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ORIGINAL ARTICLE

Kinetics of destruction of *Escherichia coli* and *Pseudomonas fluorescens* inoculated in ewe's milk by high hydrostatic pressure

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Ewe's milk standardized to 6% fat was inoculated with Escherichia coli *and* Pseudomonas fluorescens *at a concentration of* 10^7 *and* 10^8 *cfu ml*$^{-1}$ *respectively, and treated by high hydrostatic pressure. Treatments consisted of combinations of pressure (50–300 MPa), temperature (2, 10, 25 and 50°C), and time (5, 10 and 15 min). Violet red bile agar and crystal-violet-tetrazolium count were used to determine* E. coli *and* P. fluorescens *respectively. Pressurization at low and moderately high temperatures produced higher* P. fluorescens *inactivation than treatments at room temperature, while pressurization at only moderately high temperature produced high* E. coli *inactivation; low and room temperatures produced similar reductions. On* E. coli, *reductions of 6·5 log units were produced with 300 MPa for 15 min at 50°C, while on* P. fluorescens, *reductions of 5·9 log units were produced with 250 MPa for 15 min at 50°C. Both micro-organisms showed a first-order kinetics of destruction in the range 0–30 min, with* D-*values (at different temperatures and pressures from 150 to 300 MPa) between 2·5 and 18·8 min for* E. coli, *and 2·8–23·3 min for* P. fluorescens. *A baroprotective effect of ewe's milk (6% fat) on both micro-organisms was observed, in comparison with other studies using different means and similar pressurization conditions.*

Introduction

Nowadays, there are two main factors that have stimulated the industry to investigate new non-thermic processing methods, such as ionizing radiation, electric or magnetic fields, light pulses and high hydrostatic pressure (HHP) (Mertens and Knorr 1992). Firstly, there is increasing consumer demand for high nutritional and sensory quality, minimally processed, additive-free, and microbiologically safe foods; secondly, the interest shown by the industry in developing products with different aromas, textures and functional properties from the existing ones and the possibility to lengthen the commercial life of the products. HHP (100–1000 MPa) is a physical process which can allow food

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manufacturers to inactivate micro-organisms but not the majority of enzymes that are important for cheese ripening (Cheftel 1991, Earnshaw 1992, Hayashi 1989, Rönner 1994). The application of HHP is uniform and instantaneous throughout a food product. It is unique since the effects do not follow a concentration gradient (Hoover et al. 1989). The basis is that pressure will accelerate processes leading to a volume decrease, whereas any synthesis involving a volume increase will be inhibited. HHP has certain advantages over more traditional food processing methods, such as lack of chemical additives, novel textures, treatment of solid (cheese) or very viscous (honey) foods, and operation at low, ambient or moderately high temperatures so that natural flavour and taste can be preserved without damaging food constituents (e.g. vitamins) (Mertens 1993). Hite (1899), first explored the possibilities of adapting HHP as a food processing method. He and his coworkers (Hite et al. 1914) examined a wide range of foods and beverages for the potential use of HHP processing, but until a decade ago, HHP had not received sufficient attention to be used in the food industry. Some authors have studied the effect of HHP on micro-organisms in cultured or buffered media (Styles et al. 1991), meat (Carlez et al. 1993, Shigehisa et al. 1991), a cow's UHT milk (Styles et al. 1991), liquid cream (Raffalli et al. 1994) and other foods and drinks (Ogawa et al. 1992), but very few studies have been carried out on ewe's milk (Gervilla et al. 1997a,b). The extensive consumption of cheeses from non-pasteurized milk in many countries (in order to obtain a product with pronounced flavour and aroma) makes the study of ewe's milk relevant. The present study deals with pressure inactivation of two bacterial species: *Escherichia coli* and *Pseudomonas fluorescens*. *E. coli* belongs to the Enterobacteriaceae family and is part of the flora in the intestine of humans and warm-blooded animals. Because of its typical habitat, *E. coli* is considered to be a good index of direct or indirect contamination of faecal origin. Storage of raw milk under refrigeration causes psychrotrophic bacteria to grow, especially *Pseudomonas* spp. Ewe's milk is frequently stored under refrigeration for several days to reduce collection costs, because of low production volumes per farm (Núñez et al. 1984). *P. fluorescens* is an indicator of *Pseudomonas* spp., the major components of the spoilage flora of refrigerated milk. When *P. fluorescens* grows in ewe's milk during refrigerated storage, casein hydrolysis is enhanced and milk coagulation characteristics are changed (Uceda et al. 1993). The main objective of this work was to study the effect of different treatment conditions (pressure, time and temperature) on the destruction of *E. coli* and *P. fluorescens*, inoculated in ewe's milk at a rate of about 10^7–10^8 cfu ml^{-1}. 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 strains

Escherichia coli 405 CECT and *P. fluorescens* 378 CECT were obtained as freeze-dried cultures in thermosealed vials from the Spanish Type Culture Collection (CECT, University of 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 (LB; Oxoid Ltd., Basingstoke, Hampshire, UK) at 37°C for 24 h and *P. fluorescens* in tryptone soy broth (TSB; Oxoid Ltd.) at 30°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 used in the initial rehydration. These broth cultures were used to inoculate nutrient agar (NA; Oxoid Ltd.) for *E. coli* and tryptone soy agar (TSA; Oxoid Ltd.) for *P. fluorescens*, which were maintained at 4°C and transferred monthly to provide stock cultures. For each experiment, a tube of stock media (TSA for *E. coli* and NA for *P. fluorescens*) was used by growing *E. coli* in LB at 37°C for 24 h and growing *P. fluorescens* in TSB at

30°C for 24 h to achieve an approximate concentration of 10^9 and 10^{10} cfu ml^{-1} in each broth respectively.

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 (International Dairy Federation 1987). Ash content was determined by gravimetric analysis after the sample had been calcined in an oven at 550°C (International Dairy Federation 1964). Fat content was determined by the Gerber method (International Dairy Federation 1991). Total nitrogen was calculated using the digestion block method, a modification of the Kjeldahl method (International Dairy Federation 1993). The pH was measured by using a pH meter (micro-pH 2001; Crison Instruments SA, Alella, Spain) (Richardson 1985).

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 within 60–90 min from collection at 75 ± 1°C for 1 min in a continuous tubular heat exchanger (Garvia SA, Barcelona, Spain) with a capacity of 50 l h^{-1}. Pasteurized milk was collected in 11 sterile bottles, adjusted to pH 6·7 (by adding 1 N NaOH and/or 1 N HCL) and refrigerated at 4°C. Then, 10 ml of each broth culture (with 10^9 and 10^{10} cfu ml^{-1}) were separately added to 1 l of pasteurized ewe's milk to obtain approximately 10^7 and 10^8 cfu ml^{-1} of *E. coli* and *P. fluorescens* respectively. 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 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 (*c.* 2 l) which can reach 500 MPa in 2 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 (ethyleneglycol–water mixture) within the walls of the vessel. The chamber water temperature was measured by a thermocouple. Samples were kept at atmospheric pressure for 5–10 min in the chamber until temperature equilibrium was established.

The response of *E. coli* and *P. fluorescens* to treatments at different conditions of pressure (50, 100, 150, 200, 250 and 300 MPa), temperature (2, 10, 25 and 50°C), and time (5, 10 and 15 min) was studied. To determine the population kinetics of *E. coli* and *P. fluorescens*, pasteurized ewe's milk was inoculated to approximately 10^7–10^8 cfu ml^{-1} (each micro-organism separately). Assays were performed at 150, 200, 250 and 300 MPa, temperatures (2, 10, 25 and 50°C), and different times (0–30 min). Linear regression of *E. coli* and *P. fluorescens* counts was computed for each temperature. An estimate of the decimal reduction value (D = the time in min 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

Approximately 10 h elapsed from pressurization of the samples until performance of the microbiological assays; the samples were kept at 4°C for this period to avoid the growth of inoculated strains and possible post-pressurization stress. After treatment each samples was analysed using the appropriate decimal dilutions in Ringer solution (9 ml).

Violet red bile agar (VRBA; Oxoid Ltd.) was used to determine the number of *E. coli* in treated and control samples. The plates were incubated at 37°C for 24 h. Purple colonies, with a halo of the same colour, were counted. At the same time, plate count agar (PCA; Oxoid Ltd.) was used to determine possible contamination of samples; the plates were incubated at 30°C for 48 h.

To determine the number of *P. fluorescens* in treated and control samples, Bacto Pseudomonas Agar F (BPAF; Difco Laboratories, Detroit, Michigan, USA) and Irgasan® DP300 (2,4,4'-trichloro-2'-hydroxy diphenyl ether; Ciba-Geigy SA, Barcelona, Spain) were used. Irgasan® DP300 is an antibacterial that is selective for the isolation of *Pseudomonas* spp. The BPAF was sterilized and cooled to 65°C before 10 ml of Irgasan® DP300 solution (0·04% in 95% ethanol) was added aseptically. Then, the medium was shaken to evaporate all the ethanol (Schiemann 1979). The plates were incubated at 30°C for 48 h. All plates of BPAF were observed under ultraviolet light at 365 nm to detect fluorescence and to confirm that the colonies were *P. fluorescens* (Piton and Richard 1985). A crystal-violet-tetrazolium (CVT) count, which permits the differentiation between Gram-positive and Gram-negative bacteria, was used as confirmation and to differentiate possible Gram-positive strains that grew in BPAF medium and that were also resistant to pasteurization and to HHP treatments. The CVT count technique involved the addition of 1 ppm of crystal violet to standard PCA before sterilization and 50 ppm 2,3,5-triphenyltetrazolium chloride (ICN Biomedicals, Inc., Aurora, Ohio, USA) to the agar just before the plates were poured. The plates were incubated at 30°C for 48 h before they were counted. With low dilutions (0·5 ml per plate), the relatively large amount of milk partially neutralized the effect of the crystal violet. This effect was overcome by diluting 9 ml of milk with 9 ml of sterile water containing 10 ppm of crystal violet. One millimetre of this mixture was added to each of two plates, and the total count on the two plates represented the contamination per millilitre (ml^{-1}). Gram-negative colonies were distinctly red. Very few of the micro-organisms surviving pasteurization grew on the plates, and colonies were generally very small and uncoloured or lightly coloured (Olson 1963). To determine possible contamination of samples, a PCA assay was made; the plates were incubated at 30°C for 48 h. Each dilution of VRBA, BPAF, CVT count, and PCA were plated twice.

Statistical treatment of data

Each experiment was replicated three times with duplicate analysis in each replication. An analysis of variance was performed using the general linear models (GLM) procedure of Statistical Analysis System (SAS; SAS Inst., Cary, North Carolina, USA). Duncan's new multiple range test and Student's Newman–Keuls test were used to obtain equivalent comparisons among sample means. Evaluations were based at a level of significance $\alpha = 0{\cdot}05$ ($P < 0{\cdot}05$). *D*-values were determined as the inverse of the slope of the survivor regression curve.

Results

Composition of ewe's milk before standardization was: total solids 18·26% ± 1·71%, fat 7·55% ± 1·32%, total nitrogen 5·73% ± 0·15%, ash 1·14% ± 0·09%. The pH was 6·66 ± 0·09.

Differences between the numbers of micro-organisms that were isolated from PCA and selective media from the same samples were never more than >0·2 log units, which indicates that the contamination was negligible from pasteurized milk and was not important during the process of sample preparation. To eliminate the possibility of a temperature effect *per se*, inoculated milk samples were held at 1 and 53°C for 30 min. No reduction in initial counts was observed under these conditions, which were slightly more extreme than the maximum time and temperature conditions of pressurized samples.

In the overall context of the experiments, an increase in pressure resulted in an increase in inactivation. Also, as treatment time increased, the inactivation increased. However, when pressure and temperature were applied together there was greater inactivation at the temperature extremes (50°C for *E. coli* and 2 and 50°C for *P. fluorescens*), compared to treatment at more moderate temperatures (10 and 25°C).

E. coli proved to be more pressure-resistant than *P. fluorescens* (Fig. 1). It was necessary to apply pressures of 200 MPa at 2, 10 and 25°C for 15 min in order to obtain reductions of approximately 1 log unit of *E. coli*. At 50°C with pressures of 50, 100 and 150 MPa for 5–15 min, reductions of only 0–0·5 log units were reached.

At 200 MPa (and above 250 and 300 MPa), the pressure exerts a considerably ($P < 0·05$) destructive effect at the four pressurization temperatures applied. The pressure treatment was most effective at 50°C when 5–6·5 log reductions were obtained with 300 MPa for 5–15 min respectively. The analysis of variance of the number of surviving cells of *E. coli* shows that in all the combinations of HHP treatment carried out as a whole, the pressure applied caused significant differences ($P < 0·05$) between 300, 250, 100 and 50 MPa, but not ($P > 0·05$) between 200 and 150 MPa (increased destruction: 300 > 250 > 200 ≥ 150 > 100 > 50 MPa); there were significant differences ($P < 0·05$) between 50 and 10°C and these two temperatures separately with 25 or 2°C, but not ($P > 0·05$) between 25 and 2°C (increased destruction 50 > 25 ≥ 2 > 10°C); there were significant differences ($P < 0·05$) between all treatments times applied (increased destruction 15 > 10 > 5 min). The analysis of the *F*-value shows that pressure was the main factor; it explains more than 50% of the variability of the statistical model on destruction of *E. coli* by HHP.

P. fluorescens (Fig. 2) proved to be more sensitive to HHP treatment than *E. coli*. However, with pressures of ≤200 MPa the logarithmic reductions were very similar to those obtained with *E. coli* at all temperatures, except at 25°C, when *E. coli* proved to be more sensitive at 200 MPa at 10 and 15 min. Treatment of *P. fluorescens* with pressures of 250 MPa for 15 min at 2, 10, 25 and 50°C gave reductions of 4, 3, 3·5 and 6 log units respectively. As occurred with *E. coli*, it is at 200 MPa and above that *P. fluorescens* suffered a considerable ($P < 0·05$) descent in its initial population at the four temperatures. At 50°C, with only 150 MPa for 15 min, the population of *P. fluorescens* fell by approximately 2·5 log units; a considerable ($P < 0·05$) decrease compared to *E. coli*, which decreased by barely 0·5 log units. Also, at 50°C with 300 MPa at the three treatment times, the total destruction of its initial population was reached, reductions ≥8·5 log units. The analysis of variance of the number of surviving cells of *P. fluorescens* indicated that in all combinations of pressurization carried out, the pressures studied caused significant differences ($P < 0·05$) between 300, 250, 200 and 50 MPa, but not ($P > 0·05$) between 150 and 100 MPa (increased destruction 300 > 250 > 200 > 150 ≥ 100 > 50 MPa); there were significant differences ($P < 0·05$) between all temperatures assayed (increased destruction 50 > 2 > 25 > 10°C); and there were significant differences ($P < 0·05$) between all treatment times applied (increased destruction 15 > 10 > 5 min). Analysis of the *F*-value shows that pressure was the main factor, accounting for 60%, then temperature, accounting for 28% of variability of the statistical model on destruction of *P. fluorescens* by HHP.

Fig. 3 shows that on *E. coli* and *P. fluorescens*, the tendency to achieve major survival at 250 MPa for 15 min occurs at 10°C. On the other hand, maximum lethality occurred at 50°C on *E. coli* and 2 and 50°C on *P. fluorescens*. Without taking into account the level of lethality that each microorganism showed (Fig. 3), the two tendency curves (according to the temperature) depict very similar profiles of bacterial destruction.

Study of kinetics to destruction on E. coli *and* P. fluorescens

Table 1 shows destruction kinetics (*D*-values) at 2, 10, 25 and 50°C with different

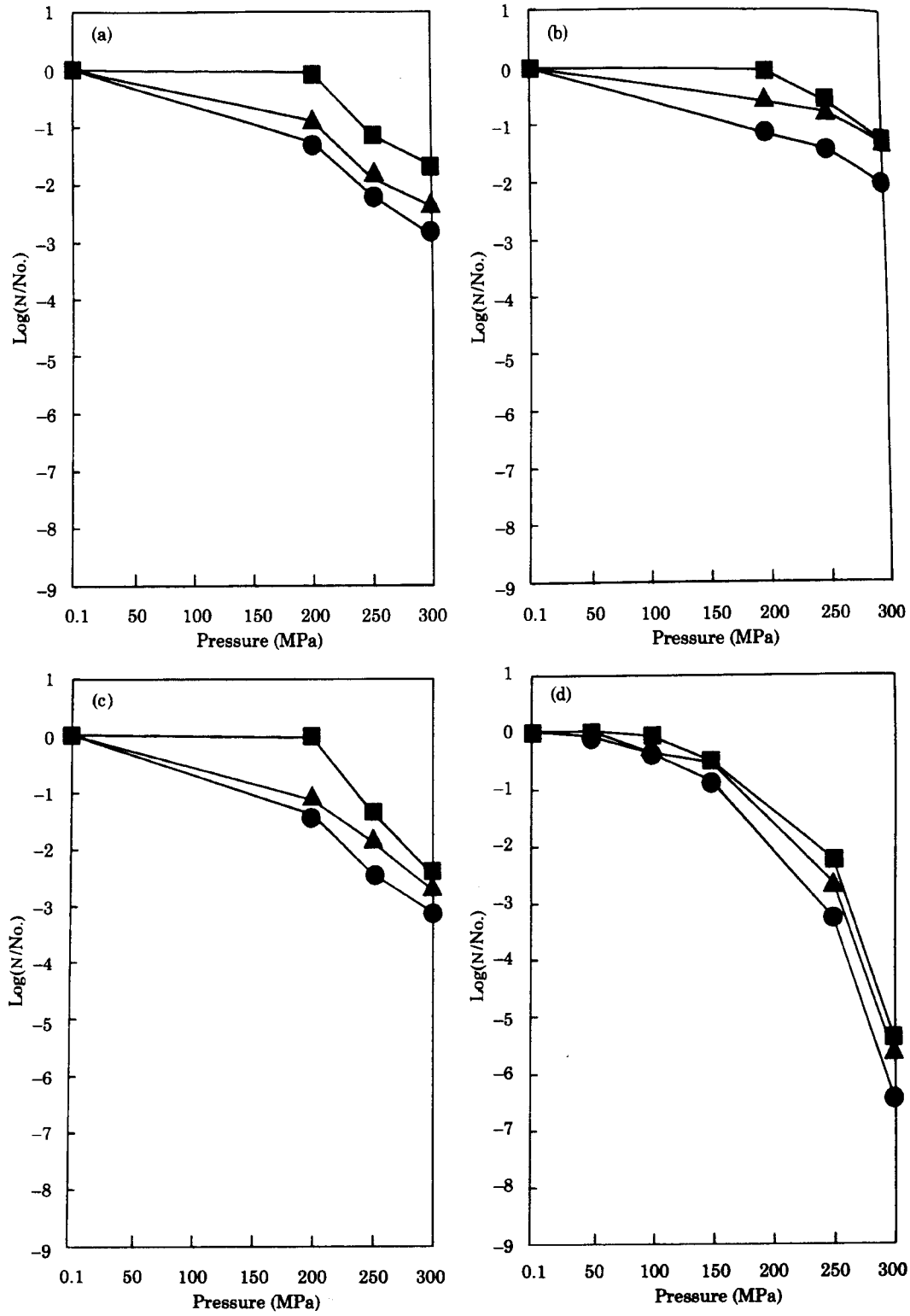

Figure 1. Effect of high hydrostatic pressure on *Escherichia coli* 405 CECT inoculated in ewe's milk. Pressure for 5 min (■), 10 min (▲) and 15 min (●) at 0·1 logarithm of initial counts (control); (a) 2°C, (b) 10°C, (c) 25°C and (d) 50°C.

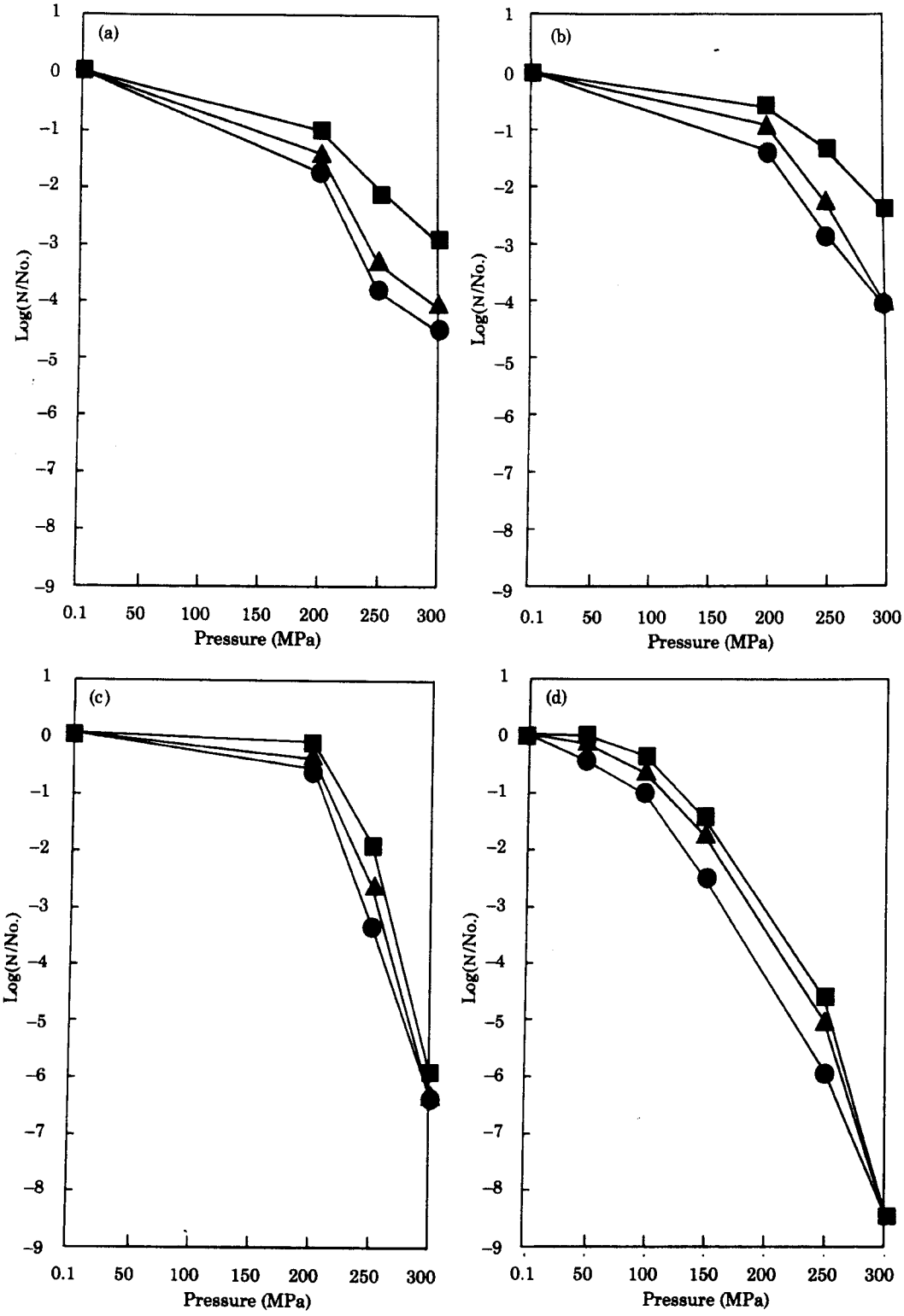

Figure 2. Effect of high hydrostatic pressure on *Pseudomonas fluorescens* 378 CECT inoculated in ewe's milk. Pressure for 5 min (■), 10 min (▲) and 15 min (●) at 0·1 logarithm of initial counts (control); (a) 2°C, (b) 10°C, (c) 25°C and (d) 50°C.

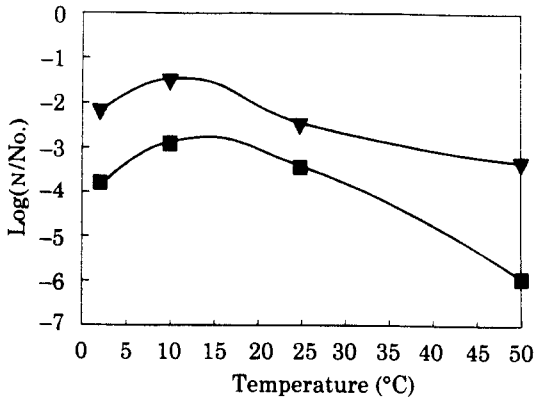

Figure 3. Effect of high hydrostatic pressure as a function of temperature at 250 (MPa) for 15 min on *Escherichia coli* 405 CECT (▼) and *Pseudomonas fluorescens* 378 CECT (■), inoculated in ewe's milk.

pressure treatments on *E. coli* and *P. fluorescens*, with an initial population of 10^7 and 10^8 cfu ml^{-1} respectively. At 50°C, there was a large difference in the *D*-values obtained when pressures of 150 and 300 MPa were used (18·8 and 2·5 min), showing that increased pressure does not correspond to a linear decrease in decimal reduction time; an increase of pressure 1:2 (150:300 MPa) reduces the *D*-value to 7·4:1 (18·8:2·5 min). Supposing that our food (ewe's milk, 6% fat) was contaminated with an initial load of 10^7 cfu ml^{-1} of *E. coli* and we applied one HHP treatment with $D_{150\,MPa/50°C} = 18·8$ min and another with $D_{300\,MPa/50°C} = 2·5$ min, it would take 131·6 and 17·7 min to reduce the *E. coli* population to zero respectively; obviously an increase of pressure would be more effective than prolonging the time of HHP treatments at lower pressures.

With *P. fluorescens* at 50°C, the increase of 100 MPa (from 150 to 250 MPa) produced a decrease in the time of decimal reduction from 6·4 to 2·8 min. This decrease was not as large as the one that occurred with *E. coli*, whereas at 25°C, an increase of only 50 MPa (from 200 to 250 MPa) caused a very big decrease in the decimal reduction time, from 23·3 to 4·6 min. With an initial population of 10^8 cfu ml^{-1} of *P. fluorescens* (in ewe's milk, 6% fat) and a HHP treatment of $D_{200\,MPa/25°C} = 23·3$ min and $D_{250\,MPa/25°C} = 4·6$ min, it would take 186 min and 36·6 min to eliminate the initial population totally, respectively.

Table 1. *D*-values of *Escherichia coli* 405 CECT and *Pseudomonas fluorescens* 378 CECT, inoculated in ewe's milk for high hydrostatic pressure inactivation

Micro-organisms	Pressure (MPa)	Temp. (°C)	*D*-value[a] (min)	Equation[a]	r^2
E. coli	150	50	18·80	$Y = 7·1 - 0·0532 \times t$[b]	0·946355
	200	25	9·51	$Y = 7·3 - 0·1052 \times t$	0·936428
	250	2	6·83	$Y = 7·3 - 0·1464 \times t$	0·938166
	250	10	11·14	$Y = 7·5 - 0·0898 \times t$	0·965823
	250	25	6·40	$Y = 6·8 - 0·1562 \times t$	0·937918
	250	50	4·86	$Y = 6·6 - 0·2056 \times t$	0·955687
	300	2	5·35	$Y = 7·2 - 0·1871 \times t$	0·924152
	300	10	8·13	$Y = 7·3 - 0·1231 \times t$	0·961158
	300	25	5·19	$Y = 6·8 - 0·1926 \times t$	0·945338
	300	50	2·53	$Y = 6·7 - 0·3952 \times t$	0·956177
P. fluorescens	150	50	6·35	$Y = 8·2 - 0·1576 \times t$	0·916685
	200	25	23·26	$Y = 8·5 - 0·0431 \times t$	0·986554
	250	2	3·87	$Y = 7·7 - 0·2584 \times t$	0·930408
	250	10	5·27	$Y = 7·9 - 0·1898 \times t$	0·969665
	250	25	4·58	$Y = 8·1 - 0·2182 \times t$	0·928388
	250	50	2·75	$Y = 7·3 - 0·3634 \times t$	0·981496
	300	2	3·38	$Y = 7·4 - 0·2962 \times t$	0·966963
	300	10	3·56	$Y = 7·6 - 0·2808 \times t$	0·972931

[a] Data are the means ($n = 6$) of three indepndent experiments.
[b] $Y = \log_{10}$ cfu ml^{-1} (where t is time of treatment of pressurization).

We designed an experiment to confirm the total inactivation of micro-organisms (*E. coli* and *P. fluorescens*) by pressure and detect, if present, sublethally-damaged micro-organisms. From each treatment where no microbial growth was detected, the sample was incubated at 37°C for 24 h (*E. coli*) and 30°C for 24 h (*P. fluorescens*), and afterwards plated in its selective media (each one at optimal conditions of incubation). Results were analysed and no microbial growth was detected in any case (where initially no microbial growth was detected) confirming total inactivation and the absence of sublethally-damaged micro-organisms (data not shown).

Discussion

Butz and Ludwig (1986) showed reductions of 4, 6 and ≥7·5 log units on *E. coli* in sterile saline TS when applying pressures of 300 MPa at 40°C for 10, 20 and 25 min respectively. These reductions are found among those obtained in this study when we apply pressures of 300 MPa for 15 min at 25 and 50°C. Takahashi (1992) using 1/500 M PBS pH 7·0 obtained reductions of close to 4 and ≥8 log units on *E. coli* when pressurizing with 200 MPa for 20 min at 20 and −20°C respectively. In similar conditions we obtained less reductions, for example, treatments with 200 MPa for 15 min and 2°C produced decreased of 1·4 and 1·3 log units respectively. In other pressurization treatments carried out by Takahashi (1992) using similar to our own conditions, greater lethality on *E. coli* was always achieved than we did, with these results, it could be said that ewe's milk (6% fat) exercised a baroprotector effect on *E. coli*. The results of Takahashi (1992) indicated that *E. coli* was killed by HHP considerably at low temperature (−20°C), whereas our treatments at low temperature (2 and 10°C) produced a similar or lesser destruction than at room temperature (25°C). At any rate, the cause of this great inactivation produced at −20°C could be due to the process described in the water phase diagram (Kalichevski et al. 1995); at 200 MPa and −20°C, water is on the limit of liquid and solid phases, and the effect of fusion and crystallization of the water (intra- and extracellular) could harm the micro-organisms. Ludwig et al. (1992) studied the inactivation of *E. coli* by HHP up to 500 MPa in the temperature range between 1 and 50°C for 0 to 80 min in physiological NaCl solution. This study showed *E. coli* to be very sensitive to HHP, as greater destruction of *E. coli* was observed in all pressurization combinations than was found in the current study. Like us, Ludwig et al. (1992) obtained first-order kinetics of destruction using pressures of 200–500 MPa at 25, 40 and 50°C, at least in the range 0–30 min. Ludwig et al. (1992) also studied temperature dependence of *E. coli* inactivation with treatments of 200 MPa for 15 min; the maximum survival was obtained at room temperatures (20–30°C), however, with treatments of 250 MPa for 15 min, we obtained maximum survival at 10°C (Fig. 3). Other authors such as Hauben et al. (1997), studied variability of *E. coli* strains by HHP treatments in potassium phosphate buffer (pH 7·0, 10 mM). The *E. coli* K-12 strain MG1655, in the majority of pressurization combinations similar to ours, suffered a considerably greater decrease to the *E. coli* shown in our tests; only those treatments carried out at 50°C seem to show similar results to our own. On the other hand, studies have been carried out on *E. coli* in foods (Patterson et al. 1995, Isaacs et al. 1995, Gervilla et al. 1997a,b). Isaacs et al. (1995) studied survival curves for *E. coli* in whole milk at different pressures, temperatures and times. Their results showed that in the stationary phase of growth *E. coli* was more baroresistant, and to obtain important reductions (>4 log units in the stationary phase) in a reasonable time (5–10 min) it was necessary to apply pressures of ≥400 MPa. Patterson et al. (1995) studied the effect of HHP treatments on various micro-organisms in 10 mM phosphate-buffered saline pH 7·0, UHT milk and raw poultry meat. Of the pathogens tested *E. coli* O157:H7 strains NCTC 12079, H631 and H1071 showed very high resistance to HHP treatments, even when they were treated in buffered solution. The results when pressurizing with 600 MPa

at 20°C for 5–30 min showed that *E. coli* was inactivated more in buffered solution than in poultry meat and least in UHT milk.

The different responses obtained when comparing similar pressurization of *E. coli* lead us to think that there are diverse factors that considerably influence the response of micro-organisms to HHP. One factor is the substrate or food in which the micro-organism finds itself when pressurized; it is well known that certain constituents exist in foods or substrata and changes during pressurization may exercise a baroprotector effect or *vice versa* (Knorr et al. 1992, Maggi et al. 1994). A second important factor is the level of baroresistency there can be between different species of the genus and strains of the same species (*E. coli*), as well as the conditions of growth and possible states of prepressurization stress. These factors should be considered when making recommendations to the industry and results of the same micro-organism studied in different substrata or foods should not be extrapolated.

Few studies have been carried out on species of the genus *Pseudomonas*, probably because this micro-organism, in different substrata and aqueous solutions, proved to be quite barosensitive and easily inactivated (Gervilla et al. 1997b). However, as we have previously been able to confirm with *E. coli*, it is necessary to study the particular behaviour of the micro-organisms of interest in the particular substrata or food studied. In a study carried out by Gervilla et al. (1997b) on *P. fluorescens*, reductions of ≥ 7 log units were obtained in the majority of HHP treatments, with pressures of 400–500 MPa at 2, 10, 25 and 50°C for 5–15 min. In this study the great barosensitivity of *P. fluorescens* in ewe's milk was shown; however, we believed it would be interesting to also complete the whole range of interactions (pressure, temperature and time) for this micro-organism, with the aim of finding the minimum combinations capable of destroying a significant number of *P. fluorescens* cells, and also to find a series of *D*-values (Table 1), in order to be able to compare the results of our HHP treatments with other conventional HHP and thermal treatments.

Carlez et al. (1993) studied the effect of HHP on various micro-organisms in minced beef muscle, among these, *P. fluorescens*, with pressures from 50 to 400 MPa for 20 min at 4, 20, 35 and 50°C. Complete inactivation (≥ 6 log units) was observed at pressures above 200 MPa at all treatment temperatures, whereas in ewe's milk, with 200 MPa for 15 min at 2, 10 and 25°C, we obtained reductions of 1·8, 1·4 and 0·6 log units, and at 50°C with 250 MPa for 15 min a reduction of 5·9 log units was obtained. In all the comparable treatments, *P. fluorescens* proved to be considerably more sensitive to HHP in minced beef than in ewe's milk (6% fat). A decimal reduction time calculated was 23·8 min at 20°C with 150 MPa for *P. fluorescens*, whereas in ewe's milk we obtained a similar result: 23·3 min at 25°C with 200 MPa. As in our study, the results from Carlez et al. (1993) coincide in that low temperature (4°C) was more effective than room temperature (20°C), that milk constituents therefore appear to partly protect *P. fluorescens* against HHP inactivation, and that *P. fluorescens* was more sensitive to pressure than *Citrobacter freundii* (another Enterobacteria like *E. coli*) although both micro-organisms are Gram-negative. Another study carried out by Carlez et al. (1994) shows the sensitivity and post-pressurization growth for *Pseudomonas* spp. at 20°C for 20 min with 200–450 MPa in minced meat. They obtained reductions of 2 and 4 log units when applying 200 and 300 MPa respectively for *Pseudomonas* spp., whereas our results for *P. fluorescens* showed reductions of 0·6 and 3·4 log units at 25°C for 15 min with 200 and 250 MPa respectively. If we compare the results of Carlez et al. (1993, 1994), it is possible to see that within the *Pseudomonas* genus there may be more baroresistant species than *P. fluorescens*, and that their results coincide (in both studies); *Pseudomonas* was the least resistant micro-organism to HHP treatments. Ludwig and Schreck (1997) also studied *Pseudomonas aeruginosa* in aqueous medium, at 200 MPa with different times and temperatures. A considerable decrease in the number of *P. aeruginosa* was seen as reductions of about 3, 5 and 8 log units were obtained with 200 MPa for 15 min at 30, 10 and

1°C respectively. The effect of temperature shows more destruction at 50 > 40 > 1 > 10 > 30°C, showing as in this study, that low and moderately high temperature were more lethal than room temperature for *Pseudomonas*.

It is generally felt that for micro-organisms the primary site pressure damage is the cell membrane (Earnshaw 1992), as seems to occur with *E. coli* (Isaacs et al. 1995), which causes loss of the permeability of the cellular membrane and extracellular substances (substrata or food) penetrate in the intracellular space, provoking diverse alterations depending on the substances that penetrate in the moment of pressurization. On the other hand, some micro-organisms, according to the pressurization temperature, follow a destruction curve similar to the denaturation curve of proteins under HHP treatments; some proteins are more susceptible to denaturation during pressurization at low temperatures than at room temperatures (Hawley 1971), which could explain the response of *P. fluorescens* to pressurization temperature in this study.

In general, Gram-positive bacteria are more resistant than Gram-negative ones (as for resistance to heat), and independently of the Gram-type, spherical forms (cocci) are more resistant than rod forms (bacillus) (Ludwig and Schreck 1997). The lethal effects of a given HHP treatment on vegetative micro-organisms is strongly influenced by the constituents of the media or food, and by the pH and water activity. Other studies to determine the effect of HHP treatments on other micro-organisms in ewe's milk are being undertaken, such as assays to determine the effect on the ewe's milk constituents.

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