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Effect of High Hydrostatic Pressure on *Listeria innocua* 910 CECT Inoculated into Ewe's Milk

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ABSTRACT

Ewe's milk standardized to 6% fat was inoculated with *Listeria innocua* 910 CECT at a concentration of 10^7 CFU/ml and treated by high hydrostatic pressure. Treatments consisted of combinations of pressure (200, 300, 350, 400, 450, and 500 MPa), temperature (2, 10, 25, and 50°C), and time (5, 10, and 15 min). To determine numbers of *L. innocua*, listeria selective agar base with listeria selective supplement and plate count agar was used. Low-temperature (2°C) pressurizations produced higher *L. innocua* inactivation than treatments at room temperatures (25°C). Pressures between 450 and 500 MPa for 10 to 15 min were needed to achieve reductions of 7 to 8 log units. The kinetics of destruction of *L. innocua* were first order with *D*-values of 3.12 min at 2°C and 400 MPa and 4 min at 25°C and 400 MPa. A baroprotective effect of ewe's milk (6% fat) on *L. innocua* was observed in comparison with other studies using different media and similar pressurization conditions.

Key words: High hydrostatic pressure, *Listeria innocua*, kinetics of destruction, ewe's milk

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Listeria monocytogenes has been described as the causative agent of cases of foodborne illness associated with the consumption of milk, cheese, and other milk products. *L. monocytogenes* is important to the dairy industry because of its ability to grow under a wide variety of food-processing conditions. The pH range for growth is 4.4 *(11)* to 9 *(28)*; temperature conditions between 1°C to 45°C permit its development, with an optimum between 30 and 37°C *(28)*. Its resistance to NaCl is high: it is able to live in media containing up to 10% NaCl (a_w = 0.935) *(8)*. Other limiting conditions on microbial growth (vacuum, freezing, ultraviolet energy, etc.) show the ability of *L. monocytogenes* to survive adverse conditions *(12, 21, 34)*. Thermal treatment is the most frequent process technology applied to milk. From this point of view, many authors differ about the resistance of *L. monocytogenes* to conventional pasteurization (71.7°C for 16 s), because the effectiveness of the treatment (thermal conductivity) is affected by the composition of the food to be treated *(2, 7, 9, 10)*.

At present, it is necessary to search for alternatives to conventional treatments that are capable of providing safety for foods without the addition of preservatives. Likewise, it is necessary not to modify the physicochemical, nutritional, and organoleptic characteristics of raw foods by changes induced by treatments. It is well known that thermal treatments in milk lead to undesirable side effects such as off flavors, nonenzymatic browning, vitamin denaturation, protein precipitation, etc. *(1, 20, 25, 30)*. In recent years high hydrostatic pressure (HHP) treatment of foods has been developed with pressures ranging from 100 to 600 MPa *(4, 14)*, which have produced important microbiological reductions *(15, 22, 33)*. Some authors have studied the effect of HHP on *Listeria* spp. in meat *(3)*, cow's UHT (ultra-high-temperature-treated) milk *(30)*, liquid cream *(26)*, and cultured or buffered media *(31)*, but no work on ewe's milk has been undertaken. The extensive consumption of cheeses from nonpasteurized milk in many countries makes the study of ewe's milk relevant. Furthermore, ewe's milk (whole or skimmed) has a thermoprotective effect on *Listeria* spp. *(23)*.

A nonhuman pathogen, *Listeria innocua*, was chosen for this work. As is known, due to the phylogenetic proximity of *L. innocua* to *L. monocytogenes* (a human pathogen), *L. innocua* is an indicator microorganism for *L. monocytogenes (6, 23, 24)*.

The main objective of this work was to study the effect of different treatment conditions (pressure, time, and temperature) on the destruction of *L. innocua* 910 CECT inoculated into ewe's milk at a rate of about 10^7 CFU/ml. Also an attempt was made to determine the *D*-value (decimal reduction time) and estimate the application conditions of HHP technology.

MATERIALS AND METHODS

Bacterial strain

Listeria innocua 910 CECT was obtained from the Spanish Type Culture Collection (CECT; University of Valencia, Valencia, Spain). Rehydration was undertaken in 3 ml of brain heart infusion broth (BHI) (Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C for 24 h. Subsequently 1 ml of the culture was inoculated into 9 ml of BHI at 37°C for 24 h and used in each experiment. The stock culture was maintained on brain heart infusion agar (BHIA)

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(Oxoid) at 4°C and transferred monthly. *L. innocua* was inoculated into BHI at 37°C for 24 h; concentrations were obtained of 10^9 CFU/ml. Ten milliliters of this culture was added to 1-liter milk samples to achieve an approximate concentration of 10^7 CFU/ml in the milk samples.

Composition and physicochemical analyses of milk

The total solids content was determined by drying at 102 ± 2°C in an oven until a constant weight was reached *(17)*. Ash content was determined by gravimetric analysis after the sample had been calcinated in an oven at 550°C *(16)*. Fat content was determined by the Gerber method *(18)*. Total nitrogen was calculated using the digestion block method, a modification of the Kjeldahl method *(19)*. The pH was measured by using a pH meter (micro-pH 2001, Crison Instruments S.A., Alella, Spain) *(27)*.

Preparation and inoculation of milk samples

Milk from Manchega ewes was obtained from the dairy farm of the Veterinary Faculty, Universitat Autònoma de Barcelona, Spain. Raw milk was collected from the first milking in the morning and standardized to 6% fat. Standardized milk was pasteurized 60 to 90 min after collection at 85 ± 1°C for 1 min in a continuous tubular heat exchanger (Garbia SA, Barcelona, Spain) with a capacity of 50 liters/h. Pasteurized milk was collected in 1-liter sterile bottles, adjusted to pH 6.7 (by adding 1 N NaOH and/or 1 N HCl) and refrigerated at 4°C.

Ten milliliters of BHI with 10^9 CFU/ml of *L. innocua* was inoculated into 1 liter of pasteurized ewe's milk to obtain approximately 10^7 CFU/ml. The milk was gently shaken by hand for 5 min and then 30 ml of inoculated milk was pipetted into disinfected 30-ml polyester bottles. As much air as possible was expelled from the bottles and the caps were sealed with Teflon film.

High-pressure processing

The samples were pressurized by using discontinuous high hydrostatic pressure equipment (ACB, Nantes, France), with a pressure chamber of 10 cm diameter and 25 cm height (ca. 2 liters) which can reach 500 MPa in 4 min. Samples were submerged in water, which acted as the hydrostatic fluid medium. The chamber temperature was determined by means of a thermoregulating system which circulated heating and/or cooling fluid (ethylene glycol-water mixture) within the walls of the vessel. The chamber water temperature was measured by a thermocouple. Samples were kept for 5 to 10 min at atmospheric pressure in the chamber until temperature equilibrium was established.

Time, temperature, and pressure parameters were selected on the basis of previous unpublished studies. The response of *L. innocua* was studied to treatments at different conditions of pressure (200, 300, 350, 400, 450, and 500 MPa), temperature (2, 10, 25, and 50°C), and time (5, 10, and 15 min). To determine the population kinetics of *L. innocua*, pasteurized ewe's milk was inoculated to approximately 10^8 CFU/ml. Assays were performed at 400 MPa, two temperatures (2 and 25°C), and different times (0 to 45 min). Linear regression of *L. innocua* counts was computed for each temperature. An estimate of the decimal reduction value (D = the time in minutes necessary to kill 90% of the microbial population at a certain temperature and pressure) was obtained by finding the absolute value of the inverse of the slope. Each treatment was individually performed three times.

Microbiological assays

The time elapsed from pressurization of the samples until performance of the microbiological assays was approximately 10 h and the samples were kept at 4°C during this period to avoid the growth of inoculated strains and possible postpressurization stress.

After treatment each sample was analyzed using the appropriate decimal dilutions in Ringer solution (9 ml).

To determine the quantity of *L. innocua* in treated and control samples, 0.1-ml aliquots were pipetted from dilutions in Ringer solution and surface-plated onto Oxford formulation listeria selective agar base (LSA) in Oxford formulation listeria selective supplement (S) (both Oxoid). The plates were incubated at 37°C for 48 h. Colonies of *Listeria* spp. were identified as gray colonies surrounded by black zones caused by esculin hydrolysis on LSA + S (according to Oxoid recommendations). Selected colonies were confirmed as *Listeria* spp. by the following criteria: gram-positive, non-spore-forming, oxidative-negative, and catalase-positive. At the same time plate count agar (PCA) (Oxoid) was used to determine any possible contamination of samples; the plates were incubated at 30°C for 48 h. Each dilution was plated twice on LSA + S and PCA.

Statistical treatment of data

Each experiment was replicated three times with duplicate analysis in each replication. A descriptive analysis was made of the variables which were of interest using the statistics package SPSS; subsequently an analysis of variance (ANOVA) was performed using the general linear models procedure of Statistical Analysis System (SAS). Duncan's new multiple range test was used to obtain pairwise comparisons among sample means. Evaluations were based on a 5% significance level ($P < 0.05$). D-values were determined as the inverse of the slope of the survivor regression curve.

RESULTS

The composition of the ewe's milk before fat standardization was total solids, 18.24% ± 1.87%; fat, 7.73% ± 1.63%; total nitrogen, 5.71% ± 0.16%; ash, 1.22% ± 0.14%; the pH was 6.68 ± 0.09.

The response of *L. innocua* 910 CECT to pressures from 200 to 500 MPa and under different conditions of time and temperature is shown in Figure 1. In general increased inactivation occurred with longer exposure and greater magnitude of pressure. The sensitivity of *L. innocua* was not noticeable at 200 MPa. At this pressure a reduction of 1 log unit was observed at 50°C for 10 and 15 min. The increment of 300 to 350 MPa showed the greatest initial decrease (about 2 log units) at both 2 and 10°C independently of applied time. At 25°C this increment was detected in the range between 400 and 450 MPa, while at 50°C the greatest initial decrease of population was observed from 200- to 300-MPa treatments.

At 500 MPa, viable cells of *L. innocua* were not detectable within 5 min at any treatment temperature, and the reduction of population was about 7 to 8 log units. At 450 MPa, combinations of 2, 10, and 25°C for 10 min led to a reduction by 5 to 6 log units to about 10^1 to 10^2 CFU/ml within 5 and 10 min respectively; moreover, extension of the pressurization time to 15 min at these temperatures resulted in no growth of the culture. At 50°C, different combinations of pressure and time showed the same lethal effect as mentioned; 5 min at 450 MPa, 10 min at 400 MPa, or 15 min at 350 MPa resulted in no viable cells counts. It is noticeable that *L. innocua* was less sensitive at 25°C compared to the other temperatures used (Figure 2). This effect was more
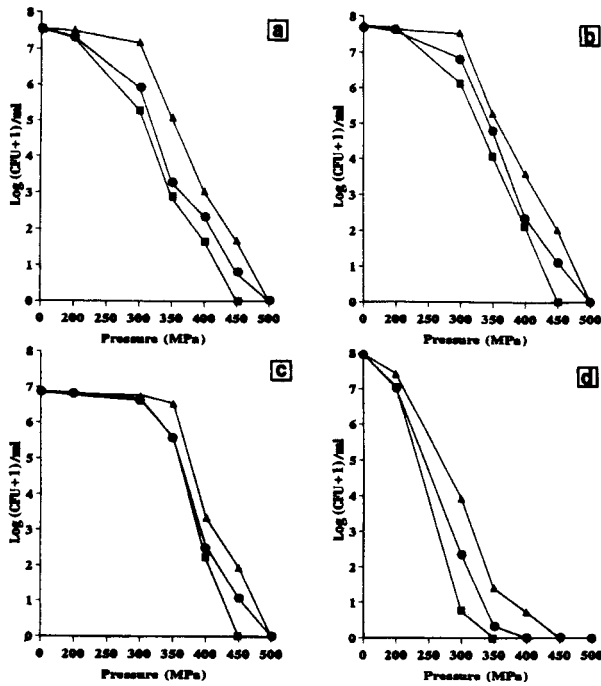
FIGURE 1. *Effect of high hydrostatic pressure on* Listeria innocua *910 CECT inoculated in ewe's milk. Pressure for (▲) 5 min, (●) 10 min, (■) 15 min. Pressure at 0, logarithm of initial counts (control); a, 2°C; b, 10°C; c, 25°C; d, 50°C.*

marked at 350 MPa and longer pressure application times (10 and 15 min).

The analysis of variance (ANOVA) shows that there were significant differences between pressures of 200, 300, and 350 MPa ($P < 0.05$), but not between 400, 450, and 500
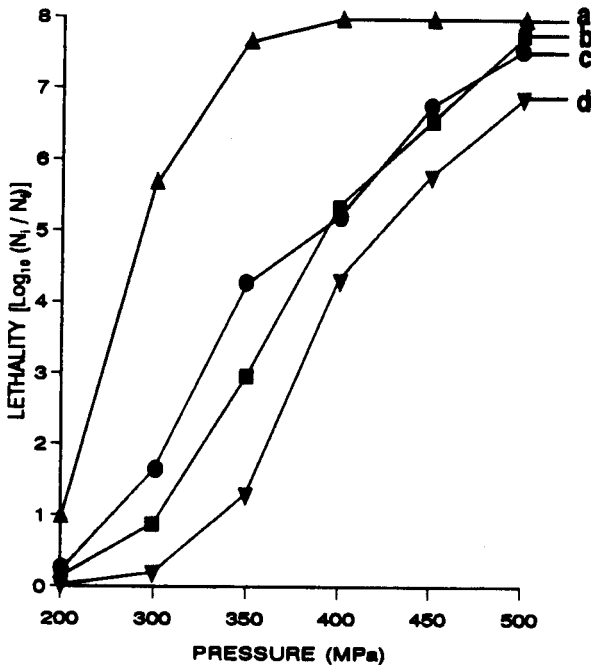

FIGURE 2. *Effect of high hydrostatic pressure at different temperatures for 10 min on* Listeria innocua *910 CECT inoculated in ewe's milk. (●) 2°C, (■) 10°C, (▼) 25°C, (▲) 50°C. Destruction limit, a, 50°C; b, 10°C; c, 2°C; d, 25°C.*
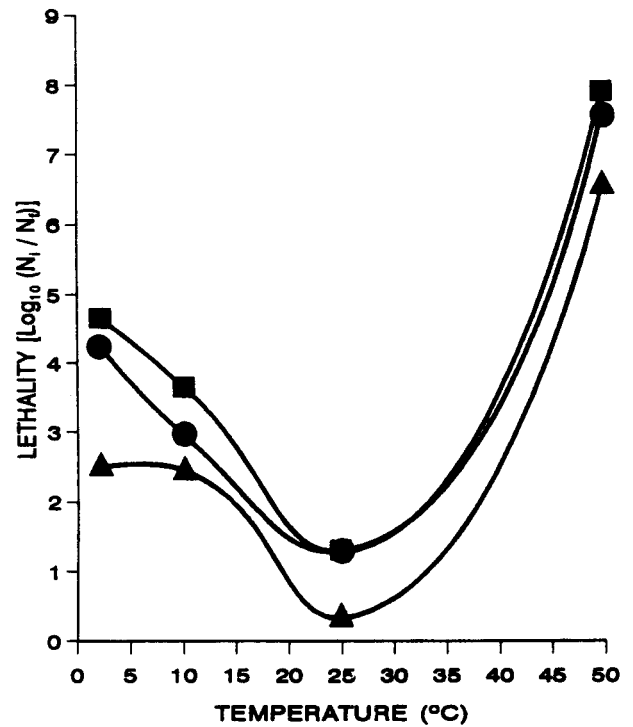

FIGURE 3. *Effect of high hydrostatic pressure as a function of temperature at 350 MPa and different times on* Listeria innocua *910 CECT inoculated in ewe's milk. (▲) 5 min, (●) 10 min, (■) 15 min.*

MPa ($P < 0.05$). There were significant differences between all the temperatures and treatment times studied ($P < 0.05$). From the analysis of the F-value we should emphasize that the pressure was the main factor. Pressure per se explains the 73% of the variability of the statistical model.

With respect to temperature, in the range between 20 and 30°C the assays at 300, 350, 400, and 450 MPa showed maximum survival of the strain (data not shown). In the range of temperature mentioned, the maximum survival varied as a function of pressure and time of treatment applied. Figure 3 shows that the tendency to achieve maximum survival at 350 MPa is near 23°C.

Study of kinetics of destruction of L. innocua *910 CECT*

Initial counts were about 7.7 log CFU/ml. The range of time in which the major destruction rate was observed was situated between 0 and 10 min for both temperatures assayed. With 10 min of treatment, reductions of 5 and 4.4 log units were observed at 2 and 25°C, respectively. The population reduction shown with an initial count of 7.7 log CFU/ml was confirmed by follow-up assays, in which 10^3 CFU/ml of *L. innocua* was inoculated. The same conditions of pressure and temperature were used, applying times of 5 min. In these treatments, the same reduction was obtained (data not shown).

Ten hours after pressurization, milk samples were analyzed to confirm the irreversibility of the cellular damage due to HHP. Two samples of each treatment were analyzed simultaneously. The first of them was directly incubated (37°C for 48 h) and the second was cultured (LSA + S) and

immediately incubated (37°C for 48 h). From the observation of plates of the latter samples, treatments with no growth were detected. To confirm these results, samples previously incubated corresponding to those treatments were plated (LSA + S) and incubated (37°C for 48 h). In these duplicates, no growth was observed in any case. This confirms the total destruction of the strain and eliminates the question of the possible effects of postpressurization stress (data not shown).

Destruction kinetics studies (D-values) show that the destruction limit (7.7 log units) at 2°C was achieved at 20 min of treatment, while at 25°C it was achieved at 30 min. In both cases, survival for longer treatment times was not detected.

The number of survivors decreased progressively and linearly. The equations obtained by linear regressions were as follows: at 2°C, log CFU/ml = 6.3 − 0.32 × t (where t is time of treatment) and r^2 = 0.9448; at 25°C, log CFU/ml = 6.8 − 0.25 × t and r^2 = 0.9372.

By analogy with thermal treatments and with first-order microbial destruction, the following D-values were obtained: $D_{400 \text{ MPa/2°C}}$ = 3.12 min and $D_{400 \text{ MPa/25°C}}$ = 4 min.

DISCUSSION

Raffalli et al. (26) studied the effect of HHP on L. innocua in liquid cream (35% fat) at room temperature (25 to 26°C). The logarithmic reductions obtained were lower than those obtained in ewe's milk (6% fat). The decimal reduction value of L. innocua found by Rafalli et al. (26) was $D_{450 \text{ MPa/25°C}}$ = 7.4 min, while we obtained $D_{400 \text{ MPa/25°C}}$ = 4 min. This shows the baroprotective effect of fat in microorganisms (31). This protective effect has also been observed in thermal treatments (23).

Carlez et al. (3) studied the effect of HHP on L. innocua in minced meat and drew similar conclusions about the effect of temperature in HHP treatment. Refrigeration temperatures (2 to 4°C) and temperatures of 40 to 50°C provided a greater bactericidal effect on L. innocua than room temperatures (20 to 25°C). Decimal reductions obtained in minced meat were higher than those observed in ewe's milk (6% fat) comparing similar pressurization treatments. On the other hand, the assays undertaken at moderately high temperatures (50°C) in minced meat (3) provided lower logarithmic reductions than in our study. This could be explained by the greater thermal transmission coefficient in liquid media compared to solid media, and the fact that the center of the solid sample needs more time to reach the treatment temperature.

Some proteins are more susceptible to denaturation during pressurization at low temperatures than at room temperatures (13), which could explain the response of L. innocua to the pressurization temperature in this study. For most microorganisms it is accepted that the primary site of pressure damage is the cell membrane, so that the permeability of the cell membrane is affected (5). These malfunctions are due to crystallization of phospholipids and denaturation of membrane proteins (4), although the true causes of the cellular damage in Listeria spp. are not well known. In our

experiments, L. innocua showed higher resistance to HHP at room temperature (25°C), and the resistance was more pronounced in the range between 20 and 30°C. At low temperatures (2 and 10°C) L. innocua showed greater sensitivity to pressure, as observed by other authors (3).

The study of inactivation of L. innocua, a gram-positive bacterium, compared with studies on the effect of HHP on other microorganisms, supports the general conclusion reached by various authors (3, 29, 32) that gram-positive microorganisms are more resistant to pressure than gram-negative microorganisms. In general, gram-positive microorganisms need the application of 500 and 600 MPa at 25°C for 10 min to achieve inactivation, while gram-negative microorganisms are inactivated with treatments of 300 MPa at 25°C for 10 min.

D-values in thermal treatments at temperatures between 64 and 71.7°C are situated between 0.95 and 0.09 s (2, 23). We found the pressure D-values (minutes) were higher for the destruction of Listeria spp. than in thermal treatments (seconds), but HHP has the advantages that the pressures, temperatures, and times applied produce lesser physicochemical modifications in milk than thermal treatments (4). Low-temperature HHP treatments were effective in the destruction of L. innocua, preventing the undesirable effects of thermal treatments (1, 20, 25, 30). We think that HHP could be a good alternative to pasteurization treatments.

MacDonald and Sutherland (23) demonstrated that gram-negative microorganisms have greater thermal sensitivity than Listeria spp., and were able to conclude that there are no significant differences in the thermal sensitivities of L. monocytogenes and L. innocua. They showed that fat exerts a thermoprotective effect, which is more pronounced in ewe's milk than in cow's and goat's milk. These authors (23) obtained a reduction of 2.9 log units of L. innocua in ewe's milk (5% fat) at 65°C for 15 min, while in our study a reduction of 4.7 log units of L. innocua in ewe's milk (6% fat) was obtained at 350 MPa 2°C for 15 min. Other studies to determine the baroprotective effect of ewe's milk on other microorganisms are being undertaken, such as assays to determine the effect of HHP on the ewe's milk constituents.

ACKNOWLEDGMENTS

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