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EFFECT OF CONCURRENT HIGH HYDROSTATIC PRESSURE, ACIDITY AND HEAT ON THE INJURY AND DESTRUCTION OF *LISTERIA MONOCYTOGENES*

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ABSTRACT

The effect of concurrent use of high hydrostatic pressure, heat and acidity on Listeria monocytogenes Scott A and CA was investigated. In general, lethality was enhanced when cells were pressurized at higher temperatures or lower pH. Strain CA demonstrated an additional 3-log₁₀ reduction when pressurized at pH 4.0 as compared with pH 6.0 at 353 MPa, 45C for 10 min. Scott A was reduced an additional 1 log₁₀ by increasing the temperature from 25C to 45C with pressurization at 252 MPa, pH 6.0 for 30 min. Exposure to 404 MPa at 45C for 30 min demonstrated complete injury or death of CA cells with an initial concentration of >10⁸ CFU/mL. At least an 8-log₁₀ reduction was observed for both L. monocytogenes strains Scott A and CA when exposed to the combined treatments of 252 MPa, 45C, pH 4.0 for 30 min.

INTRODUCTION

Hydrostatic pressure can have a disruptive effect on living organisms. Pressures in the kilobar range can result in the loss of viability of cells through physiological or biochemical effects (Marquis 1976; Chong and Cossins 1983; Hoover 1993). First examined as a food processing method in 1899, renewed interest has been generated to incorporate hydrostatic pressure as a food processing method to lessen or eliminate thermal processing or the use of chemical preservatives in some products.

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Food processing and preservation using combined methods involves applying hurdle technology so that microbiological safety and stability of foods results from several factors acting sequentially or concurrently to inhibit the growth of undesirable microorganisms (Leistner 1992). If the factors effect different targets within a cell, then each factor contributes to disruption of cellular homeostasis. The application of several different hurdles to processing or preservation of food often allow for the development of foods with less process damage and greater consumer appeal.

Concurrent exposure to high hydrostatic pressure (HHP), heat and mild acidity for yeast was described by Pandya *et al.* (1995). Increasing the temperature during pressurization was shown to reduce the pressure requirements for yeast inactivation. Both *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* with initial concentrations of 1×10^8 cfu/mL were completely inactivated with 10-min pressurizations at 304 MPa and 25C at pH 4.0. Decreasing the pressure to 253 MPa and raising the temperature to 45C achieved the same effect. The effect of HHP in conjunction with pH reduction on *Listeria monocytogenes* was reported by Mackay *et al.* (1995). Reducing the suspension buffer pH from 7.1 to 5.3 resulted in progressive enhancement of lethality from HHP at 304 MPa by up to 1.8 log₁₀ cfu/mL. The effect of HHP at 23C on suspensions of *L. monocytogenes* Scott A and CA in pH 7.0 buffer and milk was reported by Styles *et al.* (1991). Exposure to 343 MPa in buffer produced a 7-log₁₀ reduction in Scott A, while the same exposure to cells suspended in milk yielded a 3-log₁₀ reduction. In this study, we determined the degree of injury and inactivation to *L. monocytogenes* Scott A and CA exposed to concurrent high hydrostatic pressure, mild heat and acidity.

MATERIALS AND METHODS

Cultures and Media

The two strains of *L. monocytogenes* studied were Scott A (serotype 4b; from R. Twedt, FDA, Cincinnati, OH) and CA (serotype 4b; from E. Marth, University of Wisconsin, Madison). Cells were cultured in Tryptic soy broth (Difco, St. Louis, MO) plus 0.6% yeast extract (Difco; TSBY) at 35C for 18 h. Cells were harvested by centrifugation at 6,000 x g for 10 min and resuspended in phosphate-buffered saline (PBS; 0.1 M dibasic anhydrous sodium phosphate, 0.1 M monobasic sodium phosphate monohydrate, plus 0.85% w/v NaCl; Kaplan and Colowick 1955). Sample dilutions were performed by serial dilutions in PBS. Working cultures were maintained on slants of Tryptic soy agar plus 0.6% yeast extract (TSAY) and were held at 2C for up to 30 days (Farber and Pagotto 1992).

Cells used for sample trials were twice subcultured. The first inoculum was from an agar slant into 9 mL TSBY. Cultures were grown overnight and 2 mL was used to inoculate 200 mL of TSBY in 250-mL screw-capped centrifuge bottles for

a second overnight culture. Cells were harvested by centrifugation, washed in 100 mL PBS, reharvested and suspended in 20 mL PBS. These washed cells were stored at 4C for use within 6 h.

The nonselective plating medium was TSAY. Trypticase phosphate agar (TPA; Difco) was supplemented with 0 to 8% w/v NaCl (TPAN) to determine the optimal salt level to distinguish injury. Control and pressure-treated cell suspensions (202 MPa, 45C, pH 5.2 and 6.0, 10 min) were plated on each medium. The criterion for selection of the optimal salt level was to define the salt concentration that suppressed the nonpressurized cells minimally while suppressing the pressurized cells maximally, relative to the nonsupplemented TPA (Crawford *et al.* 1989). TPAN achieved differentiation via both reduced water activity ($a_w=0.973$) and intolerance of injured cells to salt (Busch and Donnelly 1992). Maximum recovery of injured cells was achieved with *Listeria* recovery agar (LRA), a medium designed to promote recovery of stressed and injured cells of *L. monocytogenes* from processed food products (Busch and Donnelly 1992). Thus, two media systems were used to determine injury; comparison of growth after pressurization on TPAN and TSAY and growth on TSAY and LRA.

Injury was determined by pressurizing cells in isotonic nutrient-free phosphate-buffered citrate (PBC; 0.1 M citric acid, 0.2 M dibasic sodium phosphate; Kaplan and Colowick 1955) and then plating on repair medium (LRA), standard growth medium (TSAY) and selective medium (TPAN). Injury was calculated as the percent difference in surviving cells observable on TSAY and TPAN or LRA and TSAY as:

$$\% \text{ Injury} = 1 - \left[\frac{\text{(Selective medium counts)}}{\text{LRA counts}} \right] \times 100\%$$

based on the method of Meyer and Donnelly (1992).

High Pressure Treatments

Experimental samples were aseptically transferred to polyester film pouches (Kapak Corp., Minneapolis, MN), heat-sealed and pressurized at various time (10 or 30 min), temperature (25 or 45C), pH (4.0, 4.6, 5.2 or 6.0) and pressure (151 to 404 MPa) combinations using an isostatic press (Autoclave Engineers, Erie, PA) with a maximum operating pressure of 412 MPa. After removal from the chamber, samples from trials conducted at 45C were immediately cooled in a 20C waterbath. Following HHP, the sample pouches were sanitized with disinfectant solution and aseptically cut open. The cell suspensions were serially diluted and plated in duplicate on the appropriate medium with a Spiral Plater (Model C, Spiral Systems,

Inc.; Cincinnati, OH) (Chain and Fung 1991) and incubated at 35C. Plates were read on a Laser Counter (Model 500 A, Exotech, Inc.; Gaithersburg, MD). All experiments were replicated on separate dates.

The effect of exposing *L. monocytogenes* to acidic buffer at 45C without pressure was determined by suspending the cells in pH 4.0 and 6.0 PBC. These samples were then held at 45C in a circulating waterbath for 30 min, removed to a 20C waterbath, cooled to 25C and plated.

RESULTS AND DISCUSSION

HHP Lethality

The effect of exposing cells to the most severe combinations of the nonpressure parameters (pH 4.0, 45C, 30 min) had little or no effect on the survival of *L. monocytogenes* Scott A or CA (data not shown). TPAN counts were reduced 0.8 log₁₀ cfu/mL for CA and 0.9 log₁₀ cfu/mL for Scott A. All other plate count differences were < 0.5 log₁₀.

Work done by Styles *et al.* (1991) suggested that exposures to 151 MPa at ambient temperature had little effect on *L. monocytogenes* suspended in medium of neutral pH. In the present study, the lethality of strains Scott A and CA was slight when cells were pressurized at 151 MPa at 45C for 10 and 30 min in PBC at pH 4.0, 4.6, 5.2 and 6.0 (Fig. 1A). The greatest reduction was seen with cells pressurized at pH 4.0. Cell suspensions of Scott A were reduced by approximately 2 log₁₀ after 10 min and 2.9 log₁₀ after 30 min. CA populations declined approximately 1.8 log₁₀ after 10-min exposures and 2.4 log₁₀ after 30-min exposures. A trend of increasing lethality with increased acidity and pressure-treatment time was observed with both strains.

Lethality was enhanced for both strains by increasing the pressurization treatment to 202 MPa at 45C (Fig. 1B). Pressurization for 10 min gave similar results for each pH examined, with reductions of approximately 1.5 log₁₀ cfu/mL. Both Scott A and CA plate counts were reduced by more than 3 log₁₀ cfu/mL when pressurized for 30 min at pH 4.0. CA cells pressurized at pH 4.6 or 5.2 were reduced by approximately 2.5 log₁₀ and at pH 6.0, 1.5-log₁₀ reductions were observed. Similar results were seen with Scott A samples.

Increasing the pressure to 252 MPa at 25C (Fig. 1C) increased lethality for samples in pH 4.0 buffer. A 30-min exposure reduced CA samples 4.0-log₁₀ cfu/mL and Scott A by more than 5 log₁₀. Results from both strains pressurized at pH 4.6, 5.2, or 6.0 were similar to results seen with pressurization at 202 MPa at 25C for 30 min. Increasing the temperature to 45C and pressurizing at 252 MPa for 30-min at pH 4.0 completely inactivated both strains with initial populations of greater than 1 × 10⁸ cfu/mL (Fig. 1D). The cell suspensions pressurized at 252 MPa and the three other pH values gave lethality results at 45C that were within 1 log₁₀ of the 25C results for CA and Scott A strains.

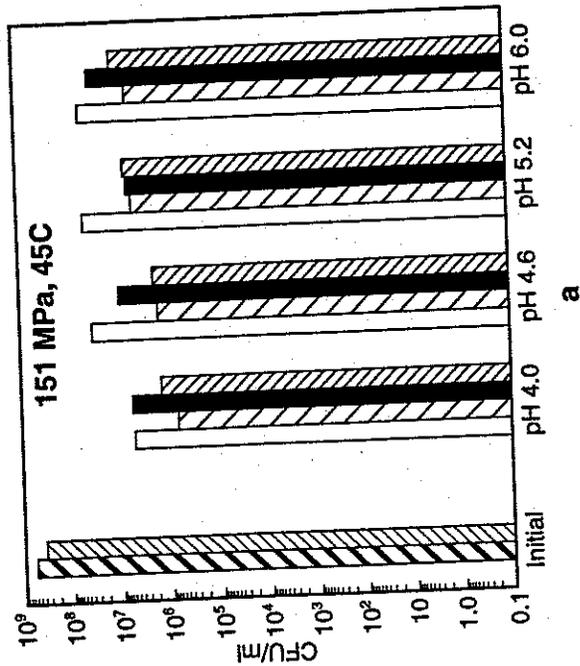
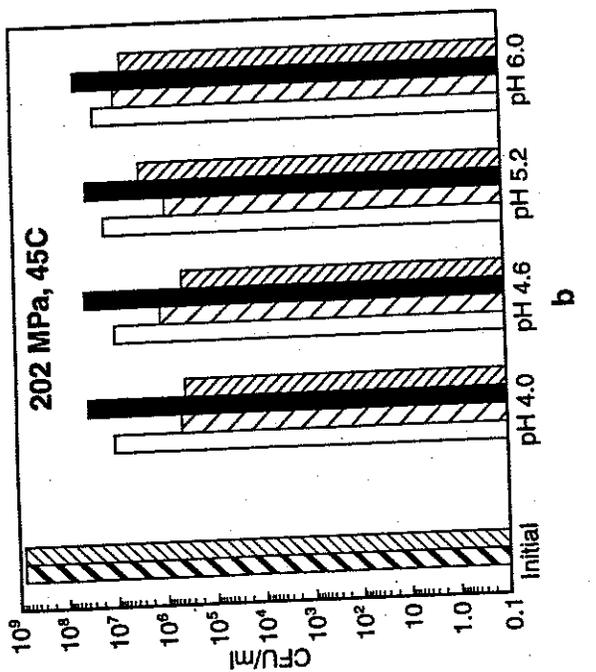
Pressurization of samples at 303 MPa, 25C (Fig. 2A) for 10 min at pH 4.0 reduced the CA population by 5.5- \log_{10} cfu/mL and the Scott A samples by 5.0 \log_{10} . Pressurization at all other pH values reduced both strains by approximately 3.0 \log_{10} . Increasing pressurization time to 30 min enhanced lethality, especially for samples pressurized at pH 4.0. Sterility was achieved for both CA and Scott A samples with initial populations of greater than 1×10^8 cfu/mL. Lethality decreased with an increase in pH. Pressurization for 30 min at pH 6.0 resulted in only a 3.0- \log_{10} reduction in cfu/mL for both strains.

When temperature was increased to 45C during pressurization at 303 MPa, sterility was again achieved for CA and Scott A strains when treated for 30 min at pH 4.0 (Fig. 2B). However, the results from several of the buffer suspensions showed a small decrease in lethality with higher temperature. For strain CA, exposure for 10 min to 45C at pH 4.0 decreased cfu/mL by 4.5 \log_{10} and at 25C by 5.5 \log_{10} . For Scott A this effect was seen at pH 4.6 and 5.2 and 30-min pressurization. At 25C, samples were reduced 6.0 \log_{10} and 5.0 \log_{10} , respectively. When pressurized at 45C, samples were reduced by only 4.0 \log_{10} at both pH values. Lethality observed with other time and pH combinations were within 1.0 \log_{10} cfu/mL when pressurized at either 25 or 45C.

When compared to the results at 303 MPa, the lethalties observed for pressurization at 353 MPa (25C) were enhanced for both CA and Scott A strains (Fig. 2C). After 10 min at pH 4.0, 7.0- \log_{10} reductions in cfu/mL were achieved for the CA samples and 6.5- \log_{10} reductions were observed for the Scott A samples. This shows approximately a 1.5- \log_{10} cfu/mL increase in lethality from the additional 50 MPa pressure at pH 4.0 for both strains. Sterilization was achieved after 30 min at pH 4.0 for CA and Scott A, and for CA after 30 min at pH 4.6 from an initial population of 5×10^8 cfu/mL. Pressurization of Scott A samples for 30 min at pH 4.6 resulted in 6.5- \log_{10} reductions. Treatments at pH 5.2 and 6.0 at 353 MPa showed greater reductions in cfu/mL for both strains by as much as 2.5 \log_{10} as compared to results at 303 MPa.

Pressurization at 404 MPa (25C) at pH 4.0 for 10 min achieved sterility for both strains with initial populations for $>1 \times 10^8$ cfu/mL (Fig. 2D). Pressurization at pH 4.6 reduced CA by 7.0 \log_{10} and Scott A by 6.0 \log_{10} . CA samples were reduced 6.0 \log_{10} at pH 6.0 while Scott A was only reduced by 4.0 \log_{10} at pH 6.0. Sterility was achieved for both strains at all four pHs when pressurization-exposure time was increased to 30 min.

Sterilization was achieved for both strains of *L. monocytogenes* at pH 4.0 and 4.6 after pressurization at 404 MPa (45C) for 10 and 30 min (data not shown). There were 6- \log_{10} reductions in cfu/mL for CA at pH 5.2 after 10 min at 45C, with sterility being achieved after 30-min exposures to the same parameters. Reductions of approximately 4.5- \log_{10} were achieved at pH 6.0



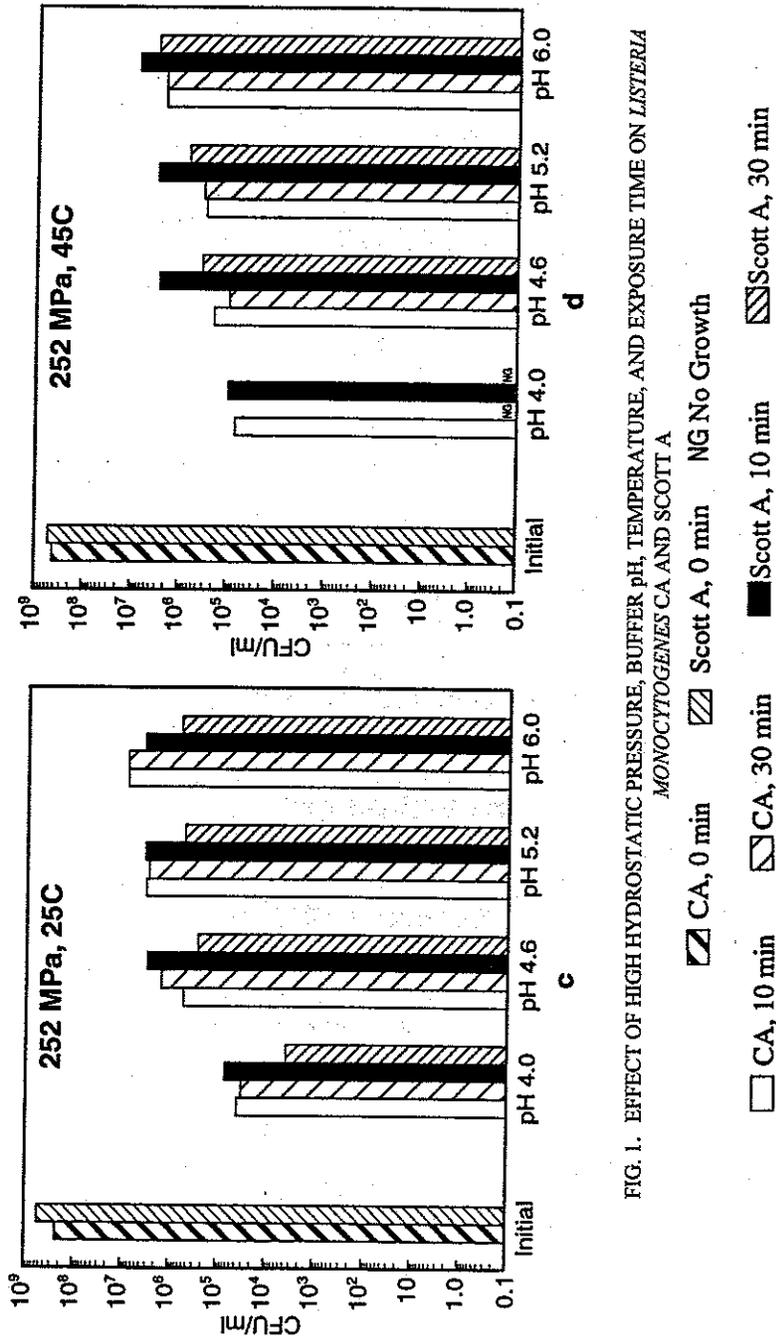
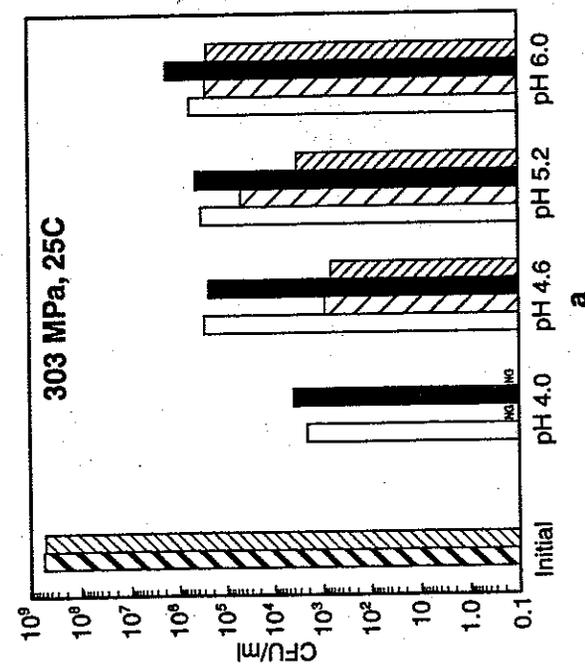
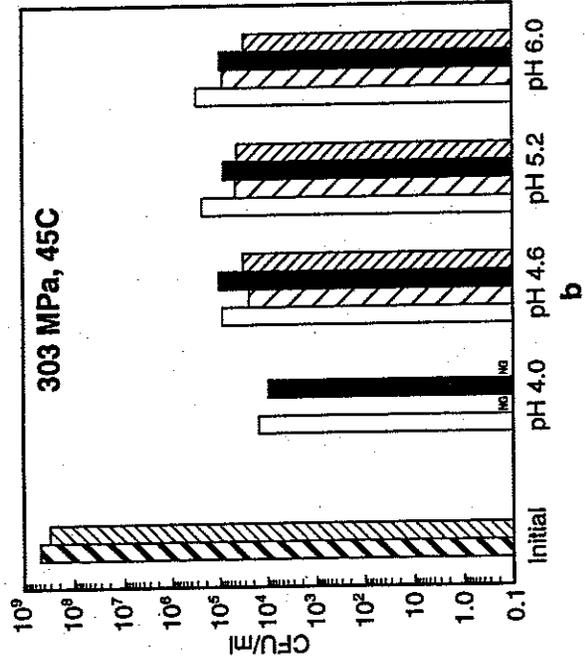


FIG. 1. EFFECT OF HIGH HYDROSTATIC PRESSURE, BUFFER pH, TEMPERATURE, AND EXPOSURE TIME ON *LISTERIA MONOCYTOGENES* CA AND SCOTT A



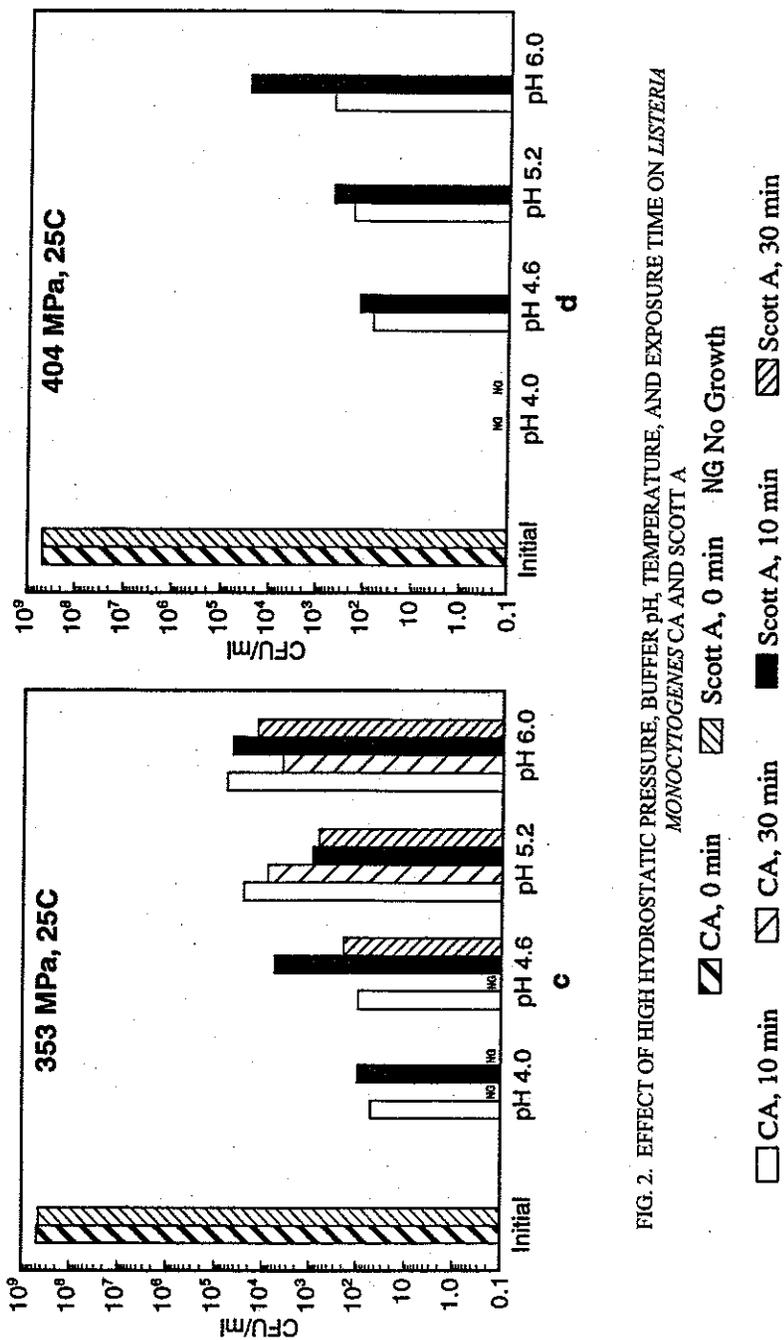


FIG. 2. EFFECT OF HIGH HYDROSTATIC PRESSURE, BUFFER pH, TEMPERATURE, AND EXPOSURE TIME ON *LISTERIA MONOCYTOGENES* CA AND SCOTT A

CA, 0 min Scott A, 0 min NG No Growth

CA, 10 min CA, 30 min Scott A, 10 min Scott A, 30 min

after 10-min exposures, with no survivors being observed after 30-min exposures. Sterility was achieved when the Scott A samples were pressurized at pH 5.2 for 10 or 30 min. When pressurized at pH 6.0, reductions of approximately 4-log_{10} were seen after 10-min pressurizations at 404 MPa and 45C. The 30-min exposures gave 7.0-log_{10} reductions.

This study has indicated that HHP applied in conjunction with mild heat and acidity can be an effective method for inactivating *L. monocytogenes* in buffer systems. For example, the combined effect of 252 MPa at 45C at pH 4.0 sterilized suspensions of *L. monocytogenes* strains CA and Scott A from an initial concentration of greater than 5×10^8 cfu/mL. Recent work by Patterson *et al.* (1995) showed similar findings, including 1×10^4 cfu/mL reductions at 374 MPa after 15 min at 20C in phosphate buffer (pH 7.0). The authors reported a protective effect against pressure for cells in milk, but not in poultry meat. Styles *et al.* (1991) reported similar findings of protection from milk, but not in clam juice. This work has shown that the lethal effect of HHP on *L. monocytogenes* can be enhanced by reducing the pH of the suspension menstra and/or increasing the temperature during pressurization.

HHP Injury

The optimal salt level to distinguish injury was determined using TPA amended with NaCl. The concentration of NaCl with the greatest degree of injury and with little effect on unpressurized cells was observed with TPA supplemented with 4% salt (TPAN). All unpressurized counts on this medium remained within 1.0log_{10} cfu/mL of the counts on TPA. These findings on salt levels inhibiting injured CA cells were in agreement with literature on salt-supplemented media for enumerating *L. monocytogenes* injured by other forms of stress (Dallimier and Martin 1988; Busch and Donnelly 1992; Meyer and Donnelly 1992).

Pressurized cells showed approximately 10 times greater growth on LRA as compared to TSAY. LRA was designed to provide enhanced recovery of injured *L. monocytogenes* cells. Therefore, pressure-induced injury of *L. monocytogenes* CA was examined by comparing growth on TPAN, TSAY, and LRA.

The injury data after 10 min at 353 MPa and 45C (Fig. 3A) showed that at pH 6.0 an initial concentration of 7×10^8 cfu/mL was reduced by approximately 5log_{10} when plated on TPAN and by 3-log_{10} when using TSAY. This corresponds to an injury rate of 99.4%. Results obtained with LRA showed that there was a 2.5-log_{10} reduction, corresponding to a 86.4% rate of injury. The same conditions, except at pH 5.2 showed no survivors on TPAN as compared to a 5.5-log_{10} reduction in cfu/mL when plated on TSAY (100% injury). Counts on LRA were reduced by 3-log_{10} demonstrating an 88.3% injury rate as compared with growth on TSAY.

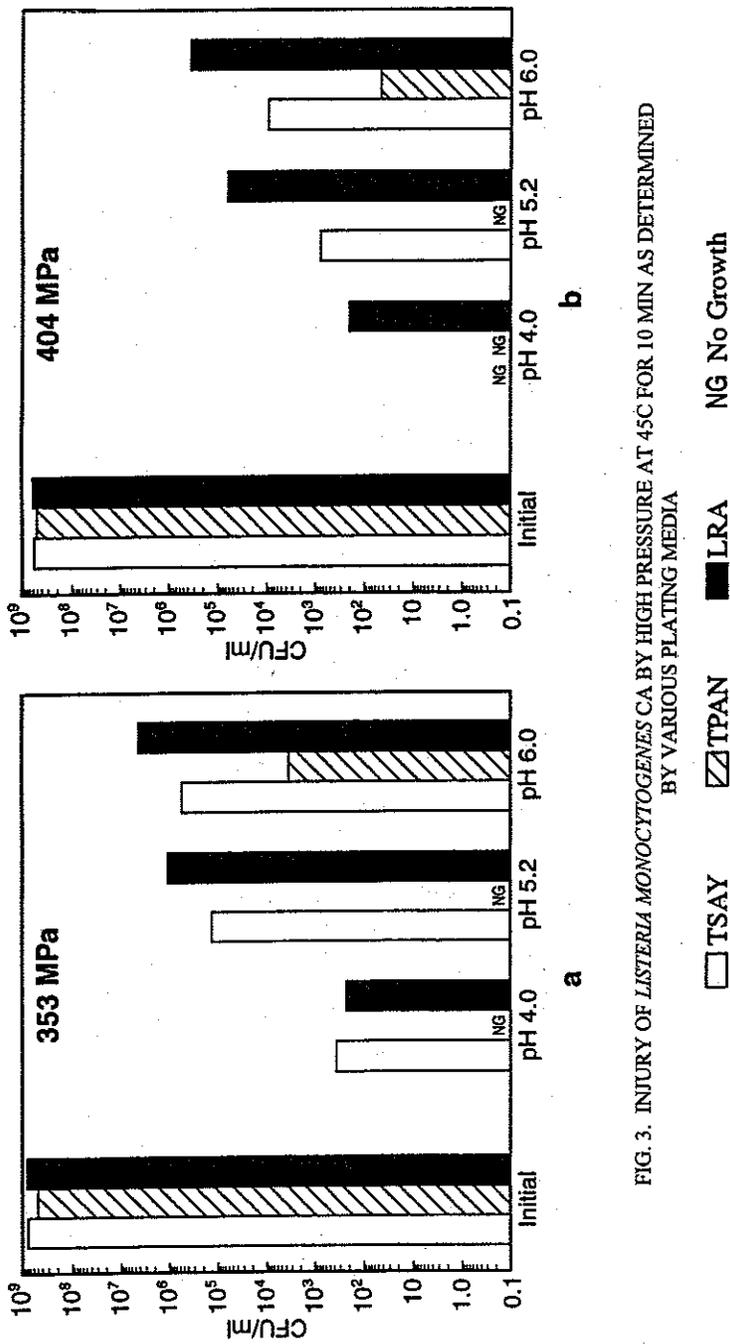


FIG. 3. INJURY OF *LISTERIA MONOCYTOGENES* CA BY HIGH PRESSURE AT 45°C FOR 10 MIN AS DETERMINED BY VARIOUS PLATING MEDIA

Increasing the pressure from 353 to 404 MPa increased the level of injury (Fig. 3B). The results after treatment at 10 min (pH 6.0) showed a 7-, 5-, and 3.5- \log_{10} reduction in numbers (cfu/mL) when plated on TPAN, TSAY, and LRA, respectively. The percentage of injury was 99.5% when plated on TPAN compared to TSAY counts and 97.4% for TSAY compared to LRA counts. At pH 5.2 the percentage of injury was 100% when plated on TPAN as compared to growth on TSAY. Bacterial counts on LRA were reduced by 4- \log_{10} at pH 5.2 while those on TSAY demonstrated a 6- \log_{10} reduction (98.8% injury). By decreasing the pH to 4.0, injury was increased to 100% for both media.

The results obtained after subjecting *L. monocytogenes* CA to 30 min at 404 MPa and 45C showed no survivors on TPAN or TSAY at any of the three pH levels tested (data not shown). LRA counts were reduced 4.5, 6, and 8 \log_{10} (0 cfu/mL) when samples were pressurized at pH 6.0, 5.2 and 4.0, respectively. This demonstrates that all cells in the population were injured, or in the case of pressurization at pH 4.0, effective elimination of all cells. No *L. monocytogenes* CA cells treated at 353 MPa or higher, suspended in PBC buffer with a pH below 6.0, were capable of growth on TPAN. Bailly *et al.* (1990) reported that heat-injured *L. monocytogenes* was incapable of growth in acidified media of pH lower than 5.2.

Injury to *L. monocytogenes* was observed to be as great as 5.0 \log_{10} (>99% injured) when comparing counts on TPAN and TSAY, and greater than 6.0 \log_{10} when comparing counts on TPAN to LRA after treatments of 353 MPa for 10 min at 45C. In general, cells observed to survive on TSAY or LRA after HHP at 353 MPa or greater for at least 10 min in PBC buffer at a pH value of 5.2 or lower were injured.

These injury data are applicable to the food industry as it considers the use of HHP as an industrial method of food processing. Foods harboring *L. monocytogenes* include foods of the low to mid-acid categories (\geq pH 4.5) intended for refrigerated distribution. Many of these foods could receive a high level of microbial inactivation with minimal process severity to the food by the application of HHP with mild heat.

ACKNOWLEDGMENTS

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