 ± 0.05^{dE}	3.42 ± 0.03^{dF}
30	4.12 ± 0.08^{eA}	2.69 ± 0.10^{eB}	2.07 ± 0.07^{eC}	5.09 ± 0.06^{dD}	3.43 ± 0.08^{eE}	2.99 ± 0.03^{eF}
60	2.89 ± 0.07^{fA}	1.77 ± 0.03^{fB}	1.32 ± 0.02^{fC}	3.54 ± 0.06^{eD}	2.63 ± 0.02^{fAE}	2.15 ± 0.02^{fBF}
90	1.34 ± 0.02^{gA}	0.00^{gB}	0	2.37 ± 0.04^{fC}	1.96 ± 0.02^{gD}	1.14 ± 0.01^{gE}

$^{a–g}$ significant survival differences at the same pressure processes; $^{A–E}$ significant survival differences among pressure processes, determined by the least significant difference test at $P < 0.05$

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Table 5. *Listeria innocua* survival at high hydrostatic pressure (HHP) treatment in raw milk (RM) at 10 and 20 °C (mean ± SD; n = 3)

Time (min)	10 °C			20 °C		
	200 MPa	250 MPa	300 MPa	200 MPa	250 MPa	300 MPa
0	7.14 ± 0.01^{aA}	7.16 ± 0.06^{aA}	7.17 ± 0.03^{aA}	7.12 ± 0.03^{aA}	7.18 ± 0.04^{aA}	7.14 ± 0.02^{aA}
5	7.15 ± 0.02^{aA}	7.15 ± 0.03^{aA}	6.91 ± 0.07^{bB}	7.16 ± 0.05^{aA}	7.15 ± 0.03^{aA}	6.96 ± 0.01^{bC}
10	7.13 ± 0.03^{aA}	7.04 ± 0.03^{bA}	6.37 ± 0.05^{cB}	7.14 ± 0.06^{aA}	7.08 ± 0.04^{aA}	6.58 ± 0.05^{cC}
15	6.97 ± 0.047^{bA}	6.58 ± 0.06^{cB}	5.62 ± 0.07^{dC}	7.08 ± 0.02^{aD}	6.72 ± 0.02^{bE}	6.16 ± 0.04^{dF}
30	6.63 ± 0.10^{cA}	6.10 ± 0.02^{dB}	4.85 ± 0.05^{eC}	6.94 ± 0.05^{aD}	$6.26 \pm 0.04c^{E}$	5.33 ± 0.07^{eF}
60	6.30 ± 0.12^{dA}	5.43 ± 0.12^{eB}	3.39 ± 0.08^{fC}	6.66 ± 0.09^{bD}	5.79 ± 0.08^{dE}	4.46 ± 0.09^{fF}
90	6.14 ± 0.06^{eA}	5.11 ± 0.04^{fB}	2.48 ± 0.12^{gC}	6.41 ± 0.06^{cD}	5.37 ± 0.05^{eE}	3.79 ± 0.02^{gF}

$^{a–f}$ significant survival differences at the same pressure processes; $^{A–F}$ significant survival differences among pressure processes, determined by the least significant difference test at $P < 0.05$

a higher survival ($P < 0.05$) of *L. innocua* than at –20 °C. In general, increased inactivation ($P < 0.05$) occurred with longer exposure time and greater magnitude of pressure. At 250 and 300 MPa, all the initially inoculated *L. innocua* microorganisms (7.16 log units) were inactivated at –20 °C after 90 min, while 1.96 and 1.14 log units survived at –30 °C, respectively. The lethality of pressures on *L. innocua* was significantly ($P < 0.05$) higher at both –20 °C and –30 °C than at 200 MPa. At –20 and –30 °C, different combinations of pressure, freezing, and time showed a higher lethal effect than 10 and 20 °C in RS. This effect was more marked at 300 MPa with a shorter pressure application time. These results showed that the freezing temperature itself has a lethal action on microorganisms to some extent.

L. innocua survived in RM due to the baroprotective effect of milk on bacterial cells, especially the high protein and fat contents of RM (Table 5). Effects of 250 MPa on *L. innocua* were significantly ($P < 0.05$) different starting from 15- and 30-min exposures at 10 and 20 °C, respectively. The inactivated *L. innocua* at 200 and 250 MPa and 20 °C after 90 min were 0.75 and 1.79 log units, respectively, and 1.02 and 2.05 log units at 10 °C, respectively. Lethality was rapid when the pressure reached 300 MPa, lethality increasing to 4.68 and 3.37 log units at 10 and 20 °C, respectively. The temperature decrease from 20 to 10 °C showed a significant ($P < 0.05$) reduction in *L. innocua* at 200 and 250 MPa starting from 15 min exposure, while these decreases were significant ($P < 0.05$) at 300 MPa at all applications.

The inactivated *L. innocua* at –30 °C and 200 and 250 MPa after 90 min were 4.72 and 5.38 log units, respectively, while 5.53 and 6.74 log units were inactivated at –20 °C, respectively (Table 6). All *L. innocua* microorganisms were inactivated at 300 MPa at –20 °C, while 6.38 log units were inactivated at –30 °C. A sig-

Table 6. *Listeria innocua* survival at high hydrostatic pressure (HHP) treatment in raw milk (RM) at –20 and –30 °C (mean ± SD; n = 3)

Time (min)	–20 °C			–30 °C		
	200 MPa	250 MPa	300 MPa	200 MPa	250 MPa	300 MPa
0	7.13 ± 0.04^{aA}	7.15 ± 0.04^{aA}	7.19 ± 0.04^{aA}	7.12 ± 0.02^{aA}	7.21 ± 0.01^{aA}	7.17 ± 0.02^{aA}
5	7.13 ± 0.05^{aA}	7.06 ± 0.05^{bB}	5.68 ± 0.080^{bC}	7.15 ± 0.05^{aD}	7.09 ± 0.02^{bE}	6.29 ± 0.06^{bF}
10	7.02 ± 0.03^{bA}	6.85 ± 0.03^{cB}	4.69 ± 0.06^{cC}	7.08 ± 0.05^{bD}	6.95 ± 0.06^{cE}	5.51 ± 0.02^{cF}
15	5.65 ± 0.03^{cA}	4.08 ± 0.03^{dB}	3.87 ± 0.02^{dC}	6.03 ± 0.03^{cD}	5.27 ± 0.02^{dE}	4.42 ± 0.03^{dF}
30	4.81 ± 0.01^{dA}	3.01 ± 0.01^{eB}	2.29 ± 0.03^{eC}	5.31 ± 0.04^{dD}	4.31 ± 0.03^{eE}	3.89 ± 0.02^{eF}
60	3.00 ± 0.02^{eA}	1.87 ± 0.02^{fB}	1.12 ± 0.02^{fC}	3.38 ± 0.02^{eD}	2.63 ± 0.02^{fE}	2.14 ± 0.02^{fF}
90	1.63 ± 0.02^{fA}	0.42 ± 0.02^{gB}	0	2.44 ± 0.02^{fD}	1.78 ± 0.09^{gE}	0.78 ± 0.01^{gF}

$^{a–g}$ significant survival differences at the same pressure processes; $^{A–F}$ significant survival differences among pressure processes, determined by the least significant difference test at $P < 0.05$

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nificant ($P < 0.05$) reduction of *L. innocua* was started after 10 min pressure application at 200 MPa, while it was significantly ($P < 0.05$) reduced at all applications at 250 and 300 MPa. The temperature increase from –30 to –20 °C showed a significant ($P < 0.05$) reduction in *L. innocua* at all pressures.

The low-temperature effects at HHP on microorganisms differed; inactivation in RS and RM was greater in the order –20 > –30 > 10 > 20 °C. *L. innocua* showed higher resistance to low pressure at 20 °C than at chilling temperature (10 °C). The inactivation values of *L. innocua* were higher in RS than in RM at all temperatures and pressures. More *L. innocua* microorganisms survived in RM due to the baroprotective effect of milk on bacterial cells. Similar results were reported in the literature (Gervilla et al. 1997; Pinho et al. 2015; Li et al. 2020). During the pressure shifts freezing processes, the sample is cooled under atmospheric pressure to –20 and –30 °C and then subjected to HHP. The freezing seems to cause stress on the membrane. The ice would result in higher damage at HHPLT with the volume increase. Patterson et al. (1995) and Gervilla et al. (2000) reported that greater inactivation occurred in the order fruit juices > PBS > RM at 20 °C by HHP. Gervilla et al. (2000) indicated that milk could also contain antimicrobial factors that can produce a synergic inhibitory effect with pressure in RM. They reported lower inactivation in UHT milk in the PBS > RM > UHT milk order. The results for *L. innocua* in RS and RM were like these results as higher inactivation was observed in RS than in RM, but the frozen application was significantly ($P < 0.05$) higher than unfrozen applications.

The inactivation was also dramatically increased ($P < 0.05$) at 250 and 300 MPa compared to 200 MPa. This would be due to more severe damage to the cell membrane and leakage of cellular components (Shimado et al. 1993; Kalchayanand et al. 2002). Interestingly, when *L. innocua* cells were treated even with a pressure of 300 MPa for 90 min at 10 and –20 °C, there would be a drastic increase in the release of internal substances. At about 300 MPa for 10 min treatment, many cells (from 0.78 to 3.12 log units) were inactivated in RS and RM depending on temperature.

L. innocua was completely (7.16 log units) inactivated after 90 min at –20 °C in both RS and RM at 300 MPa. On the contrary, *L. innocua* in RS was reduced to 4.86, 4.09, and 6.02 log units at the same pressure at 10, 20, and –30 °C, respectively, and in RM to 4.68, 3.37, and 6.38 log units, respectively. Pinho et al. (2015) reported that *L. innocua* and *L. helveticus* in skimmed milk were more resistant to 200 MPa reaching inactivation at 1.58 and 1.14 log units, respectively. They also reported that *P. fluorescens*, *L. innocua* and *L. helveticus* were completely inactivated (7.0 log units) at 200, 250, and 300 MPa, respectively. This difference can be explained by process temperature and time, food matrix and strain of microbial culture. Ludwig et al. (1992) reported greater destruction of *E. coli* at low temperatures (2 °C) than at 25 °C. These results were like our results when HHP showed higher destructive effects on *L. innocua* at chilling (10 °C) and freezing (–20 and –30 °C) temperatures than at room temperature (20 °C).

CONCLUSION

L. innocua showed high resistance to 200 MPa. Still, above this pressure, there was an increased microbial inactivation in a narrow pressure range (200–300 MPa), reaching the total inactivation load (7.16 log units) after 250 and 300 MPa applications in RS at –20 °C after 90 min while it was observed in RM only at 300 MPa. Inactivation of *L. innocua* was higher at the temperature increase from –30 to –20 °C. At the same time, it was decreased with the temperature increase from 10 to 20 °C. The HHP applications at chilling temperatures would result in high inactivation. Freezing to the ice of higher density than liquid water would result in membrane damage. Freezing to ice and connection to HHP resulted in excellent preservation of the textural characteristics of RM. HHPLT treatment can be used as a potential nonthermal technique for food preservation. Following the investigations of pressurisation combined with other synergic treatments or processes, HHPLT may be a good alternative to heat treatment for milk or other foods.

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