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Short communication

Viability loss and morphology change of foodborne pathogens following exposure to hydrostatic pressures in the presence and absence of bacteriocins

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Abstract

Cell suspensions of three pathogens were exposed to hydrostatic pressure (HP), bacteriocin mixture (nisin and pediocin) or a combination of HP + bacteriocins and changes in colony forming units (cfu) and cell-morphology by scanning electron microscopy (SEM) were studied. Cell viability loss, as determined from the reduction in cfu before and after a treatment, occurred in *Listeria monocytogenes* by all three treatments and in *Salmonella typhimurium* and *Escherichia coli* O157:H7 by HP and HP + bacteriocin combination. Cell wall and cell membrane collapse and cell lysis was indicated in *L. monocytogenes* exposed to bacteriocin or HP + bacteriocin and in *Salmonella* and *E. coli* exposed to HP or HP + bacteriocin.

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1. Introduction

Hydrostatic pressure (HP) above 200 MPa is being studied as an innovative food processing and preservation method due to its ability to kill and injure microbial cells (Kalchayanand et al., 1994; Farkas and Hoover, 2000; Ray et al., 2001a,b; Ray, 2002). Death and injury of microorganisms by HP has been theorized to be associated with the destabilization of structural and functional integrity of different macromolecules, especially in the wall and membrane of

cells. Volume compression and phase transition of cellular macromolecules during pressurization result in the disruption of H-bonds, ionic bonds and hydrophobic interactions of the macromolecules and adversely affects their structures and functions (Hoover, 1993). Results of several studies have indicated that wall, membrane and several enzymes and nucleic acids of the microbial cells could be adversely affected by HP treatment (Hoover, 1993; Smelt, 1998; Farkas and Hoover, 2000; Ray et al., 2001a,b; Ray, 2002). Kato and Hayashi (1999) showed that HP induced a phase transition of the lipid bilayer membrane, shifting the natural crystalline phase to an initial reversible gel-phase and finally to an irreversible integrated phase, as well as reduced thickness of

the bilayers. Such a change could cause loss in membrane permeability along with losses in transmembrane proton gradient, proton motive force and ATP synthesis in HP-treated microbial cells and produce cell injury and death. A recent study reported that HP inflicted wall and membrane damage in *Leuconostoc mesenteroides* Ly cells and activated an inducible autolysin that cause extensive degradation of the cell wall and cell membrane (Kalchayanand et al., 2002).

Several studies have shown that a combination of HP and bacteriocins of lactic acid bacteria have synergistic effect on cell death of Gram-positive and Gram-negative bacteria. This could be due to their damaging effect on the cell wall and cell membrane in the sensitive bacteria together with an increased sensitivity of the cells, injured by pressurization, to bacteriocins (Kalchayanand et al., 1992, 1994, 1998a,b; Huben et al., 1996; Alpas et al., 1999). It has also been demonstrated that pediocin AcH and nisin together have synergistic bactericidal effect against many species of Gram-positive bacteria, and when they are used during pressurization, the bacterial cell death is greatly enhanced (Hanlin et al., 1993; Bennik et al., 1997; Kalchayanand et al., 1998a,b; Mulet-Powell et al., 1998). At present, very little information is available on the morphological changes that occur associated with the cell death of foodborne pathogens following exposure to HP and bacteriocins. We determined viability loss and related morphological changes following exposure of three foodborne pathogens to HP in the presence and absence of pediocin AcH and nisin and report the results here.

2. Materials and methods

2.1. Growth and sample preparation of three pathogens

Three pathogens used from our collection were *Listeria monocytogenes* Scott A, *Escherichia coli* O157:H7 and *Salmonella typhimurium* MH 2413. These cells were grown separately in tryptic soy broth (Difco, MI), supplemented with 0.6% yeast extract (TSY-broth) at 37 °C for 4 h to exponential phase, harvested by centrifugation and resuspended in fresh

TSY broth to about 10^9 colony forming units (cfu)/ml and used for further studies. The studies involved measurement of optical density (OD) at 600 nm spectrophotometrically, enumeration of cfu/ml and scanning electron microscopy (SEM) of cells before and after treatment with either a mixture of two bacteriocins or HP or a combination of HP and bacteriocins by the methods described below. These results were analyzed to determine cell lysis, viability loss and changes in cell morphology by the three treatment methods. In an initial study, for a comparison, cell suspensions of a Gram-positive spoilage bacterial strain, *L. mesenteroides* Ly, and the pathogen *L. monocytogenes* Scott A were exposed to the two bacteriocins, pediocin AcH and nisin A and examined for changes in OD_{600 nm} during incubation for 120 min at 25 °C. *L. mesenteroides* Ly has been shown to lyse by both bacteriocins (Bhunja et al., 1991; Kalchayanand et al., 1992).

2.2. Bacteriocin preparation and treatment

The two lactic acid bacterial strains, *Lactococcus lactis* subspecies *lactis* ATCC 11454 and *Pediococcus acidilactici* LB42-923, from our stock, were used to produce nisin A and pediocin AcH, respectively (Biswas et al., 1991; Yang et al., 1992). The strains were grown separately in trypticase yeast extract glucose (TCG) broth in a fermentor for 16–18 h at 30 °C (for nisin) or 37 °C (for pediocin) by the method of Yang et al. (1992). Following fermentation, the culture broths were heated at 75 °C for 15 min to kill the cells and inactivate the proteolytic enzymes. Partially purified nisin and pediocin were obtained by the pH-induced adsorption–desorption of the molecules on the surface of the producer cells, dialyzed and freeze-dried by the method developed by Yang et al. (1992) and described in a recent publication (Kalchayanand et al., 2002). The potency of each dried preparation was assayed against *Lactobacillus plantarum* NCDO955 and expressed as activity units (AU)/mg of dry preparations and stored at –20 °C (Biswas et al., 1991; Yang et al., 1992). Prior to an experiment, the required amounts of the dried preparations were dissolved in sterile deionized water, assayed for AU/ml and added to cell suspension either separately or as a mixture according to the need. To prepare a mixture, solutions of nisin and

pediocin, each in equal potency, were added at 7:3 ratio. The cell suspensions were incubated for 120 min to determine cell lysis and viability loss.

2.3. Pressurization of pathogens

Cell suspensions of the three pathogens were transferred in 2-ml portions in 2-ml capacity cryovials (Simport Plastic, Beloell, Canada). In the samples to be pressurized in the presence of bacteriocin, nisin and pediocin mixture at a 7:3 ratio were added to cell suspensions to a final concentration of 3000 AU/ml and transferred to cryovials in 2-ml portions. The cell suspensions in cryovials, in duplicate for each treatment for each pathogen, were pressurized at 345 MPa for 5 min at 25 °C in a hydrostatic pressure unit (Engineered Pressure System, Andover, MA; 4 in. ID and 14 in. high-pressure chamber). The pressure come-up time (about 3 min) and pressure come-down time (about 1 min) and adiabatic heating (about 3 °C/100 MPa) were not considered in the measurements. The pressurized cell suspension from each vial were examined for changes in OD_{600 nm} and cfu/ml in TSY-agar medium immediately after pressurization and following 120 min incubation at 25 °C to determine cell lysis and viability loss.

2.4. Determination of cell lysis and viability loss

The cell suspensions of each pathogen were used to prepare four sets of samples as follows: untreated control, treated with bacteriocin mixture, treated with HP alone and treated with the combination of bacteriocin and HP.

At specified times, each set is examined for changes in OD_{600 nm} in a spectrophotometer and enumerated for cfu/ml. Enumeration was done by pour plating original suspensions or serially diluting aliquots with TSY-agar medium and incubating the agar plates at 37 °C for 2d. The OD_{600 nm} and cfu/ml of the treated samples were compared with the respective untreated controls. A reduction in OD following a treatment was regarded as an indication of cell lysis. Similarly, a reduction in cfu/ml following treatment was regarded as cell viability loss (Bhunia et al., 1991; Kalchayanand et al., 1994, 1998a,b, 2002).

2.5. SEM

The control and treated cells of each pathogen were examined by SEM to visualize any morphological change that occurred in the cell following exposure to bacteriocins and pressure. The methods used for SEM were slightly different from that described in a previous publication and briefly described here (Kalchayanand et al., 2002). The cell suspensions were fixed with 3% glutaraldehyde in Na-cacodylate buffer (100 mM, pH 7.1) and sent by overnight express to the Control Microscopy Research Facility at the University of Iowa. There, the cells were pelleted, washed to remove glutaraldehyde and resuspended in the same buffer. A drop from each suspension was transferred to a poly-L-lysine-treated silicon wafer chips, which were kept for 30 min in a hydrated chamber for the cells to adhere. The attached cells were post fixed by immersing the chips in 1% osmium tetroxide (OsO₄) in cacodylate buffer for 30 min, rinsed in the same buffer and dehydrated in ethanol in ascending concentrations (%) of: 50, 70, 95 (2 ×) and 100 (2 ×), for 10 min each. The chips were mounted on aluminum stubs and coated with gold-palladium in a sputter coater (Emitech K550, Ashford, Kent, England). The chips were viewed at 3 kV accelerating voltage in a Hitachi S-4000 field emission scanning electron microscope (Hitachi Inst., San Jose, CA) and secondary electron image of cells for topography contrast were collected at several magnifications.

3. Results and discussion

3.1. Cell lysis by pediocin AcH and nisin

An initial study was conducted to determine the relative ability of the two bacteriocins (pediocin AcH and nisin) to lyse the cells of the pathogens, as determined by a reduction in the OD. The nonpathogenic *L. mesenteroides* Ly cells, which are known to be lysed by both bacteriocins at a relatively low concentration (1280 AU/ml), were used as control. The results for the two Gram-positive bacteria are presented in Table 1. The two Gram-negative pathogens, which are normally not sensitive to the two bacteriocins, were not included. During 120-min incubation with either pediocin or nisin, the OD for

Table 1
Reduction in optical density (OD_{600 nm}) of two bacterial strains following treatment with pediocin AcH and nisin A

Bacterial strain	Bacteriocin used ^a	Incubation time (min)	OD _{600 nm} (%)
<i>L. mesenteroides</i> Ly	Pediocin	0	0.23 (100) ^b
	AcH	120	0.02 (9)
	Nisin A	0	0.22 (100)
		120	0.02 (9)
<i>L. monocytogenes</i> Scott A	Pediocin	0	0.28 (100)
	AcH	120	0.21 (75)
	Nisin A	0	0.28 (100)
		120	0.01 (4)

^a The exponentially growing cells were exposed to the bacteriocins (1280 AU/ml) and examined for changes in OD during incubation at 25 °C.

^b Initial OD_{600 nm} was 100%.

L. mesenteroides reduced to 9% of the original. It has been shown before that such a reduction in OD is associated with lysis of the cells (Bhunja et al., 1991; Kalchayanand et al., 2002). The OD of *L. monocytogenes* Scott A cells, under identical conditions, was reduced to 75% by pediocin and 4% nisin. This is due to differences in potency between the two bacteriocins toward growing and non-growing cells. While nisin is extremely potent against growing cells, pediocin is effective against both growing and non-growing cells of Gram-positive bacteria (Motlogh et al., 1992; Ray et al., 2001a,b). This is one of the reasons why the two together have synergistic bacte-

ricidal effect against many pathogenic and spoilage bacteria (Hanlin et al., 1993; Bennik et al., 1997; Mulet-Powell et al., 1998).

3.2. Changes in OD and cfu of the three pathogens

The cell suspensions of the three pathogens in TSY broth were treated with either a mixture of two bacteriocins (pediocin and nisin) or pressurized with or without the bacteriocin mixture and incubated at 25 °C up to 120 min. The OD and cfu/ml were determined initially and after the end of incubation and compared with the respective controls (Table 2). The OD and cfu in the controls increased during 120-min incubation, suggesting growth of all three pathogens. During incubation with the bacteriocin mixture, the Gram-positive *L. monocytogenes* had a reduction in both OD (from 0.24 to 0.18) and cfu/ml (from 9.53 to 7.11 log cycles), indicating cell lysis and viability loss, respectively. In contrast, the two Gram-negatives showed an increase in OD and cfu/ml due to growth. This is because the Gram-negative under normal conditions are resistant to bacteriocins of lactic acid bacteria, including pediocin and nisin (Ray et al., 2001a,b). Pressurization alone at 345 MPa for 5 min at 25 °C did not change the OD of *L. monocytogenes*, but reduced it in *E. coli* and *Salmonella* strains, especially after 120-min incubation. The cfu/ml was reduced by about 1 log cycle but by over 7 log cycles in the two Gram-negatives. Previous studies also have

Table 2

Changes in optical density (OD_{600 nm}) and log₁₀ colony forming units (cfu/ml) of three pathogens following treatment with either bacteriocins (B) or hydrostatic pressure (HP) or their combination

Pathogens (strains)	Min at 25 °C ^a	Control ^b		Bacteriocin ^c		HP		HP + B	
		OD	cfu/ml	OD	cfu/ml	OD	cfu/ml	OD	cfu/ml
<i>L. monocytogenes</i> (Scott A)	0	0.24	9.53	NT ^d	NT	0.27	8.40	0.27	3.00
	120	0.62	10.60	0.18	7.11	0.24	8.20	0.19	1.86
<i>E. coli</i> O157:H7 (932)	0	0.54	8.20	NT	NT	0.49	1.34	0.51	ND ^e
	120	0.80	9.30	0.72	9.28	0.32	0.90	0.28	ND
<i>S. typhimurium</i> (MH2413)	0	0.42	8.90	NT	NT	0.36	1.00	0.38	ND
	120	0.56	9.20	0.51	9.20	0.30	0.90	0.34	ND

^a Minutes incubated at 25 °C following a treatment with bacteriocin mixture, HP or HP + B.

^b cfu/ml in log₁₀, each data is an average of two samples. Control cells multiplied during 2 h incubation at 25 °C as evidenced from these increase in OD and cfu/ml.

^c Bacteriocins used were a mixture of pediocin AcH and nisin A in 3:7 ratio to a final concentration of 3000 AU/ml.

^d NT: not tested, since they are same as the respective controls.

^e ND: no cfu was detected in 2 ml of undiluted cell suspensions.

reported a greater viability loss of Gram-negative bacteria than Gram-positive bacteria following pressurization under similar conditions (Farkas and Hoover, 2000; Ray et al., 2001a,b; Ray, 2002). Pressurization in the presence of the bacteriocin mixture and subsequent incubation reduced the OD in all three pathogens. The cfu/ml was reduced by over 6–7 log cycles in *L. monocytogenes* as compared to 1–2 log cycles either by pressurization alone or bacteriocin treatment alone. No cfu for both Gram-negatives was detected in 2-ml original cell suspension. This is because pressurization inflicts sublethal

injury in the cell wall and cell membrane of Gram-positive and Gram-negative survivors, which become susceptible to the bacteriocins (Kalchayanand et al., 1992). Similar results have been reported by other researchers (Kalchayanand et al., 1994).

3.3. Scanning electron micrographs of the pathogens

The SEM-generated photomicrographs of the control and treated cells of the three pathogens, presented in Figs. 1–3, show the changes in cell morphology and topography. The control *Listeria* cells have nor-

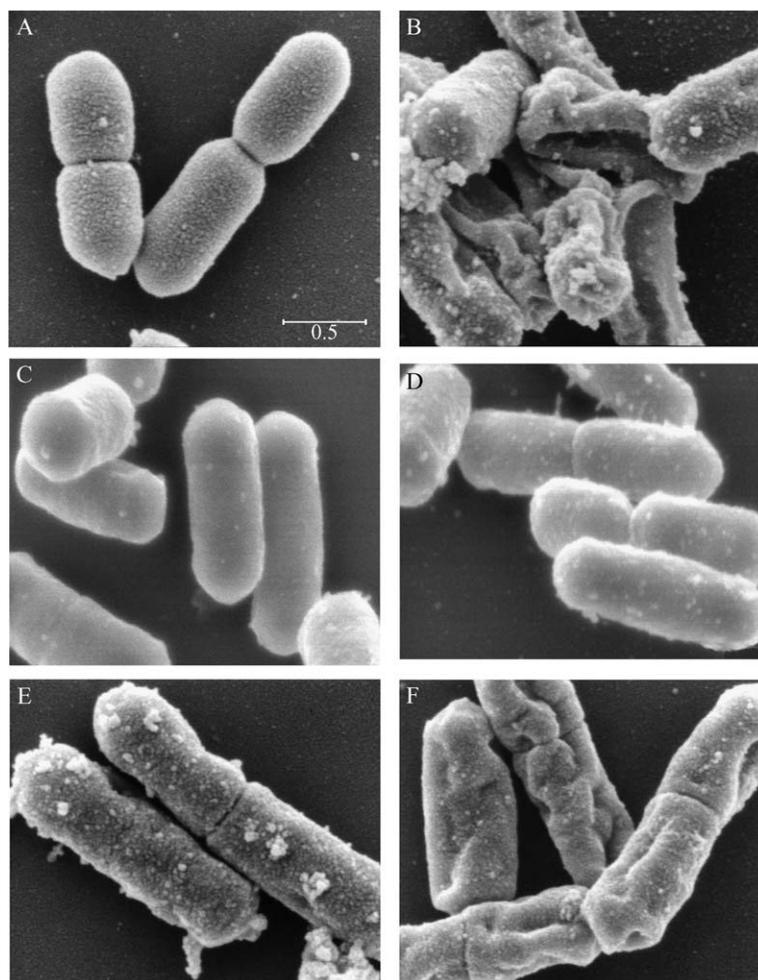


Fig. 1. Scanning electron photomicrographs of *Listeria monocytogenes* Scott A cells: (A) untreated control; (B) following 120 min incubation at 25 °C with nisin and pediocin; (C) immediately after hydrostatic pressure (HP) treatment; (D) following 120 min incubation at 25 °C after HP treatment; (E) immediately after HP treatment in bacteriocin mixture; and (F) following 120 min incubation at 25 °C after HP and bacteriocin mixture treatment. Bar (A) in micrometers.

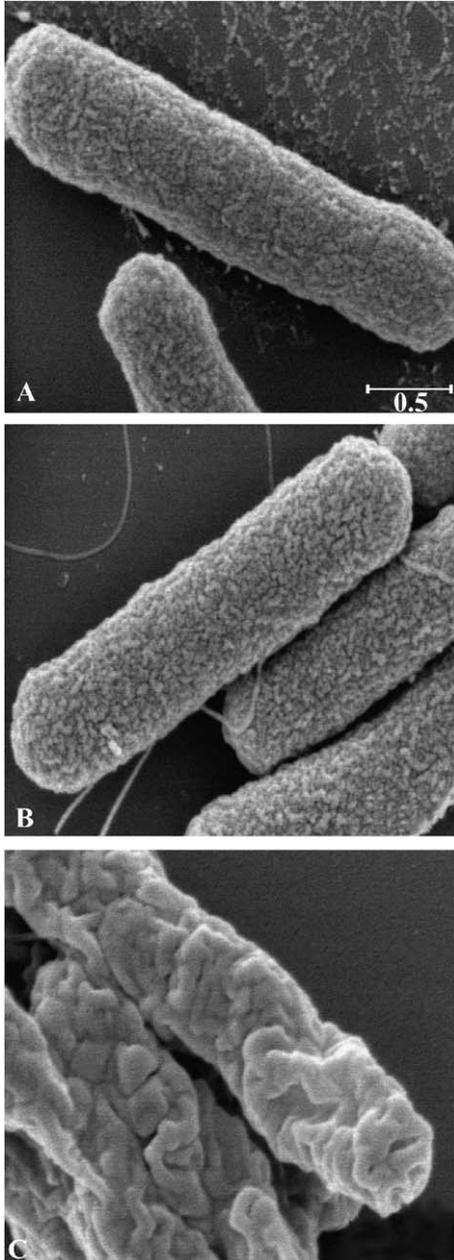


Fig. 2. Scanning electron photomicrographs of *Salmonella typhimurium* MH2413 cells (A) untreated control; (B) following 120 min incubation at 25 °C with nisin and pediocin; (C) immediately after hydrostatic pressure (HP) treatment. Treatment with a combination of HP and bacteriocin mixture produced cell morphology similar to C. Bar (A) in micrometers.

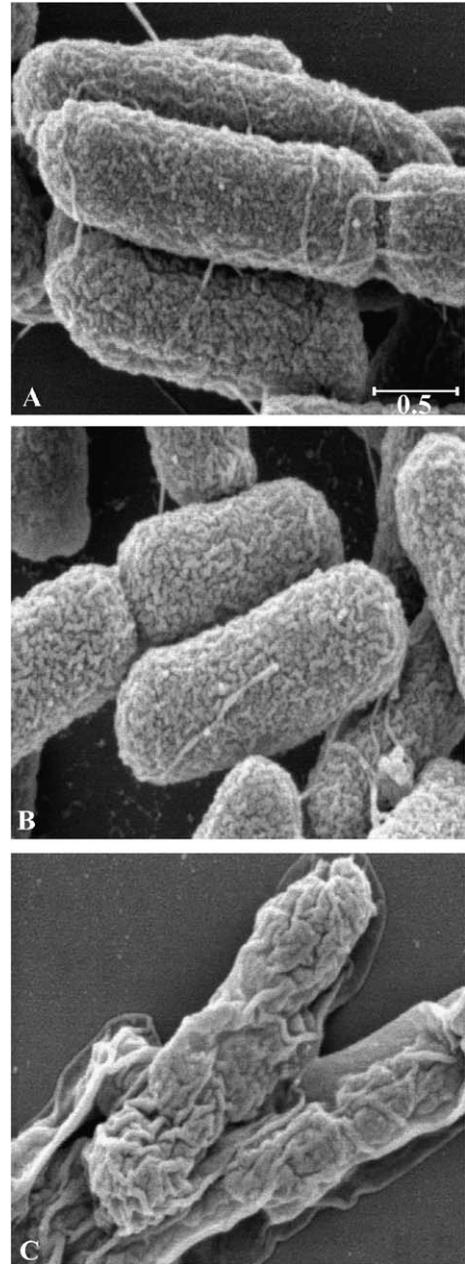


Fig. 3. Scanning electron photomicrographs of *Escherichia coli* O157:H7 #932 cells (A) untreated control; (B) following 120 min incubation at 25 °C with nisin and pediocin; (C) immediately after hydrostatic pressure (HP) treatment. Treatment with a combination of HP and bacteriocin mixture produced cell morphology similar to C. Bar (A) in micrometers.

mal granular texture (Fig. 1A). The bacteriocin-treated cells appeared collapsed (Fig. 1B); it could be associated with the damage in the cell wall and cell membrane and subsequent lysis and reduction in OD (Table 2). Immediately after pressurization, the *Listeria* cells did not show much difference in morphology other than non-granular surface (Fig. 1C; although over 1 log cycle or 90% of the cells were dead; Table 2); after 120-min incubation, at 25 °C, the cell surface appeared rough in appearance (Fig. 1D), which is quite different from the normal cells (Fig. 1A). Pressurization in the presence of the bacteriocin mixture showed partial collapse immediately (Fig. 1E), which increased greatly after 120-min incubation (Fig. 1F) resulting in the reduction in OD (Table 2). In a previous study with *L. mesenteroides* Ly, which has an inducible autolytic enzyme, bacteriocin treatment, pressurization or their combination not only produced cell death and cell lysis, but they also triggered the autolytic enzyme, which by hydrolyzing the wall, disintegrated the cells (Bhunja et al., 1991; Kalchayanand et al., 2002). In *L. monocytogenes* Scott A, these treatments did not cause disintegration of the cell probably due to absence of inducing autolytic enzyme in the strain. Treatment of *E. coli* O157:H7 and *S. typhimurium* with the bacteriocin mixture for 120 min did not produce any morphological changes as compared to the respective control (Figs. 2A,B and 3A,B, respectively). These results justified their unaltered OD and cfu/ml in Table 2. Photomicrographs of the cells of both strains, immediately after pressurization, showed extensive changes on the cell surface topography and morphology, indicating damage to the cell envelope (outer- and inner-membranes; Figs. 2C and 3C). Incubation of these cells for 120 min further enhanced these changes (data not presented). These findings concur the results of viability loss and OD reductions of the two strains in Table 2. Pressurization in the presence of the two bacteriocins produced similar but more extensive changes than the pressurized cells (data not presented).

4. Conclusion

The results of these studies provided a direct confirmation that viability loss of *L. monocytogenes* following treatment with pediocin and nisin or HP is

associated with the damages inflicted in the cell envelope (wall and membrane); pressurization in the presence of the two bacteriocins produced higher level of cell death by enhancing these damages. We observed similar effects in another Gram-positive bacterial strain *L. mesenteroides* Ly (Bhunja et al., 1991; Kalchayanand et al., 2002). In the two Gram-negative pathogens, *E. coli* O157:H7 and *S. typhimurium*, pressurization produced very high amounts of cell death and extensive changes in the cell envelope. Although the bacteriocin mixture did not cause either viability loss or any change in cell morphology of the two Gram-negative bacteria, when present during pressurization, they enhanced the viability loss as well as changes in cell morphology.

This observation is in agreement with the previous assumptions that, although normally the Gram-negative bacterial cells are insensitive to bacteriocins of lactic acid bacteria, pressure-induced sublethal injury in the cell envelope, make many survivors sensitive to the bacteriocins (Kalchayanand et al., 1992, 1994). These results could be used in designing processing parameters to efficiently eliminate foodborne pathogens from food by a combination of HP and bacteriocins.

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