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Hydrostatic Pressure and Electroporation Have Increased Bactericidal Efficiency in Combination with Bacteriocins

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We report here that both ultrahigh hydrostatic pressure (UHP) and electroporation (EP) induced sublethal injury to bacterial cells surviving the treatments. The injured cells of both gram-positive and -negative bacteria became sensitive to the bacteriocins pediocin AcH and nisin. Bacteriocins in combination with either UHP or EP have greater antibacterial effectiveness than UHP or EP alone.

Ultrahigh hydrostatic pressure (UHP) and pulsed electric field (PEF), because of their antimicrobial effectiveness, are being investigated as possible nonthermal methods of food preservation (5, 7, 13, 15, 16). While UHP can be applied to both solid and liquid foods, PEF to date shows effects only in liquid foods. In UHP processing, a food is exposed to isostatic pressure which enables it to retain size and shape, along with color, flavor, and nutrients (3, 5, 16). UHP also improves the texture of some foods and potentially destabilizes some undesirable enzymes (3, 4, 13, 15). Both UHP and PEF destroy microbial cells by destabilizing the structural and functional integrity of the cytoplasmic membrane (2, 9, 10, 15). The amount of cell death is directly proportional to the level of pressure and its duration for the UHP method and the voltage and the total pulse time for the PEF method (2, 10, 15).

To increase the antimicrobial efficiency of UHP and PEF methods, the hurdle concept of food preservation (two or more antimicrobial agents at suboptimal levels are more effective than one at the optimal level without affecting the acceptance quality of a food) can be useful, especially when a food needs to be stored under an adverse condition (14). Limited studies have revealed that UHP has increased antimicrobial efficiency in combination with low heat, low pH, lysozyme, chitosan, or CO_2 (4, 15). The mechanism(s) by which this greater microbial destruction is obtained has not been explained. It could be due to sublethal injury of cells by the UHP. Many stresses impose sublethal injury to microbial cells, which become sensitive to different physical and chemical environments to which the normal cells are resistant (19). There are suggestions that the hurdle concept can be combined with sublethal injury to increase the microbial destruction level (22). We are currently studying the effectiveness of several biopreservatives to increase the antimicrobial efficiency of UHP and electroporation (EP; a form of PEF) methods. Previous studies in our laboratory have revealed that antibacterial peptides or bacteriocins of lactic acid bacteria are bactericidal to sublethally injured gram-positive and gram-negative bacteria (11, 20–22). In this study, we determined that both UHP and EP induce sublethal injury on bacterial cells and have increased bactericidal efficiency in combination with bacteriocins.

Enumeration of viable and injured cells. Three pathogens, *Listeria monocytogenes* Scott A, *Escherichia coli* O157:H7 932, and *Salmonella typhimurium* M1, from our collection were used. The cells were grown in tryptic soy broth for 16 to 18 h at 37°C, harvested, and resuspended to obtain 10^{12} to 10^{14} cells per ml either in 0.1% peptone for use in UHP studies or in PM buffer (7 mM sodium phosphate, 1 mM $MgCl_2$ [pH 6.5]) for EP studies (12). Initial studies showed that at a higher pressure, many cells die very quickly. Therefore, a high cell concentration was used to facilitate determination of the numbers of injured and dead cells in the combination studies of UHP plus bacteriocins. The cell suspensions were maintained at 4°C before and after UHP and EP treatments prior to enumeration of CFU. To determine the size of the viable population, a cell suspension was serially diluted in 0.1% peptone water and pour plated in tryptic soy agar (TSA). To determine the number of sublethally injured cells in a population, a cell suspension was serially diluted and surface plated simultaneously on prepoured plates of a nonselective agar (TSA) and a selective agar (modified Oxford medium for *L. monocytogenes*, violet red bile for *E. coli*, and xylose-lysine deoxycholate for *S. typhimurium*). The plates were incubated at 37°C for up to 2 days, and CFU were enumerated.

UHP and EP treatments. For UHP treatment of a strain, small plastic vials (Cryovials; 2-ml capacity) were completely filled with cell suspensions in duplicate. When necessary, purified pediocin AcH (identical to pediocin PA-1 [8]), nisin, or a mixture containing equal amounts of both was added to cell suspensions to give final concentrations of 5,000 activity units (AU)/ml (23). Purification of the two bacteriocins and assay of AU were done by previously described methods (1, 23). For a comparison, 1 IU of nisin was found to be equivalent to 100 AU of our preparation of either nisin or pediocin AcH (17). The vials were individually put in plastic bags and vacuum sealed. Then, the vials were put in the chamber (6 by 18 in. [15.24 by 45.72 cm]) of the hydrostatic pressure unit (Harwood Engineering, Walpole, Mass.). Liquid (oil) was pumped into the chamber until the desired pressure (30,000 to 50,000 lb/in^2) was reached, held for the desired time (approximately 1 min), and then released to drop the pressure to atmospheric pressure (14.7 lb/in^2). Since the pump was controlled manually, the times to attain a particular end pressure from atmospheric pressure and to drop back to atmospheric pressure differed

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TABLE 1. Enumeration of three pathogens before and after UHP and EP treatments on nonselective and selective agar media

Treatment[a] and bacterial strain	Enumeration time	Concn of cells (log_{10} CFU/ml)		
		In medium[b]		Dead and injured after treatment[c]
		Nonselective	Selective	
UHP				
L. monocytogenes Scott A	Before UHP	11.90 ± 0.08	11.80 ± 0.07	
	After UHP	5.00 ± 0.05	2.00 ± 0.10	6.9 and 3.0
E. coli O157:H7 932	Before UHP	11.70 ± 0.03	11.20 ± 0.01	
	After UHP	8.00 ± 0.05	5.00 ± 0.04	3.7 and 3.0
S. typhimurium M1	Before UHP	13.40 ± 0.04	11.90 ± 0.01	
	After UHP	8.50 ± 0.01	2.00 ± 0.10	4.9 and 6.5
EP				
L. monocytogenes Scott A	Before EP	13.80 ± 0.02	13.50 ± 0.01	
	After EP	11.00 ± 0.04	10.70 ± 0.01	2.8 and 0.3
E. coli O157:H7 932	Before EP	13.40 ± 0.02	13.20 ± 0.02	
	After EP	9.00 ± 0.02	7.70 ± 0.02	4.4 and 1.3
S. typhimurium M1	Before EP	12.90 ± 0.03	10.90 ± 0.08	
	After EP	8.60 ± 0.01	6.30 ± 0.01	4.3 and 2.3

[a] The cells were subjected to 50,000 lb/in^2 for 1 min. The times to attain the increase from 14.7 to 50,000 lb/in^2 and the drop to 14.7 lb/in^2 were about 23 and 4 min, respectively. Cells were subjected to 12.5 kV/cm at a 25-μF capacitance.

[b] The nonselective medium was TSA for all strains, and the selective media were modified Oxford medium agar for *L. monocytogenes*, violet red bile agar for *E. coli*, and xylose-lysine deoxycholate agar for *S. typhimurium*. Each item of data represents the mean ± standard error of two to four readings.

[c] The differences in numbers of CFU in TSA before and after treatment were considered to indicate the number of dead cells, and the differences between TSA (nonselective) and selective agar after treatment were considered to indicate the number of injured cells (18).

between experiments. The temperature of the liquid remained almost unchanged at room temperature (22°C) at high pressures. Following pressure treatment, the vials were removed and stored at 4°C, and CFU were enumerated within 2 h. For PEF treatment, the cell suspensions were subjected to EP in a Gene Pulser (Bio-Rad Laboratories, Richmond, Va.). Cell suspensions (200 μl) were placed in cuvettes (0.2 cm). When required, purified bacteriocins were added to cell suspensions as before to a final concentration of 5,000 AU/ml. Electroporation was done at 12.5 kV/cm at a 25-μF capacitance with a single pulse at 4°C. The samples were stored at 4°C prior to enumeration of CFU.

The CFU in cell suspensions of the three pathogens before and after UHP (50,000 lb/in^2 for 1 min) and EP treatments were enumerated on both TSA and a selective medium to determine the levels of viability loss and sublethal injury among the survivors (Table 1; all results are presented in logarithmic units [U] and each item of data is an average of two to four separate counts). Viability loss or cell death estimated from the CFU on TSA before and after the treatments ranged from 3.7 to 6.9 U by UHP and 2.8 to 4.4 U by EP. Before the treatments, the numbers of CFU on the selective media were lower than the corresponding numbers of CFU on TSA and differences ranged from 0.1 (for *L. monocytogenes*) to 2.0 (for *S. typhimurium*) U. The lower CFU counts on selective media could be due to inherent sensitivity of the strains to the selective compounds in the media and/or due to stress imposed during preparation and holding of cell suspensions at 4°C prior to enumeration (18, 19). After treatment, the differences in counts between selective and nonselective media ranged from 3.0 to 6.5 U by UHP and from 0.3 to 2.3 U by EP. The inability of some of the cells surviving a treatment to form colonies on

TABLE 2. Enumeration of three pathogens on TSA plates after UHP treatment in the absence and presence of bacteriocins

Bacterial strain	Treatment[a]	Concn of cells (log_{10} CFU/ml) at[b]:		
		30,000 lb/in^2	40,000 lb/in^2	50,000 lb/in^2
L. monocytogenes Scott A	UHP	9.40 ± 0.10 (12.50 ± 0.05)[c]	8.90 ± 0.01 (12.50 ± 0.05)	7.80 ± 0.04 (12.70 ± 0.03)
	UHP + ped	7.90 ± 0.10	7.70 ± 0.04	6.00 ± 0.03
	UHP + nis	8.90 ± 0.02	8.10 ± 0.03	4.50 ± 0.01
	UHP + ped + nis	7.80 ± 0.01	7.10 ± 0.01	3.40 ± 0.02
E. coli O157:H7 932	UHP	11.10 ± 0.01 (12.80 ± 0.06)	10.70 ± 0.02 (12.80 ± 0.06)	9.30 ± 0.01 (14.10 ± 0.02)
	UHP + ped	10.30 ± 0.04	9.90 ± 0.04	7.50 ± 0.02
	UHP + nis	8.90 ± 0.03	8.70 ± 0.02	5.70 ± 0.05
	UHP + ped + nis	9.70 ± 0.01	9.60 ± 0.02	6.80 ± 0.06
S. typhimurium M1	UHP	10.60 ± 0.04 (13.90 ± 0.01)	9.70 ± 0.02 (13.90 ± 0.01)	7.60 ± 0.02 (13.90 ± 0.01)
	UHP + ped	9.80 ± 0.01	8.10 ± 0.08	5.50 ± 0.02
	UHP + nis	9.70 ± 0.06	6.80 ± 0.04	4.00 ± 0.01
	UHP + ped + nis	9.80 ± 0.01	8.00 ± 0.05	5.60 ± 0.02

[a] ped, pediocin AcH; nis, nisin. The bacteriocins were used separately or in combination at a final concentration of 5,000 AU/ml.

[b] The pressures were applied for about 1 min. The times to attain highest pressure from 14.7 lb/in^2 and then drop to 14.7 lb/in^2 were, respectively, 7 and 3 min for 30,000, 10 and 4 min for 40,000, and 20 and 9 min for 50,000 lb/in^2. Each item of the data is the mean ± standard error of four readings.

[c] The data in parentheses indicate CFU per milliliter prior to a treatment. The difference in the numbers of CFU before and after a treatment was used to determine viability loss or cell death.

TABLE 3. Enumeration of three pathogens on TSA after EP in the absence and presence of bacteriocins

Bacterial strain	Treatment[a]	Concn of cells (log_{10} CFU/ml)[b]
L. monocytogenes Scott A	Control	13.70 ± 0.01
	EP	11.00 ± 0.02
	EP + ped	10.50 ± 0.01
	EP + nis	7.80 ± 0.05
	EP + ped + nis	8.20 ± 0.01
E. coli O157:H7 932	Control	12.00 ± 0.30
	EP	8.50 ± 0.03
	EP + ped	8.20 ± 0.20
	EP + nis	7.80 ± 0.02
	EP + ped + nis	7.90 ± 0.01
S. typhimurium M1	Control	11.70 ± 0.09
	EP	8.60 ± 0.08
	EP + ped	8.00 ± 0.07
	EP + nis	7.90 ± 0.05
	EP + ped + nis	8.00 ± 0.01

[a] For explanations, see footnote *a* in Table 2. EP was applied at 12.5 kV and a 25-μF capacitance in a single pulse at 4°C.

[b] CFU per milliliter before treatment. Each item of the data is the mean ± standard error of two readings.

a selective medium while retaining the ability to form colonies on a nonselective medium is a manifestation of sublethal injury and results from the inability of these injured cells to multiply in the selective environment (18, 19). These cells subsequently die in the presence of selective agents (22). These results have demonstrated that both UHP and EP inflict lethal as well as sublethal injury to gram-positive and gram-negative bacterial cells.

Sublethally injured bacterial cells are reported to become sensitive to bacteriocins of lactic acid bacteria (11, 22). To determine if the two treatments have increased bactericidal efficiency in the presence of bacteriocin(s), the pathogens were subjected to UHP and EP treatments in the presence of pediocin AcH, nisin, or their combination. In the UHP treatment alone, as the pressure increased from 30,000 to 50,000 lb/in^2, the viability loss or cell death increased from 3.1 to 4.9 U for *L. monocytogenes*, 1.7 to 4.8 U for *E. coli*, and 3.3 to 6.3 U for *S. typhimurium* (Table 2). However, in the presence of the bacteriocins, either individually or in combination, a greater reduction in viability occurred. The highest reductions with the UHP (50,000 lb/in^2) and bacteriocins were as follows: 9.3 U for *L. monocytogenes* with the pediocin AcH and nisin combination and 8.4 U for *E. coli* and 9.9 U for *S. typhimurium* with nisin. Similarly, EP alone reduced the viability of the three strains by 2.7 to 3.5 U (Table 3). In the presence of the bacteriocin(s), the greatest reductions were 5.9 U for *L. monocytogenes*, 4.2 U for *E. coli*, and 3.8 U for *S. typhimurium*. Under the test conditions used in this study, the bactericidal efficiency was greater in UHP treatment than in EP treatment, even at 30,000 lb/in^2 for 1 min, and viability losses were greater when bacteriocins were present in UHP and EP treatments. In a previous report, we proposed that pediocin AcH and nisin, in combination, could have increased antibacterial efficiency against gram-positive bacteria (6). We also see a similar effect with UHP-treated, but not EP-treated, *L. monocytogenes*. However, for the two gram-negative strains, nisin alone produced the greatest effect. In the present study, very large cell populations but relatively low bacteriocin concentrations were used. The final concentrations in AU per milliliter of the bacteriocins were kept at the same level in all studies. For an ideal condition to study the possible additive effects of bacteriocins, one needs to consider the pH and ionic environment of the suspending medium and relative cell and bacteriocin concentrations; one of the bacteriocins should be at a concentration to produce its maximum bactericidal effect in a population. In the present study, these conditions were different from those in the previous study (6).

The results presented here have shown that both UHP and EP treatments caused viability loss and sublethal injury to cells of the three pathogens. Because of the sensitivity of injured cells to bacteriocins, an increase in viability loss (or cell death) occurs when UHP or EP treatment is given in the presence of a bacteriocin. Similar results have been observed with other pathogenic and spoilage bacteria (data not shown). Thus, nonthermal treatments, such as UHP and EP, in combination with biopreservatives, such as bacteriocins of food grade lactic acid bacteria, can be used to increase bactericidal efficiency and enhance the safety and shelf life of foods.

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