3.5. High Pressure Inactivation of Microorganisms Inoculated into Ovine Milk of Different Fat Contents. (Gervilla y col., 2000). *Journal of Dairy Science,* 83: 674-682.

High Pressure Inactivation of Microorganisms Inoculated into Ovine Milk of Different Fat Contents

R. Gervilla, V. Ferragut, and B. Guamis
Unitat de Tecnologia dels Aliments (C.e.R.T.A.),
Facultat de Veterinària, Universitat Autònoma de Barcelona,
08193 Bellaterra, Barcelona, Spain

ABSTRACT

High hydrostatic pressure inactivation of *Escherichia coli*, *Pseudomonas fluorescens*, *Listeria innocua*, Staphylococcus aureus, and *Lactobacillus helveticus* were studied. These microorganisms were inoculated at a concentration between 10^7 and 10^8 cfu/ml in Ringer solution and in ovine milk adjusted to 0, 6, and 50% fat content to evaluate the baroprotective effect of fat content on inactivation of microorganisms. Treatments of pressurization consisted of combinations of pressure (100 to 500 MPa) and temperature (4, 25, and 50°C) for 15 min. Gram-negative microorganisms were more sensitive than were Gram-positive ones (more destruction *P. fluorescens* > *E. coli* ≥ *List. innocua* > *Lb. helveticus* > *S. aureus*). Pressurizations at low temperature (4°C) produced greater inactivation on *P. fluorescens*, *List. innocua*, and *Lb. helveticus* than at room temperature (25°C), whereas for *E. coli* and *S. aureus* the results were opposite. Ovine milk per se (0% fat) showed a baroprotective effect on all microorganisms, but percentage of fat (6 and 50%) did not show a progressive baroprotective effect in all pressurization conditions or for all microorganisms.

(**Key words:** high hydrostatic pressure, bacteria, ovine milk, fat content)

Abbreviation key: **HHP** = high hydrostatic pressure, **0FC** = 0% fat content, **6FC** = 6% fat content, **50FC** = 50% fat content.

INTRODUCTION

Recently, food industries and consumers have increased demand for foods with high nutritional and sensory qualities and with guarantees of safer handling. New alternatives to conventional treatments (preservatives and thermal processes) such as electric or magnetic fields, ionizing radiation, light pulses, and high hydrostatic pressure (**HHP**) (19) are being developed to satisfy these demands. The use of HHP (up to 1000 MPa) for food preservation was pioneered by Hite (9) and Hite et al. (10). Several authors have studied the effect of HHP on microorganisms in buffered media (27), meat (3), ovine milk (5, 6, 7, 8), cheese (1), and other foods (20, 23).

Ovine milk production is increasing in Mediterranean countries; it is used most often for cheese making (90% of total production). Increased popularity of raw milk cheeses has led to increased study of microbial inactivation by HHP treatment of ovine milk.

The present study deals with pressure inactivation of five bacterial species. *Escherichia coli* CECT 405 is considered to be a good index of direct or indirect contamination of fecal origin; *Pseudomonas fluorescens* CECT 378 is an indicator for *Pseudomonas* spp., the major components of the spoilage flora of refrigerated milk; *Listeria innocua* CECT 910 is an indicator for *Listeria monocytogenes*, which is a human pathogen and possible contaminant of milk and dairy products; *Staphylococcus aureus* CECT 534 is a pathogenic microorganism, which has been involved in a number of food-poisoning outbreaks in milk and dairy products; and *Lactobacillus helveticus* CECT 414 is a microorganism of technological interest in the cheese production, which can be used as a starter culture in the manufacture of some fermented dairy products and is representative of genus *Lactobacillus*.

The main objective of this work was to study the effect of different treatment conditions (pressure and temperature) on the inactivation of *E. coli*, *P. fluorescens*, *List. innocua*, *S. aureus*, and *Lb. helveticus* inoculated in Ringer solution and in ovine milk containing different percentages of fat to evaluate its baroprotective effect against HHP treatments.

MATERIALS AND METHODS

Bacterial Strain

Escherichia coli CECT 405, *P. fluorescens* CECT 378, *List. innocua* CECT 910, *S. aureus* CECT 534, and *Lb. helveticus* CECT 414 were obtained as freeze-dried cultures in thermosealed vials from the Spanish Type Cul-

Received April 28, 1999.
Accepted November 16, 1999.
Corresponding author: R. Gervilla; e-mail: tecn.aliments@uab.es.

ture Collection (**CECT**; Universidad de Valencia, Valencia, Spain). The vials were maintained at 4°C until use. Rehydration was undertaken in 3 ml of appropriate broth: *E. coli* in lactose broth (Oxoid Ltd., Basingstoke, Hampshire, UK) at 37°C for 24 h, *P. fluorescens* and *S. aureus* in tryptone soy broth (Oxoid Ltd.) at 30 and 37°C for 24 h, respectively, *List. innocua* in brain heart infusion broth (Oxoid Ltd.) at 37°C for 24 h, and *Lb. helveticus* in Man-Rogosa-Sharpe broth (Oxoid Ltd.) at 37°C for 24 h. Subsequently, 1 ml of each cultured broth was inoculated in 9 ml of the same broth and incubated under the same conditions as rehydration. These broth cultures were used to inoculate nutrient agar (Oxoid Ltd.) with *E. coli*, tryptone soy agar (Oxoid Ltd.) with *P. fluorescens* and *S. aureus*, brain heart infusion agar (Oxoid Ltd.) with *List. innocua*, and Man-Rogosa-Sharpe agar (Oxoid Ltd.) with *Lb. helveticus*, which were maintained at 4°C and transferred every 2 wk to provide stock cultures. For each experiment, a tube of stock medium was used for growing each microorganism in 10 ml of the same broth and conditions of rehydration to achieve an approximate concentration of 10^9 cfu/ml (stationary phase of growth).

Preparation and Inoculation of Milk Samples

Milk from Manchega ewes was obtained from the dairy farm of the Facultat de Veterinaria (Universitat Autònoma de Barcelona, Spain). Raw milk was collected from the first milking in the morning, centrifuged, and adjusted to 0% fat content (**0FC**), 6% fat content (**6FC**) or 50% fat content (**50FC**). Standardized milks and Ringer solution were pasteurized at 110 ± 1°C for 1 min in an autoclave (J. P. Selecta S.A., Abrera, Spain). Pasteurized milks and Ringer solution were collected in 1-L sterile bottles, adjusted to pH 6.7 (by adding 1N NaOH, or 1N HCl, or both), and refrigerated at 4°C.

Separately, 10 ml of each broth culture (10^9 cfu/ml) was added to 1 L of pasteurized samples to obtain approximately 10^7 cfu/ml. Samples were gently shaken by hand for 5 min and then 30 ml of inoculated samples was pipetted into disinfected 30-ml polyester bottles (Bibby Sterilin Ltd, Stone, UK). As much air as possible was expelled from the bottles, and the caps were sealed with Teflon film.

Composition and Physico-Chemical Analyses of Milk

The total solids content was determined by drying at 102 ± 2°C in an oven until a constant weight was reached (12). Ash content was determined by gravimetric analysis after the sample had been calcinated in an oven at 550°C (11). Fat content was determined by the Gerber method (13). Total nitrogen was calculated by the digestion block method, a modification of the Kjeldahl method (14). The pH was measured by using a pH meter (micro-pH 2001; Crison Instruments S.A., Alella, Spain) (25).

HHP Treatments

Samples were pressurized by using discontinuous HHP equipment (ACB, Nantes, France) with a 2-L pressure chamber. The time needed to achieve maximum pressure (500 MPa) was 2 min. The chamber and water (hydrostatic fluid medium) inside were cooled or heated to treatment temperature with a constant flow of ethyleneglycol-water (1:1) solution within the walls of the vessel. Samples were kept for 5 to 10 min at atmospheric pressure in the chamber until temperature equilibrium was established. The temperature of the samples was monitored by a thermocouple to determine the most extreme temperature conditions to which samples were subjected.

Time, temperature, and pressure parameters were selected on the basis of previous unpublished studies. The responses of *E. coli*, *P. fluorescens*, *List. innocua*, *S. aureus*, and *Lb. helveticus* to treatments were studied at different conditions of pressure (100 to 500 MPa), temperature (4, 25, and 50°C), and time (15 min). To evaluate the effect of temperature per se, inoculated samples were held at the most severe temperature-time conditions reached in the treatments (2 and 53°C for 20 min), and then inoculated samples were evaluated. Each treatment was individually performed three times.

Microbiological Assays

Samples were kept at 4°C prior to analysis (10 h approximately) to avoid postpressurization stress. Appropriate dilutions of each sample were made in Ringer solution (9 ml) for microbial determinations.

To determine *E. coli* counts, 1-ml volumes of sample or decimal dilutions were plated in duplicate on violet red bile agar (Oxoid Ltd.) and incubated at 37°C for 24 h. Purple colonies, with a halo of the same color, were counted.

To determine *P. fluorescens* counts, 0.1- or 1-ml (in direct inoculation) samples or decimal dilutions were surface plated in duplicate on crystal-violet-tetrazolium count and incubated at 30°C for 48 h. The crystal-violet-tetrazolium count technique involved the addition of 1 mg/kg of crystal violet to standard plate count agar (Oxoid Ltd.) before sterilization and 50 mg/kg 2,3,5-triphenyl-tetrazolium chloride (ICN Biomedicals, Inc., Aurora, OH) to the agar just before the plates were

poured. With low dilutions (direct inoculation), the relatively large amount of milk partially neutralized the effect of the crystal violet. This effect was overcome by diluting 9 ml of milk (sample) with 9 ml of sterile water containing 10 mg/kg of crystal violet. One milliliter of this mixture was added to each of the two plates, and the total count on the two plates represented the contamination per milliliter. Gram-negative colonies were distinctly red. Very few of the microorganisms surviving pasteurization grew on the plates, and colonies were generally very small and uncolored or lightly colored (21).

To determine *List. innocua* counts, 0.1- or 1-ml (in direct inoculations) samples or decimal dilutions were surface plated in duplicate on listeria selective agar base (Oxoid Ltd.) with listeria selective supplement (Oxoid Ltd.) and inoculated at 37°C for 48 h. Colonies of *Listeria* spp. were identified as grey colonies surrounded by black zones caused by esculin hydrolysis on listeria selective agar base with listeria selective supplement (according to Oxoid recommendations). Representative numbers (5 to 10) of suspicious colonies were selected and confirmed as *Listeria* spp. by the following criteria: Gram-positive, nonspore-forming, oxidative-negative, and catalase-positive.

To determine *S. aureus* counts, 0.1- or 1-ml (in direct inoculations) samples or decimal dilutions were surface plated in duplicate on Baird-Parker medium (Oxoid Ltd.) with egg yolk-tellurite emulsion (Oxoid Ltd.). The plates were incubated at 37°C and examined after 24 and 48 h. Representative numbers (5 to 10) of suspicious colonies (brown or black colonies, with or without clear zones) were selected from Baird-Parker plates for identification by the tube coagulase test.

To determine *Lb. helveticus* counts, 1-ml samples or decimal dilutions were plated in duplicate on Man-Rogosa-Sharpe agar (Oxoid Ltd.). To create microanaerobic conditions, a second layer of sterile Man-Rogosa-Sharpe agar was added. The plates were incubated at 37°C for 72 h.

At the same time, total counts were obtained in plate count agar at 30°C for 48 h to determine any possible contamination of samples during manipulation, comparing differences in number between plate count agar and selective madia (violet red bile agar, crystal-violet-tetrazolium, listeria selective agar with listeria selective supplement, Baird-Parker, and Man-Rogosa-Sharpe).

Statistical Treatment of Data

Each experiment was run three times with duplicate analysis in each replicate. An ANOVA was performed using the GLM procedure of SAS (26). Duncan's new multiple-range test and Student-Newman-Keuls test were used to obtain paired comparisons among sample means. Evaluations were based on a significance level of $P < 0.05$.

RESULTS

The mean (and SD) values for composition of ovine milk before standardization for fat content (wet weight basis) were total solids, 18.68% (SD = 1.71); fat, 7.63% (SD = 1.55); protein, 5.74% (SD = 0.19); ash, 1.16% (SD = 0.12); pH was 6.65 (SD = 0.08).

Higher pressure gave higher lethality; however, higher temperature (from low temperatures) in the pressurization treatments of samples did not give an increase of lethality.

Inactivation rates for *E. coli* CECT 405 increased with pressure ($400^a > 300^b > 200^c > 100^d$ MPa; numbers without common superscript differ, $P < 0.05$) and temperature ($50^a > 25^b > 4^c$°C; $P < 0.05$). The inactivation rate in Ringer solution was significantly greater than in milk but there was no significant effect of fat level (Ringer solution^a > 6FC^b ≥ 0FC^b ≥ 50FC^b milk; $P > 0.05$). From analysis of the F value, pressure and medium were the main factors, explaining 49 and 34% (respectively) of the variability of the statistical model on destruction of *E. coli* by HHP treatments.

Inactivation rates of *P. fluorescens* CECT 378 increased with pressure ($200^a > 100^b$ MPa; $P < 0.05$), but temperature effect ($50^a > 4^b > 25^c$°C; $P < 0.05$) and medium effect (Ringer solution^a > 0FC^b > 50FC^c > 6FC^d milk; $P < 0.05$) did not show a progressive response. From the analysis of the F value, pressure and temperature were the main factors, explaining 53 and 29% (respectively) of the variability of the statistical model.

For *List. innocua* CECT 910, inactivation rates increased with pressure ($400^a > 300^b > 200^c$ MPa; $P < 0.05$) and decreased progressively with the increase of fat content of medium (Ringer solution^a > 0FC^b > 6FC^c > 50FC^d milk; $P < 0.05$). Temperature effect did not show a progressive response ($50^a > 4^b > 25^c$°C; $P < 0.05$). From the analysis of the F value, pressure and medium were the main factors, explaining 67 and 20% (respectively) of the variability of the statistical model.

For *S. aureus* CECT 534, inactivation rates increased with pressure ($500^a > 400^b$ MPa; $P < 0.05$) and temperature ($50^a > 25^b > 4^c$°C; $P < 0.05$). Medium effect did not present a progressive response (Ringer solution^a > 0FC^b > 6FC^c ≥ 50FC^c milk; $P < 0.05$). No significant differences ($P > 0.05$) were found between the effect of treatments applied in 6FC and 50FC. From the analysis of the F value, pressure was the main factor and then temperature and medium, explaining 54, 24, and 20% (respectively) of the variability of the statistical model.

For *Lb. helveticus* CECT 414, inactivation rates increased with pressure ($500^a > 400^b > 300^c$ MPa; $P < 0.05$) but did not show linear response with temperature ($50^a > 4^b > 25^c$°C; $P < 0.05$) or with medium (Ringer solution^a > $0FC^b$ > $6FC^c \geq 50FC^c$ milk; $P < 0.05$). No significant differences ($P > 0.05$) were found between the effect of treatments applied in 6FC and 50FC milk. From the analysis of the F value, medium and pressure were the main factors, explaining 47 and 37% (respectively) of the variability of the statistical model.

The response to HHP treatments of microorganisms studied was very different. On the whole, *P. fluorescens* was the microorganism that showed the highest sensitivity to HHP treatments in all media studied. *Escherichia coli* and *List. innocua* showed similar sensitivity to HHP treatments. *Lactobacillus helveticus* showed greater resistance to HHP treatments than did *P. fluorescens, E. coli*, or *List. innocua*. Finally, *S. aureus* was the microorganism that showed the highest resistance to HHP treatments.

In general, treatment at 50°C was more effective (more destruction) for all media and microorganisms assayed (Figure 1). At low temperature (4°C) *P. fluorescens, List. innocua* and *Lb. helveticus* showed greater sensitivity to HHP treatments than treatments at room temperature (25°C). Conversely, room temperature was more effective on *E. coli* and *S. aureus* (except for Ringer solution) than was low temperature.

We designed an experiment to confirm the total inactivation of microorganisms by pressure and to detect partially damaged microorganisms, if present. From each treatment in which no microbial growth was detected, 1 ml of sample was directly plated on its selective medium (each one at optimal conditions of incubation), and another 1 ml of sample was stored at 37°C for 24 h and afterward plated on its selective medium and incubated at optimal conditions. Both results from each bacteria were then compared. No microbial growth was detected, and so we confirmed total inactivation and the absence of partially damaged microorganisms (data not shown).

Counts obtained from inoculated samples that had been held at extreme time-temperature conditions (without pressure application) were not different from initial counts, which showed that those temperature-time combinations per se had no effect on microbial populations.

Total counts obtained on plate count agar were never significantly different from those obtained in selective media.

DISCUSSION

Inactivation of *E. coli* by HHP has been studied by several authors under different conditions. Patterson et al. (22) studied the effect of HHP at 20°C on several microorganisms in different substrata or foods. *Escherichia coli* NCTC 12079 proved to be a strain very resistant to HHP with a greater inactivation in PBS, pH 7.0, than in poultry meat. This *E. coli* strain was more resistant to inactivation in UHT milk. We reported similar behavior (more destruction in Ringer solution than in ovine milk) (Figure 2), but we obtained greater logarithmic reductions comparing our results in Ringer solution and 6FC to their results in PBS and UHT milk (with similar HHP conditions). Ludwig et al. (17) studied the inactivation of *E. coli* by HHP in physiological NaCl solution. They obtained similar reductions to our results in Ringer solution (with similar HHP conditions) (e.g., reductions of 5 to 5.5 log units at 4°C with 300 MPa for 9 to 15 min), but they obtained greater destruction of *E. coli* at low temperatures (2°C) than at room temperature (25°). Greater destruction occurred at 25°C than at 4°C in all media and pressures assayed (Figure 1). Takahashi (29) studied *E. coli* in 2 m*M* PBS, pH 7.0, and obtained greater inactivation at low temperature (–20°C) than at room temperature (20°C). The inactivation at –20°C could have been due in part to a water-phase diagram (15); with 200 MPa at –20°C, water is on the limit of liquid and solid phases, and the effect of fusion and crystallization of the water (intra- and extra-cellular) could have harmed the microorganisms.

Few studies have been carried out on species of the genus *Pseudomonas*, probably because this microorganism proved to be quite barosensitive and easily inactivated (6, 7). However, study of the particular behavior of these microorganisms in particular substrata and food was necessary.

Carlez et al. (3) studied inactivation of HHP treatments on several microorganisms in minced beef muscle. Among those microorganisms, *P. fluorescens* proved to be considerably more sensitive to HHP in minced beef than in milks used in our study; their (3) results were comparable only with the reductions that we obtained in Ringer solution (about 5 to 7 log units) (Figure 3). As in our study, they obtained greater reductions at low temperature (4°C) than at room temperature (20°C). Ovine milk seemed to protect *P. fluorescens* against HHP inactivation when comparing the results. Although *Pseudomonas aeruginosa* was studied in physiological solution (18), which showed similar log reductions to *P. fluorescens* in Ringer solution, the effect of temperature on *P. aeruginosa* showed greater destruction at 50 > 40 > 1 > 10 > 30°C.

Carlez et al. (3) studied HHP inactivation on *List. innocua* in minced meat. We agreed with their results about the effect of temperature in HHP treatments; greater inactivation occurred at 50 > 4 > 35 > 20°C.

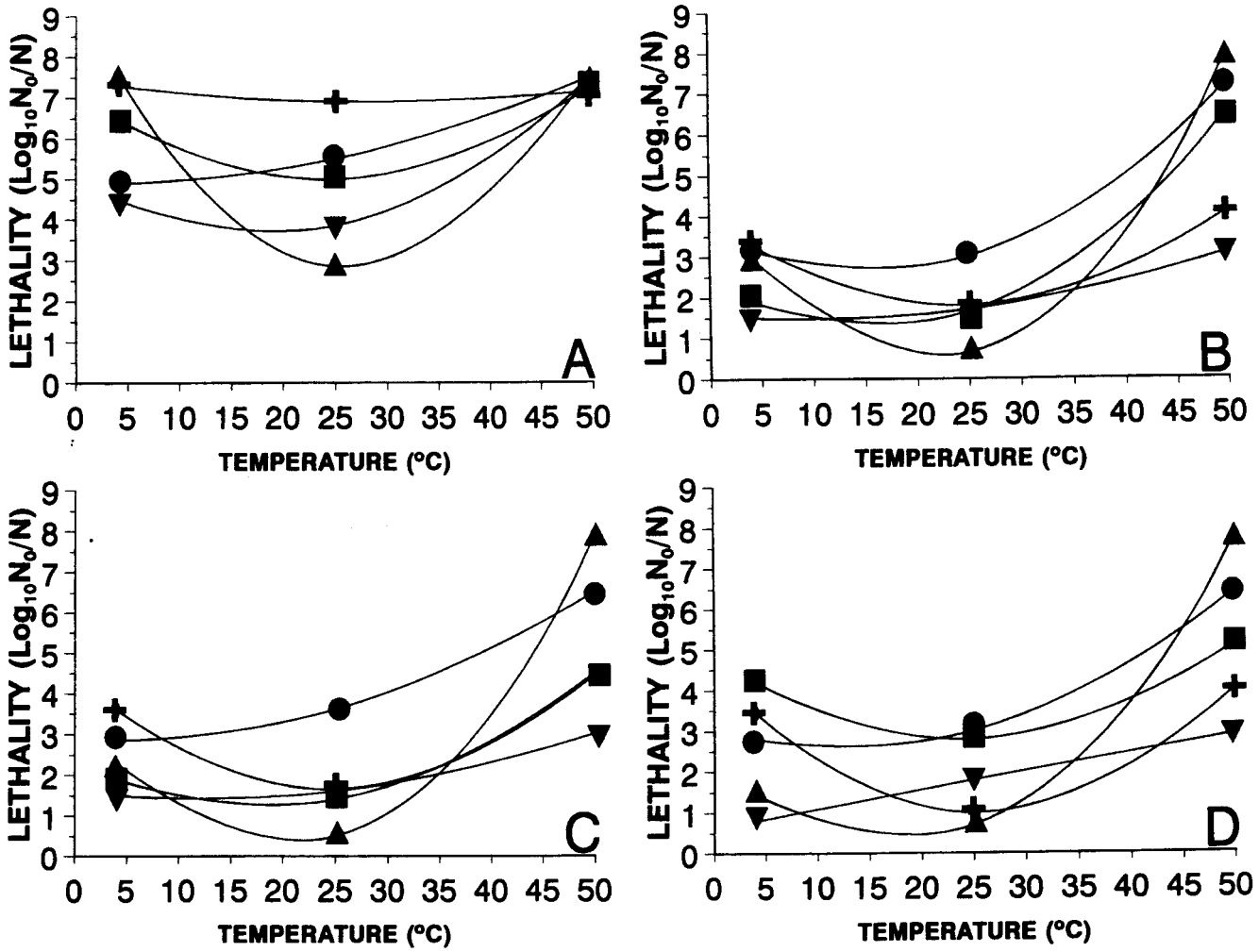

Figure 1. Tendency of high hydrostatic pressure effect as a function of temperature for 15 min on *Escherichia coli* CECT 405 at 300 MPa (●), *Pseudomonas fluorescens* CECT 378 at 200 MPa (■), *Listeria innocua* CECT 910 at 300 MPa (▲), *Staphylococcus aureus* CECT 534 at 400 MPa (▼), and *Lactobacillus helveticus* CECT 414 at 400 MPa (✚). The microorganisms were inoculated in Ringer solution (A) and ovine milk at 0% fat (B), 6% fat (C), and 50% fat (D).

Decimal reductions obtained in minced meat were less than those obtained in ovine milk, which showed a certain protective effect of ovine milk on *List. innocua* against HHP treatments. Patterson et al. (22) studied inactivation of *Listeria monocytogenes* at 20°C in different media by HHP. Greater inactivation occurred in PBS > poultry meat > UHT milk. Results in PBS were comparable to those we obtained in Ringer solution, but we obtained greater reductions in 0-, 6- and 50FC than in UHT milk and poultry meat. Styles et al. (27) also studied HHP inactivation of *L. monocytogenes* at 23°C in different media. Greater inactivation occurred in 100 m*M* PBS > raw milk > UHT milk. Our results in Ringer solution were similar to their results in PBS. The reductions obtained in 0-, 6- and 50FC were similar to those presented in raw milk but were greater than those obtained in UHT milk. It is possible that antimicrobial factors (immunoglobulins, lysozyme, lactoferrine, and lactoperoxydase system; all thermosensitive, and not present in UHT milk) produced a synergic effect with pressure in raw milk. However, Raffalli et al. (24) studied *List. innocua* at 25°C in a liquid UHT dairy cream (35% fat). They obtained reductions of 2.5 log units (400 MPa for 15 min), whereas we obtained reductions of about 4.5 to 5 log units (400 MPa for 15 min) in 0-, 6- and 50FC (Figure 4).

For *S. aureus*, several groups studied inactivation in different media by HHP treatments; all agreed that *S. aureus* was the most pressure-resistant of the microorganisms tested (2, 22, 29). Patterson et al. (22) assayed the inactivation of *S. aureus* NCTC 10652 by HHP. They obtained more log reductions in raw poultry meat

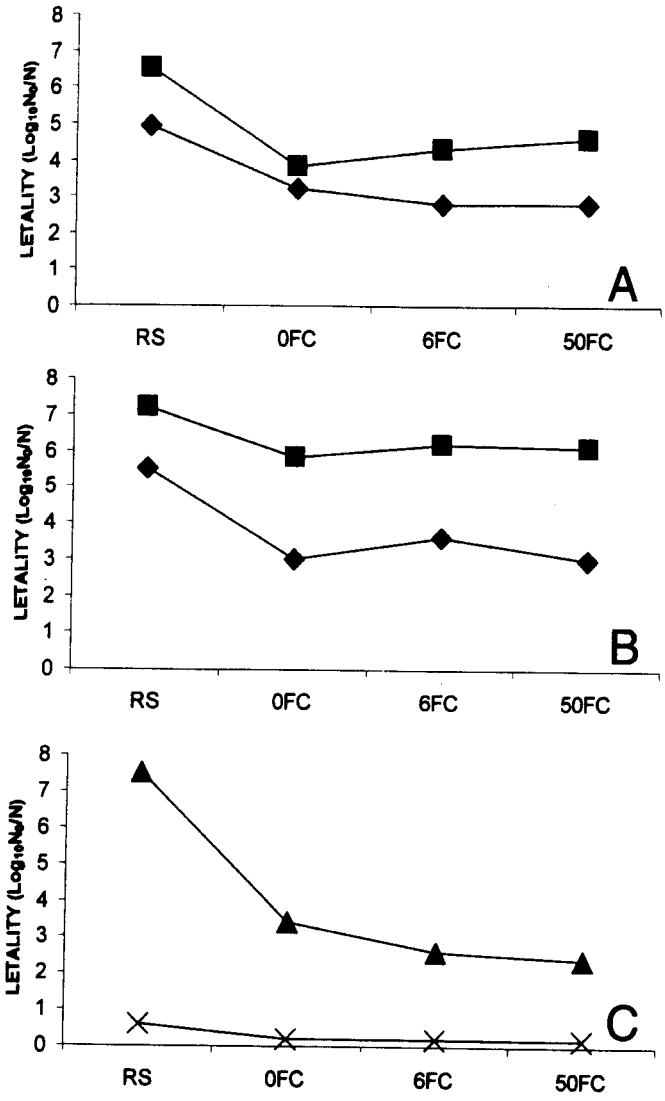

Figure 2. Effect of high hydrostatic pressure on *Escherichia coli* CECT 405 inoculated in Ringer solution (RS) and ovine milk at 0% fat (0FC), 6% fat (6FC), and 50% fat (50FC). Exposure time 15 min. A: 4°C, B: 25°C, C: 50°C. (✖) 100 MPa, (▲) 200 MPa, (◆) 300 MPa, and (■) 400 MPa.

> 10 m*M* PBS > UHT milk. Changes in pH can also occur when buffers such as PBS are pressurized, and this pH shift may have some effects on microbial inactivation. Thus, greater inactivation of *S. aureus* was produced in PBS than in raw poultry meat (22). We obtained greater log reductions in all similar conditions assayed than did Patterson et al. (22). Shigehisa et al. (28) studied HHP inactivation of *S. aureus* ATCC 25923 among several microorganisms in pork slurries. Pressures above 500 MPa were needed to obtain significant log reductions (e.g., 3.5 log units with 500 MPa at 25°C for 10 min). These results were comparable to those we obtained in 0- and 6FC at 25°C (Figure 5). Carballo et al. (2) reported HHP inactivation of *S. aureus* in high- and low-fat beef patties. In agreement with our results, they showed a certain protective effect of fat content against HHP treatments.

Lactobacillus spp. are widely present in dairy products, and these effect of HHP on *Lactobacillus* spp. could be informative in regards to development of new technologies (as HHP treatments) for the dairy industries. Kromkamp et al. (16) studied the inactivation of *Lactobacillus delbrueckii* subsp. *bulgaricus* in yogurt by HHP. They showed higher inactivation rates than those observed in our study, if we compare the results in yogurt and ovine milk (0-, 6-, and 50FC). Reductions of 4 and 5 log units (with 200 and 300 MPa for 30

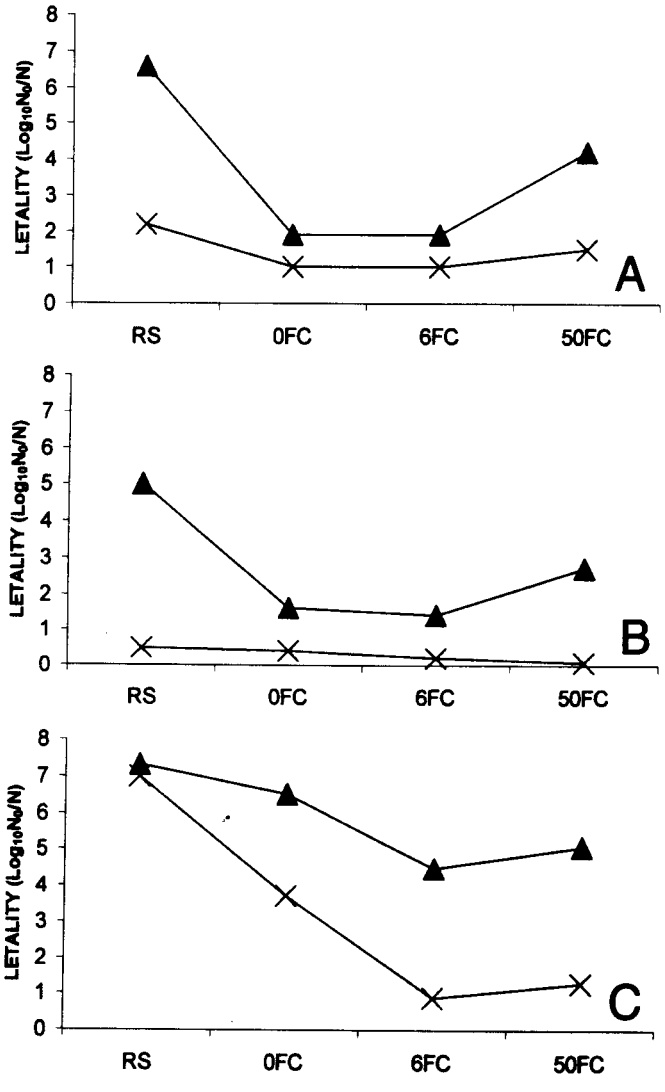

Figure 3. Effect of high hydrostatic pressure on *Pseudomonas fluorescens* CECT 378 inoculated in Ringer solution (RS) and ovine milk at 0% fat (0FC), 6% fat (6FC), and 50% fat (50FC). Exposure time 15 min. A: 4°C, B: 25°C, C: 50°C. (✖) 100 MPa, and (▲) 200 MPa.

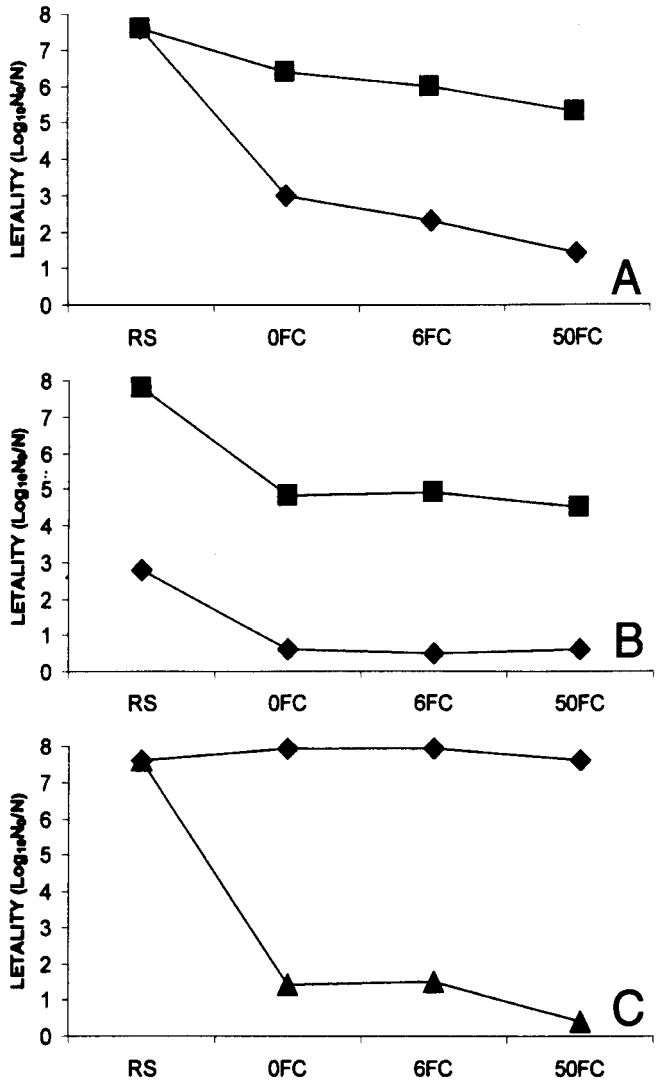

Figure 4. Effect of high hydrostatic pressure on *Listeria innocua* CECT 910 inoculated in Ringer solution (RS) and ovine milk at 0% fat (0FC), 6% fat (6FC), and 50% fat (50FC). Exposure time 15 min. A: 4°C, B: 25°C, C: 50°C. (▲) 200 MPa, (◆) 300 MPa, and (■) 400 MPa.

min at room temperature) were obtained from yogurt, whereas we needed pressures of 400 MPa (at 25°C for 15 min) to produce reductions of about 1 and 2 log units in 50- and 0FC, respectively (Figure 6). Factors like pH (4.6), fat content (0%), species of milk-producing animal (bovine), and specific microstructure of yogurt could be responsible for those inactivation differences from our results. *Lactobacillus* spp. response to HHP (at 20°C for 20 min) in minced meat was reported by Carlez et al. (4). Pressures of ≥400 MPa produced an extensive inactivation (reductions of 4 or ≥5 log units), whereas we needed pressures of 500 MPa at 25°C for 15 min to produce similar reductions (3.8, 4, and 4.8 log units in 6-, 50-, and 0FC, respectively) (Figure 6). When we compared similar HHP conditions from both referenced studies (4, 16) and our results, we observed more destruction in Ringer solution > yogurt > minced meat > ovine milk (0-, 6-, and 50FC).

Our study of inactivation of microorganisms, compared with other studies on the effect of HHP treatments, supported the general conclusion reached by various authors (3,28) that Gram-positive microorganisms are more resistant to pressure than Gram-negative microorganisms. In general, the HHP inactivation was greater on *P. fluorescens* > *E. coli* ≥ *List. innocua* > *Lb. helveticus* > *S. aureus*. The temperature effect by HHP on microorganisms was different; *P. fluorescens*, *List. innocua*, and *Lb. helveticus* showed higher resis-

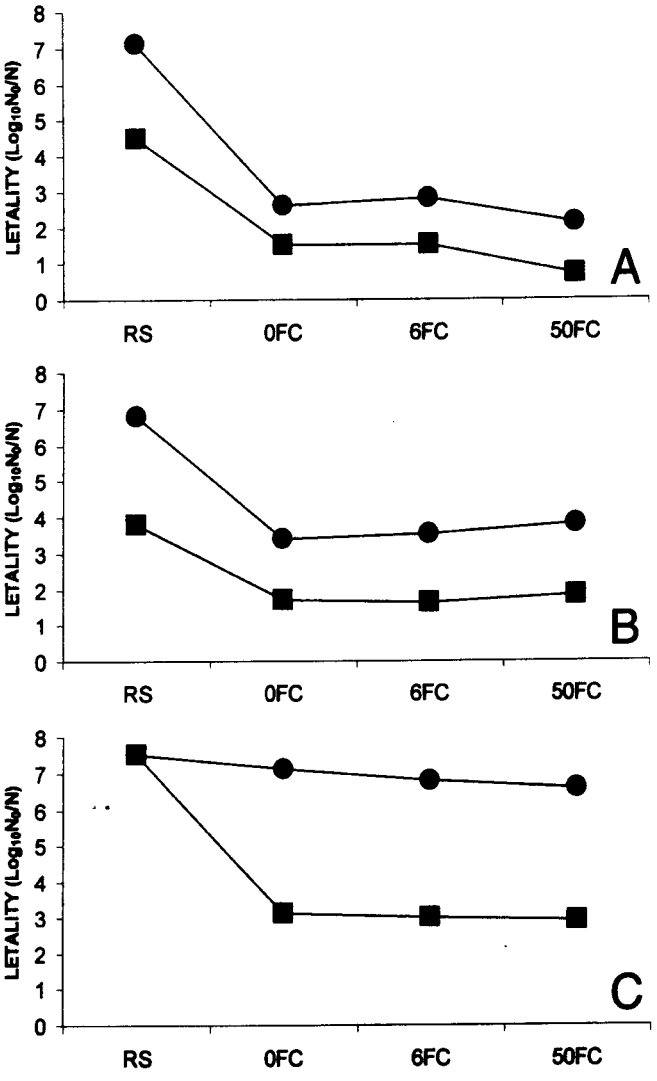

Figure 5. Effect of high hydrostatic pressure on *Staphylococcus aureus* CECT 534 inoculated in Ringer solution (RS) and ovine milk at 0% fat (0FC), 6% fat (6FC), and 50% fat (50FC). Exposure time 15 min. A: 4°C, B: 25°C, C: 50°C. (■) 400 MPa, and (●) 500 MPa.

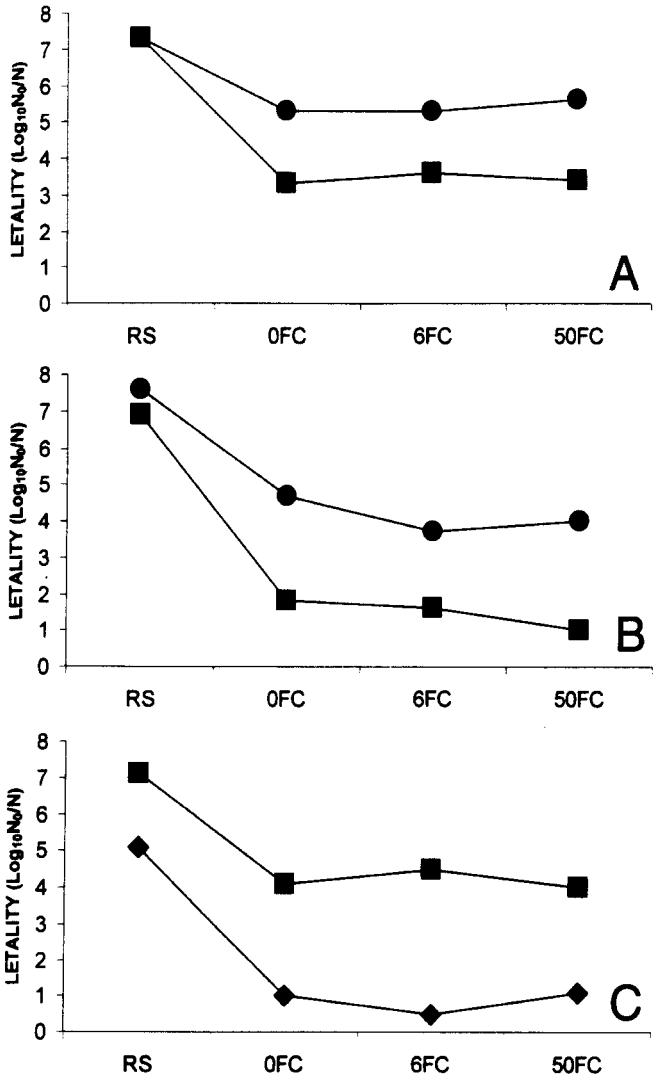

Figure 6. Effect of high hydrostatic pressure on *Lactobacillus helveticus* CECT 414 inoculated in Ringer solution (RS) and ovine milk at 0% fat (0FC), 6% fat (6FC), and 50% fat (50FC). Exposure time 15 min. A: 4°C, B: 25°C, C: 50°C. (◆) 300 MPa and (■) 400 MPa, (●) 500 MPa.

tance to HHP at room temperature (25°C) than at low temperature (4°C), whereas *E. coli* and *S. aureus* showed less resistance to HHP at room temperature than at low temperature. Ovine milk per se (0FC) showed a baroprotective effect against HHP treatments on all microorganisms and HHP conditions assayed (comparing reductions of Ringer solution and 0FC) (Figures 2 to 6). On the other hand, fat content produced (6- and 50FC) a different effect according to the pressure, temperature, and microorganism assayed. In the global context of experimental model, fat content (0-, 6- and 50FC) did not show a protective effect on *E. coli*. On *S. aureus* and *Lb. helveticus*, fat produced a baroprotective effect, but no progressive protection was seen between 6- and 50FC fat content. With *List. innocua*, the increase of fat content resulted in a progressive protection against inactivation due to HHP. On the contrary, high fat content (50FC) was more lethal than intermediate fat content (6FC) on *P. fluorescens*.

It seems that the final effect of fat content on microorganisms was the sum of a baroprotective behavior against HHP: a shock absorber effect of pressure and to avoid the interchange of substances between intra- and extra-cellular space; and a barodestructive behavior against HHP: increasing concentration of certain liposoluble substances with antimicrobial effect, interchanging triglycerides of milk with lipoproteins of cellular membrane of microorganisms (altering its permeability), and forming crystals of fat (mainly at low temperatures during HHP treatments). The final effect of fat content seems to be influenced by the conditions of HHP treatments (mainly pressure and temperature), microbial strain, percentage of fat, and animal species of milk fat.

The HHP inactivation on vegetative microorganisms is strongly influenced by the composition of the media or food. So the differences between the inactivation of microorganisms to HHP in buffer and real food systems needs to be investigated. We believe that by following the investigations of pressurization combined with other synergic treatments or processes, pressurization may well be a good alternative to pasteurization. Studies to determine the effect of HHP on other microorganisms in ovine milk on microbial inactivation are being undertaken by our group, and also the effect of ovine milk constituents.

ACKNOWLEDGMENTS

This study has been financially supported by the AIR-CT 92-0296 Project: High Hydrostatic Pressure Treatments: Its Impact on Spoilage Microorganisms, Biopolymer Activity, Functionality and Nutrient Composition of Food Systems (EC; Brussels, Belgium). We thank Pilar Pérez for her help in the University laboratory (Tecnologia dels Aliments, U.A.B., Bellaterra 08193, Spain), and the Spanish Type Culture Collection (CECT; Valencia, Spain) for providing the strains.

REFERENCES

1 Capellas, M., M. Mor-Mur, E. Sendra, R. Pla, and B. Guamis. 1996. Populations of aerobic mesophils and inoculated *E. coli* during storage of fresh goat's milk cheese treated with high pressure. J. Food Prot. 59:582–587.

2 Carballo, J., P. Fernández, A. V. Carrascosa, M. T. Solas, and F. Jiménez-Colmenero. 1997. Characteristics of low- and high-fat beef patties: effect of high hydrostatic pressure. J. Food Prot. 60:48–53.

3 Carlez, A., J. P. Rosec, N. Richard, and J. C. Cheftel. 1993. High pressure inactivation of *Citrobacter freundii*, *Pseudomonas fluorescens* and *Listeria innocua* in inoculated minced beef muscle. Lebensm. Wiss. Technol. 26:357–363.
4 Carlez, A., J. P. Rosec, N. Richard, and J. C. Cheftel. 1994. Bacterial growth during chilled storage of pressure-treatment minced meat. Lebensm. Wiss. Technol. 27:48–54.
5 Gervilla, R., M. Capellas, V. Ferragut, and B. Guamis. 1997. Effect of high hydrostatic pressure on *Listeria innocua* 910 CECT inoculated into ewe's milk. J. Food Prot. 60:33–37.
6 Gervilla, R., X. Felipe, V. Ferragut, and B. Guamis. 1997. Effect of high hydrostatic pressure on *Escherichia coli* and *Pseudomonas fluorescens* strains in ovine milk. J. Dairy Sci. 80:2297–2303.
7 Gervilla, R., M. Mor-Mur, V. Ferragut, and B. Guamis. 1999. Kinetics of destruction of *Escherichia coli* and *Pseudomonas fluorescens* inoculated in ewe's milk by high hydrostatic pressure. Food Micobiol. 16:173–184.
8 Gervilla, R., E. Sendra, V. Ferragut, and B. Guamis. 1999. Sensitivity of *Staphylococcus aureus* and *Lactobacillus helveticus* in ovine milk subjected to high hydrostatic pressure. J. Dairy Sci. 82:1099–1107.
9 Hite, B. H. 1899. The effect of pressure in the preservation of milk. Bull. West Virginia Univ. Agric. Exp. Stn. 58:15–35.
10 Hite, B. H., N. J. Giddings, and C. E. Weakly. 1914. The effects of pressure on certain microorganisms encountered in the preservation of fruits and vegetables. Bull. West Virginia Univ. Agric. Exp. Stn. 146:1–67.
11 International Dairy Federation. 1964. Determination of the ash content of milk and processed cheese products. FIL-IDF Stand. 27. Int. Dairy Fed., Brussels, Belgium.
12 International Dairy Federation. 1987. Milk, cream and evaporated milk; total solids content. FIL-IDF Stand. 21B. Int. Dairy Fed., Brussels, Belgium.
13 International Dairy Federation. 1991. Milk and milk products-Fat content. General guidance on the use of butyrometric methods. FIL-IDF Stand. 52. Int. Dairy Fed., Brussels, Belgium.
14 International Dairy Federation. 1993. Milk-nitrogen content. FIL-IDF Stand. 220B. Int. Dairy Fed., Brussels, Belgium.
15 Kalichevsky, M. T., D. Knorr, and P. J. Lillford. 1995. Potential food applications of high-pressure effects on ice-water transitions. Trends Food Sci. Technol. 6:253–259.
16 Kromkamp, J., R. M. Moreira, L.P.M. Langeveld, and P.J.J.M. van Mil. 1996. Microorganisms in milk and yoghurt: selective inactivation by high hydrostatic pressure. Pages 267–271 *in* Proc. Int. Dairy Fed. Symp. September 6–8, 1995. Heat treatments and alternative methods. Int. Dairy Fed., Brussels, Belgium.
17 Ludwig, H., C. Bieler, K. Hallbauer, and W. Scigall. 1992. The inactivation of vegetative bacteria by pressure. Pages 25–32 *in* High Pressure and Biotechnology. C. Balny, R. Hayahi, K. Heremans, and P. Masson, ed. Colloque INSERM/John Libbey Eurotext Ltd., London, England.
18 Ludwig, H., and C. Schreck. 1997. The inactivation of vegetative bacteria by pressure. Pages 221–224 *in* High Pressure Research in the Biosciences and Biotechnology. K. Heremans, ed. Leuven University Press, Leuven, Belgium.
19 Mertens, B., and D. Knorr. 1992. Developments of nonthermal processes for food preservation. Food Technol. 46:124–132.
20 Ogawa, H., K. Fukuhisa, and H. Fukumoto. 1992. Effect of hydrostatic pressure on sterilization and preservation of citrus juice. Pages 269–278 *in* High Pressure and Biotechnology. C. Balny, R. Hayahi, K. Heremans, and P. Masson, ed. Colloque INSERM/John Libbey Eurotext Ltd., London, England.
21 Olson, H. C. 1963. Selective plating technique for detecting contamination in pasteurized milk. J. Dairy Sci. 46:362.
22 Patterson, M. F., M. Quinn, R. Simpson, and A. Gilmour. 1995. Sensitivity of vegetative pathogens to high hydrostatic pressure treatment in phosphate-buffered saline and foods. J. Food Prot. 58:524–529.
23 Ponce, E., R. Pla, M. Mor-Mur, R. Gervilla, and B. Guamis. 1988. Inactivation of *Listeria innocua* inoculated in liquid whole egg by high hydrostatic pressure. J. Food Prot. 61:119–122.
24 Raffalli, J., J. P. Rosec, A. Carlez, E. Dumay, N. Richard, and J. C. Cheftel. 1994. Stress et inactivation par haute pression de *Listeria innocua* introduites dans une crème laitière. Sci. Aliment. 14:349–358.
25 Richardson, G. H., ed. 1985. Standard Methods for the Examination of Dairy Products. 15th ed. Am. Publ. Health Assoc., Inc., Washington, DC.
26 SAS User's Guide: Statistics, Version 5 Edition. 1985. SAS Inst., Inc., Cary, NC.
27 Styles, M., D. Hoover, and D. Farkas. 1991. Response of *Listeria monocytogenes* and *Vibrio parahaemolyticus* to high hydrostatic pressure. J. Food Sci. 56:1404–1407.
28 Shigehisa, T., T. Ohmori, A. Saito, S. Taji, and R. Hayashi. 1991. Effects of high hydrostatic pressure on characteristics of pork slurries and inactivation of microorganisms associated with meat and meat products. Int. J. Food Micobiol. 12:207–216.
29 Takahashi, K. 1992. Sterilization of microorganisms by hydrostatic pressure at low temperatures. Pages 303–307 *in* High Pressure and Biotechnology. C. Balny, R. Hayahi, K. Heremans, and P. Masson, ed. Colloque INSERM/John Libbey Eurotext Ltd., London, England.