Effect of High Hydrostatic Pressure on *Escherichia coli* and *Pseudomonas fluorescens* Strains in Ovine Milk

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ABSTRACT

Ovine milk that had been standardized to 6% fat was inoculated with *Escherichia coli* 405 CECT and *Pseudomonas fluorescens* 378 CECT at a rate of 10^6 and 10^7 cfu/ml, respectively, and treated with high hydrostatic pressure. Treatments consisted of combinations of pressure (300, 400, 450, and 500 MPa), temperature (2, 10, 25, and 50°C), and time (5, 10, and 15 min). Inactivation (>6 log cfu/ml) of both strains was observed at 50°C for all pressures and treatment times. A similar level of inactivation occurred at ≥450 MPa and 25°C for *E. coli* and at ≥400 MPa and 10°C for *P. fluorescens*. Destruction was lowest at 10°C for *E. coli* and at 25°C for *P. fluorescens*. The test strain of *E. coli* was more baroresistent than was the *P. fluorescens* strain.
(**Key words**: hydrostatic pressure, *Escherichia coli*, *Pseudomonas fluorescens*, ovine milk)

Abbreviation key: **BPAF** = Bacto Pseudomonas Agar F, **CVT** = crystal-violet-tetrazolium, **HHP** = high hydrostatic pressure, **PCA** = plate count agar.

INTRODUCTION

Recently, new technologies, such as electric or magnetic fields, ionizing radiation, light pulses, and high hydrostatic pressure (**HHP**) (23), are being applied for the conservation of foods. These technologies could provide a total or partial substitute for heat treatments, providing products that are free of viable microorganisms in a reasonable time without alteration of active ingredients (2). The physical process of HHP (100 to 600 MPa) allows food manufacturers to inactivate microorganisms (14, 34) without damaging food constituents, to alter the taste or flavor, and to inactivate microorganisms but not the enzymes that are important for cheese maturation (4, 7, 11, 30). The first experiments on milk were by Hite (12); Hite et al. (13) subsequently examined a wide range of foods and beverages for the potential use of HHP processing. Since then, a number of studies (3, 21, 25) have been published on the effect of HHP on microorganisms; the majority of those studies combined different factors, such as pressure, time, temperature, water activity, pH, concentrations of certain substances, and initial numbers of microorganism. All of those studies were undertaken using buffered aqueous solutions, emulsions, meat derivatives, egg products, milk, and other food products. Bovine milk is used in the majority of studies on milk (28, 34, 36), but ovine milk has not been investigated. Ovine milk is different from bovine milk in nutrient composition, both the proportion and amounts of nutrients and in physicochemical characteristics. Given that the composition of the medium can also influence pressure effects upon microorganisms (6), the behavior of different microorganisms in ovine milk under the influence of HHP was evaluated.

The present study deals with pressure inactivation of two bacterial species: *Escherichia coli* and *Pseudomonas fluorescens*. *Escherichia 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 fecal origin. Storage of raw milk under refrigeration selects for growth of psychrotrophic bacteria, especially the *Pseudomonas* spp. Ovine milk is frequently stored under refrigeration for several days to reduce collection costs, because of the low production volumes per farm (24). *Pseudomonas fluorescens* is an indicator for *Pseudomonas* spp., the major components of the spoilage flora of refrigerated milk. When *P. fluorescens* grows in ovine milk during refrigerated storage, casein hydrolysis is enhanced, and milk coagulation characteristics are changed (37). These bacteria produce enzymes that are capable of resisting the usual sterilization temperatures for products (10) and that can degrade various milk components (5, 8, 9) to cause deterioration during storage.

Temperatures between 2 and 50°C are considered to be appropriate for HHP processing of milk. Temperatures <2°C favor the formation of ice crystals in

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TABLE 1. Physico-chemical characteristics of ovine milk before standardization.

	$\bar{X}$[1]	SD
TS	18.17	1.75
Fat	7.69	1.48
Total N	5.66	0.19
Ash	1.12	0.15
pH	6.66	0.05

[1]Means of duplicate analyses of three replicate treatments of milk.

milk during decompression, which would destabilize the milk; temperatures >50°C could denature proteins and form protein complexes (20) that would adversely affect milk clotting. Treatments periods >15 min would lower productivity. Pressures >500 MPa would be difficult to obtain with economically viable equipment and large-scale milk processing.

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 (University of Valencia, Valencia, Spain). The vials were maintained at 4°C until use. The freeze-dried cultures were first rehydrated in an appropriate broth: *E. coli* in 3 ml of lactose broth (Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C for 24 h and *P. fluorescens* in tryptone soy broth (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 (Oxoid Ltd.) for *E. coli* and tryptone soy agar (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 (tryptone soy agar for *E. coli* and nutrient agar for *P. fluorescens*) was used by growing *E. coli* on lactose broth at 37°C for 24 h and growing *P. fluorescens* in tryptone soy broth at 30°C for 24 h (37). Finally, 10 ml of each broth culture (10^8 to 10^9 cfu/ml) were added to 1 L of milk to achieve an approximate concentration of 10^6 to 10^7 cfu/ml in milk samples.

Composition and Physico-Chemical Analyses of Milk

The composition of ovine milk is given in Table 1. The total solids content was determined by drying in a 102 ± 2°C heater until a constant weight was reached (17), and ash content was determined by gravimetric analysis once the sample was ashed in the oven at 550°C (16). Fat content was determined by the Gerber method (18), and total nitrogen was determined by a modification of the Kjeldahl method using the block digestion method (19). The pH was measured potentiometrically (micro-pH 2001; Crison Instruments S.A., Alella, Spain) (29).

Preparation and Inoculation of Milk Samples

Milk from Manchega ewes was obtained from a dairy farm. Raw milk was collected from the first milking in the morning and standardized to 6% fat. The standardized milk was pasteurized 60 to 90 min after collection at 85 ± 1°C for 1 min in a continuous tubular heat exchanger with a capacity of 50 L/h (Garbia SA, Barcelona, Spain). Pasteurized milk was collected in 1-L sterile bottles, adjusted to pH 6.7 with a pH meter (micro-pH 2001; Crison Instruments S.A.) (29), and refrigerated at 4°C.

Ten milliliters of lactose broth with 10^8 cfu/ml of *E. coli* and 10 ml of tryptone soy broth with 10^9 cfu/ml of *P. fluorescens* were each inoculated into 1 L of pasteurized ovine milk to obtain approximately 10^6 and 10^7 cfu/ml, respectively. Milk was gently shaken manually for 5 min, and 30 ml of inoculated milk were bottled in a sterile tubular bag (31.8 mm diameter) made of polyvinylidene chloride (Krehalon, Eyglières, France). Bags were sealed with two knots (separated by 1 cm) and thermosealed at both ends. Care was taken to leave as little air as possible between the milk and the tubing knots. The bags were vacuum-packaged in a second bag to eliminate leakage.

HHP Processing

Samples were pressurized using discontinuous HHP equipment (ACB, Nantes, France) that had a pressure chamber of 10 cm in diameter and 25 cm in height (approximately 2 L), and could reach 500 MPa in 4 min (Figure 1). Samples were submerged in water, which acted as the hydrostatic fluid medium. The chamber temperature was determined by means of a thermoregulation system that circulated heating and cooling fluid (ethylene glycol and water mixture) within the walls of the vessel. The chamber water temperature was measured with a thermocouple. Samples were kept for 5 to 10 min at atmospheric pressure in the chamber until an equilibrium temperature was established.

Time, temperature, and pressure parameters were selected according to previous unpublished studies of

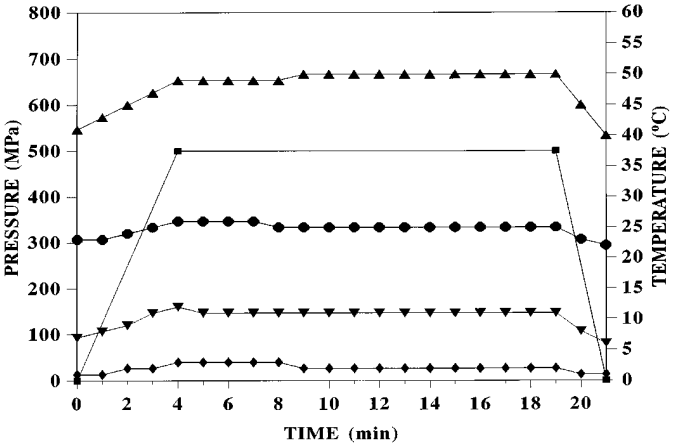

Figure 1. Variation of the chamber water temperature as a function of time during treatment at 2°C (♦), 10°C (▼), 25°C (●), and 50°C (▲) and at 500 MPa of pressure (■).

our own. The response of *E. coli* and *P. fluorescens* to treatments was assessed at different conditions of pressure [300 (*P. fluorescens* only), 400, 450, and 500 MPa], temperature (2, 10, 25, and 50°C), and time (5, 10, and 15 min). Each treatment was performed three times.

Microbiological Assays

The time that elapsed from pressurization of the samples until 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 number of *E. coli* in treated and control samples, violet red bile agar (Oxoid Ltd.) was used. The plates were incubated at 37°C for 24 h. Purple colonies, with a halo of the same color, 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 (15).

To determine the amount of *P. fluorescens* in treated and control samples, Bacto Pseudomonas Agar F (**BPAF**; Difco Laboratories, Detroit, MI) and Irgasan® DP300 (2,4,4′-trichloro-2′-hydroxy diphenyl ether; Ciba-Geigy S.A., 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) were added aseptically. Then, the medium was shaken to evaporate all of the ethanol (32). 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* (27). As confirmation and to differentiate possible Gram-positive strains that were resistant to pasteurization, resistant to HHP treatments, and grew in BPAF medium, a crystal-violet-tetrazolium (**CVT**) count was used, which permits the differentiation between Gram-positive and Gram-negative bacteria. The CVT count technique involved the addition of 1 ppm of crystal violet to standard PCA before sterilization and 50 ppm of 2, 3, 5-triphenyltetrazolium chloride (ICN Biomedicals, Inc., Aurora, OH) to the agar just before the plates were poured. The plates were incubated at 30°C for 48 h before bacteria 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 millimeter of this mixture was added to each of 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 (26). To determine the possible contamination of samples, a PCA assay was made; the plates were incubated at 30°C for 48 h (1). Each dilution of violet red bile agar, BPAF, CVT count, and PCA was plated twice.

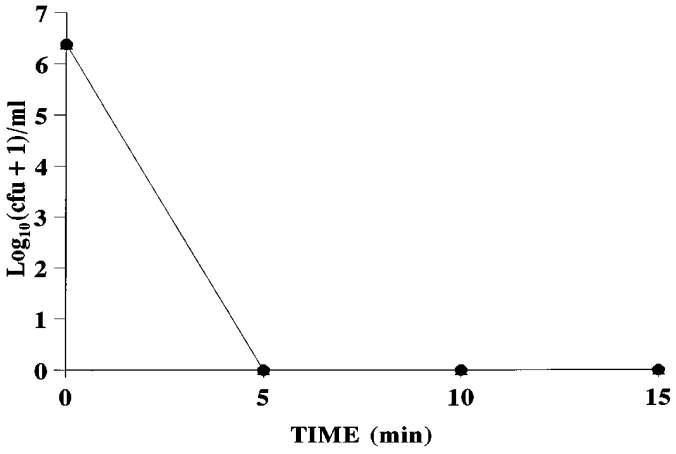

Figure 2. Effect of high hydrostatic pressure [400 MPa (▲), 450 MPa (●), and 500 MPa (■)] as a function of time at 50°C on numbers of *Escherichia coli* 405 CECT in ovine milk.

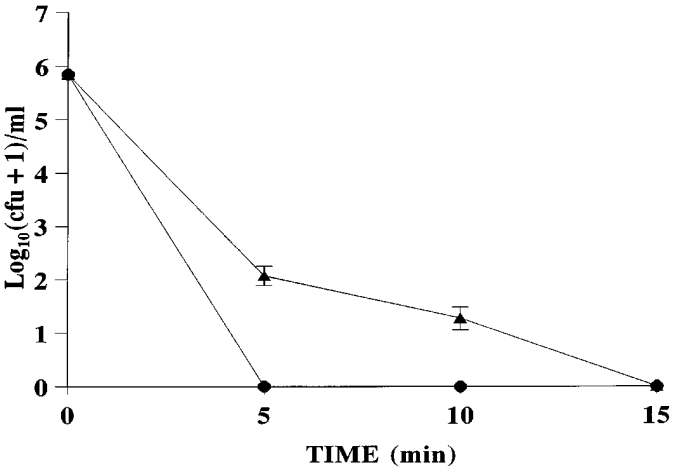

Figure 3. Effect of high hydrostatic pressure [400 MPa (▲), 450 MPa (●), and 500 MPa (■)] as a function of time at 25°C on numbers of *Escherichia coli* 405 CECT in ovine milk.

Statistical Treatments of Data

Each experiment was replicated three times with duplicate analysis in each replication. A descriptive analysis was made of the variables that were of interest using the SPSS statistical package (33); subsequently, ANOVA was performed using the general linear models procedure of SAS (31). The adjusted model in the ANOVA was

$$Y_{ijkl} = \mu + P_i + TEM_j + TI_k + \epsilon_{ijkl}$$

where Y_{ijkl} = proportion of survivors in the measurement l, μ = global mean effect, P_i = main effect of pressure on level i (i = 300, 400, 450, and 500 MPa), TEM_j = main effect of temperature on level j (j = 2, 10, 25, and 50°C), TI_k = main effect of treatment time on level k (k = 5, 10, and 15 min), and ϵ_{ijkl} = error of the measure l, together with corresponding interactions. Evaluations were based on a 5% significance level.

RESULTS AND DISCUSSION

Differences between the numbers of microorganisms that were isolated from PCA and selective media from the same samples were never >0.2 $\log_{10}$ units, which indicates that the contamination was negligible from the pasteurized milk or during the process of sample preparation.

Samples of standardized ovine milk (6% fat), inoculated with the *E. coli* strain at 10^6 cfu/ml, showed reductions of at least 6 log units during treatments at 50°C, regardless of pressure (400, 450, and 500 MPa) or time (5, 10, and 15 min) (Figure 2), At 25°C (Figure 3), the same reduction (6 log units) was produced at pressures of 450 and 500 MPa in all of the combinations of treatment times. Pressures ≥40 MPa produce morphological modifications in *E. coli* strains, such as variation in the normal cell length (38). At refrigeration temperatures of 10 and 2°C (Figures 4 and 5), treatment periods of 15 min and pressures of 450 or 500 MPa were necessary to achieve reductions of about 6 log units. No lethal effect from temperature alone was expected because the range of the applied temperatures was below the lethality for the test species. However, to eliminate the possibility of a temperature effect per se, inoculated milk samples were held at 53°C for 20 min. No reduction in initial counts was observed under these conditions, which were slightly higher than the maximum time and temperature conditions of pressurized samples. This result indicates a greater bactericidal effect at a temperature of 50°C combined with the pressure treatments that were applied.

Analysis of variance of the proportion of surviving cells of *E. coli* shows that the pressures applied caused significant differences ($P < 0.05$) between 400 and 450 MPa but not between 450 and 500 MPa, regardless of the time and temperature variables. Time differences ($P < 0.05$) were observed between 5, 10, and 15 min under any pressure or temperature condition. For all combinations of pressure and time, differences were observed ($P < 0.05$) between 2, 10, and 25°C, but not between 25 and 50°C ($P > 0.05$). Carlez et al. (3) demonstrated that *Citrobacter freundii* strains inoculated into minced beef muscle were more sensitive to high pressure when applied at tem-

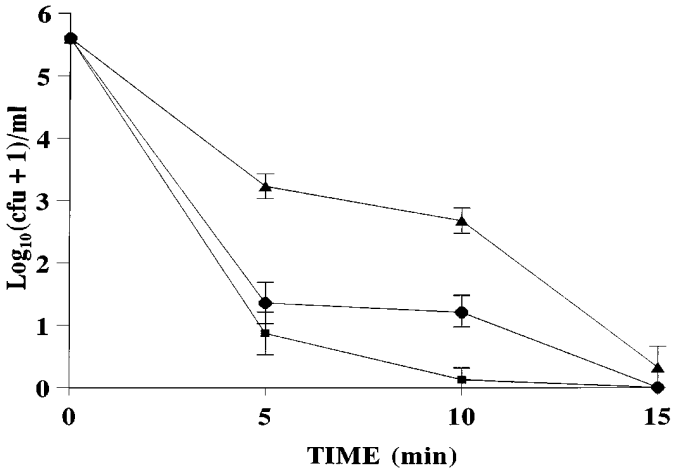

Figure 4. Effect of high hydrostatic pressure [400 MPa (▲), 450 MPa (●), and 500 MPa (■)] as a function of time at 10°C on numbers of *Escherichia coli* 405 CECT in ovine milk.

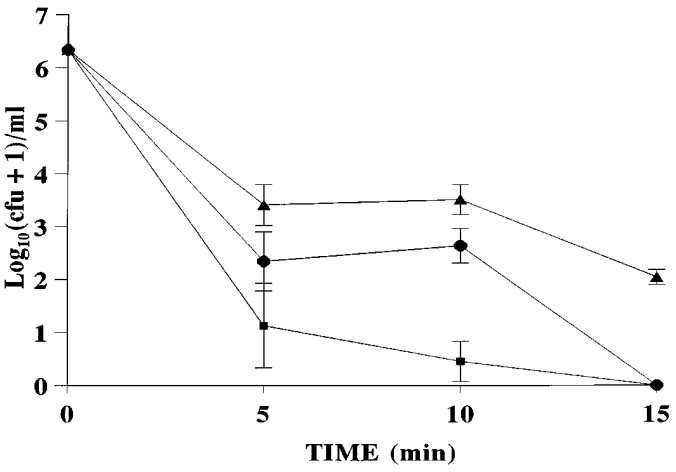

Figure 5. Effect of high hydrostatic pressure [400 MPa (▲), 450 MPa (●), and 500 MPa (■)] as a function of time at 2°C on numbers of *Escherichia coli* 405 CECT in ovine milk.

peratures of 35 and 4°C, but, at 20°C, showed higher baroresistence.

Table 2 shows the results of the destruction of the *P. fluorescens* strain inoculated at 10^7 cfu/ml. At 50°C after 5 min of treatment, destruction was total at any of the pressure and time combinations used. Substantial reduction occurred for all combinations at 10°C and for all but one combination at 2°C. The *P. fluorescens* strain was baroresistent in most combinations at 25°C. At 25°C, a pressure of 300 MPa and 10 to 15 min were necessary to produce reductions of 6 to 7 log units. These data agree with those published by Carlez et al. (3), who, working with *P. fluorescens* strains in minced beef muscle, showed that those strains were more baroresistent at temperatures of 20 and 35°C than at 4°C.

The test strain of *E. coli* was more resistant to HHP than the *P. fluorescens* strain, which is similar to results of Takahashi et al. (35), who evaluated *P. fluorescens* and *E. coli* strains in phosphate buffer. The *P. fluorescens* strain was more sensitive to pressure at low temperatures (2 and 10°C) than at near room temperature (25°C).

Figure 6 presents a comparison of the mortality of the two species at 400 MPa between 2 to 50°C during a treatment time of 5 min. Under these conditions, the destruction of *E. coli* was highest at 50°C and lowest at 10°C; *P. fluorescens*, under the same conditions, showed a greater mortality than *E. coli*. For *P. fluorescens*, destruction was slightly higher at 10 and 50°C, but the effect of different temperatures was negligible.

CONCLUSIONS

The use of pressures between 400 and 450 MPa is possible when the treatment times are extended to >15 min in order to reduce bacterial numbers to an acceptable level. Numbers of *E. coli*, when exposed to

TABLE 2. Effect of high hydrostatic pressures at different temperatures on numbers of *Pseudomonas fluorescens* 378 CECT in ovine milk.[1]

		Temperature							
Pressure	Time	2°C[2]		10°C[3]		25°C[4]		50°C[5]	
(MPa)	(min)	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
300	5	. . .[6]	. . .	. . .	. . .	14	2	0	0
300	10	. . .	. . .	. . .	. . .	7	2	0	0
300	15	. . .	. . .	. . .	. . .	7	1	0	0
400	5	2	3	0	0	5	1	0	0
400	10	0	0	0	0	2	2	0	0
400	15	0	0	0	0	5	1	0	0
450	5	0	0	0	0	5	1	0	0
450	10	0	0	0	0	7	1	0	0
450	15	0	0	0	0	4	3	0	0
500	5	0	0	0	0	7	2	0	0
500	10	0	0	0	0	1	1	0	0
500	15	0	0	0	0	2	2	0	0

[1]Data are expressed as means (n = 6) and standard deviations of final counts (colony-forming units per milliliter).

[2]Initial counts 1.1×10^7 cfu/ml.

[3]Initial counts 8.5×10^6 cfu/ml.

[4]Initial counts 1.6×10^7 cfu/ml.

[5]Initial counts 2.0×10^7 cfu/ml.

[6]Treatments were not done.

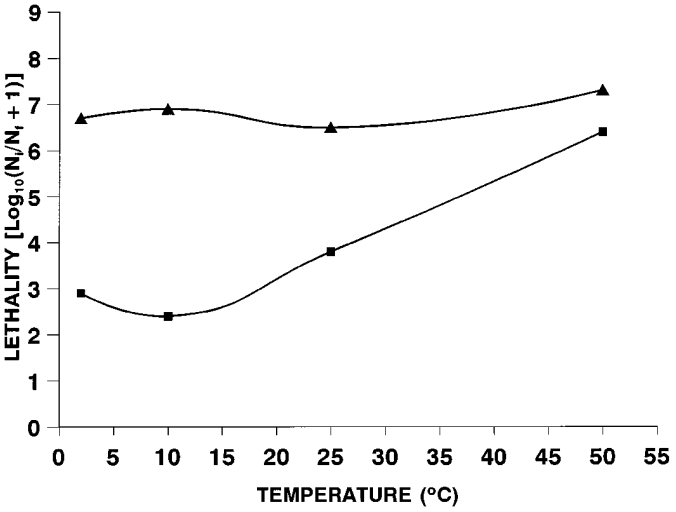

Figure 6. Lethality [$\log_{10}$ ($N_{initial}$ (N_i)/N_{final} (N_f) + 1)] of high hydrostatic pressure as a function of temperature during 5 min of treatment at 400 MPa of ovine milk that had been inoculated with *Pseudomonas fluorescens* 378 CECT (▲) and *Escherichia coli* 405 CECT (■).

500 MPa for 10 min at 2 and 10°C, were reduced by 5.5 to 6 log units and were reduced at least 6 log units at 25 and 50°C when subjected to 450 MPa for 5 min or 400 MPa for 5 min. Treatments at 2, 10, and 25°C with 400 MPa for 5 min or at 50°C with 300 MPa for 5 min reduced *P. fluorescens* by at least 6.5 and 7.3 log cycles, respectively. These levels of reduction were as good as in some thermal treatment (22).

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