Journal of Food Protection, Vol. 73, No. 12, 2010, Pages 2203–2210

Inactivation Kinetics and Virulence Potential of *Salmonella* Typhimurium and *Listeria monocytogenes* Treated by Combined High Pressure and Nisin

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MS 10-263: Received 24 June 2010/Accepted 10 September 2010

ABSTRACT

The aim of this study was to characterize the physiological and molecular changes of *Salmonella* Typhimurium and *Listeria monocytogenes* in deionized water (DIW) and nisin solutions (100 IU/g) during high pressure processing (HPP). Strains of *Salmonella* Typhimurium and *L. monocytogenes* in DIW or nisin solutions were subjected to 200, 300, and 400 MPa for 20 min. The Weibull model adequately described the HPP inactivation of *Salmonella* Typhimurium and *L. monocytogenes*. *Salmonella* Typhimurium and *L. monocytogenes* populations were reduced to less than 1 CFU/ml in DIW and nisin solutions under 400 MPa. The highest *b* value was 5.75 for *Salmonella* Typhimurium in nisin solution under 400 MPa. *L. monocytogenes* was more sensitive to pressure change when suspended in DIW than when suspended in nisin. The pressure sensitivity of both *Salmonella* Typhimurium and *L. monocytogenes* was higher in DIW solution (141 to 243 MPa) than in nisin solution (608 to 872 MPa). No recovery of HPP-injured cells in DIW and nisin solutions treated at 400 MPa was observed after 7 days of refrigerated storage. The heterogeneity of HPP-treated cells was revealed in flow cytometry dot plots. The transcripts of *stn, invA, prfA,* and *inlA* were relatively down-regulated in HPP-treated nisin solution. The combination of high pressure and nisin could noticeably suppress the expression of virulence-associated genes. These results provide useful information for understanding the physiological and molecular characteristics of foodborne pathogens under high-pressure stress.

High pressure processing (HPP), a nonthermal food preservation technology, has received much attention as an emerging technology because of the many advantages over conventional thermal processing, including homogeneous temperature distribution, low temperatures, and fewer adverse effects on sensorial properties and nutrients *(11, 20, 23, 25, 32, 39)*. During the last few decades, HPP has been applied to food products to improve microbiological safety and extend shelf life *(25, 39)*. However, for ensuring microbiological safety, the question remains whether the application of HPP can achieve complete inactivation of pathogenic and spoilage bacteria. Complex food matrices can provide a protective effect, inhibiting the HPP inactivation of contaminating bacteria that are likely to be inactivated at moderate pressures of 200 to 500 MPa *(14, 33)*. Foodborne pathogens exposed to sublethal stress may regain metabolic activity and pathogenicity under favorable environmental conditions, which is important when discussing food safety. Many researchers have suggested that HPP is more effective for bacterial inactivation when combined with other preservatives such as nisin, lysozyme, lactoperoxidase, and phenolic compounds *(2, 13, 18, 19, 31, 37, 41)*. Nisin is a natural peptide and well-known lantibiotic produced by *Lactococcus lactis* and is commonly used as a broad-spectrum antimicrobial agent *(19)*. The combined application of HPP and antimicrobials can synergistically increase the vulnerability of bacterial cells by changing the antibiotic affinity and permeability of the cytoplasmic membrane *(18, 30, 34, 35, 37, 41)*. However, little information exists on the combined effectiveness of HPP and antimicrobials. Therefore, physiological and molecular approaches are needed to understand the patterns of foodborne pathogen response to high pressure and antimicrobial stress.

Salmonella Typhimurium, a member of the *Enterobacteriaceae,* is a gram-negative, nonsporulating rod that has been associated with foodborne salmonellosis. *Salmonella* Typhimurium infection is directly associated with the expression of virulence-associated and virulence genes *(29)*. The *Salmonella* enterotoxin gene (*stn*), which encodes a 29-kDa protein, is one of the prevailing virulence-associated markers, and this protein is mainly associated with gastroenteritis *(6, 7)*. The penetration of intestinal epithelium cells by *Salmonella* Typhimurium is attributed to the protein coded by the invasion gene (*invA*) *(9)*. *Listeria monocytogenes* is a gram-positive, psychrotrophic, and intracellular foodborne pathogen that can cause gastroenteritis, meningitis, and septicemia *(12)*. Surface proteins (InlA and InlB) encoded by internalin genes (*inlA* and *inlB,* respectively) are involved in the internalization of *L.*

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monocytogenes at the first stage of intracellular infection and are responsible for cell attachment, invasion, and virulence *(4)*. The internalin genes are regulated by positive regulatory factor A (*prfA*), which is an activator that controls transcription of virulence-associated genes in *L. monocytogenes (36)*. To assess microbiological safety of HPP-treated foods, the physiological properties and pathogenic potential of HPP-injured cells must be considered in terms of the recovery behavior and transcription of virulence genes. However, there is little information on the molecular properties of foodborne pathogens in response to combined HPP and nisin treatment. The objectives of this study were to evaluate the inactivation patterns, physiological properties, and virulence potential of *Salmonella* Typhimurium and *L. monocytogenes* treated with HPP and antimicrobials as measured by kinetic parameters, flow cytometry, and real-time reverse transcriptase PCR (RT-PCR).

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains of *Salmonella* Typhimurium (KCCM 40253) and *L. monocytogenes* (KACC 12671) were provided by the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea) and the Korean Agricultural Culture Collection (KACC; Suwon, Korea), respectively. The strains were cultured aerobically in Trypticase soy broth (TSB; BD, Franklin Lakes, NJ) at 37°C for 20 h and harvested by centrifugation at 3,000 × *g* for 20 min at 4°C. The harvested cultures were resuspended in 0.1% sterile buffered peptone water (BPW) and diluted to approximately 10^8 CFU/ml for inoculation.

Sample preparation. Deionized water (DIW, pH 5.65) was used as a negative control. A stock solution of nisin (10^6 IU/g; Sigma-Aldrich Chemical Co., St. Louis, MO) was prepared by dissolving 1 g in 10 ml of 0.02 M HCl. This stock nisin solution was diluted with DIW to obtain the final working concentration of 100 IU/ml (pH 4.88). *Salmonella* Typhimurium or *L. monocytogenes* at approximately 10^7 CFU/ml was inoculated into the DIW and nisin solutions. The inoculated samples (10 ml each) were individually packaged in sterile polyethylene bags (Fisher Scientific, Fair Lawn, NJ) and sealed with an Impulse Bag Sealer (American International Electric, Whittier, CA).

High-pressure treatment. The packaged samples were subjected to 200, 300, and 400 MPa of pressure at 25°C for 0, 5, 10, and 20 min in a custom-made high-pressure processor (Ilshin Autoclave Co., Deajeon, Korea). The pressure come-up times were approximately 2.05, 2.19, and 2.56 min for 200, 300, and 400 MPa, respectively. The depressurization times were less than 20 s and was not included in the process hold time. The HPP-treated samples were used for microbial enumeration and to determine physiological properties and virulence gene expression.

Storage study. The recovery of HPP-treated cells was evaluated in a storage study immediately after HPP. The samples inoculated with *Salmonella* Typhimurium or *L. monocytogenes* were treated at 200, 300, or 400 MPa for 20 min and stored at 4°C for 7 days. Cell viability when cell numbers were below the detection limit (sensitivity ≥ 1 CFU/ml) was determined by enrichment culture in double strength TSB.

Microbiological analysis. Total viable cells of high-pressure-treated samples were directly determined by the pour plate method. The HPP-treated samples were aseptically transferred from pouches, mixed with 10 ml of 0.1% sterile BPW, and serially diluted 10-fold for plating on Trypticase soy agar (BD). The inoculated plates were aerobically incubated to determine the populations of *Salmonella* Typhimurium or *L. monocytogenes* at 37°C for 24 to 48 h.

Inactivation kinetics. The inactivation kinetic parameters were estimated by using the Weibull distribution model as follows:

$$\log\left(\frac{N}{N_0}\right) = -bt^n \quad (1)$$

where b and n are the scale and shape factors, respectively. The scale parameter (b) is equal to the reaction rate constant k (1/min) *(40)*. The pressure coefficient z_T (MPa) at constant temperature was estimated as the negative reciprocal of the slope resulting from plotting log b against pressure.

The pressure effect on the inactivation of cells can be determined at the constant temperature using the apparent activation volume:

$$\left(\frac{d\ln k}{dP}\right)_T = -\frac{\Delta V^*}{RT} \quad (2)$$

where ΔV is the apparent activation volume (cm^3/mol), R is the universal gas constant (8.314×10^{-6} m^3/MPa/K/mol), and T is the temperature (Kelvin). The ΔV^* value is the net effect of pressure reactions causing physiological changes at a constant temperature *(28)*.

Evaluation of the goodness of fit. The mean square error (MSE) was used to evaluate the goodness of fit. A smaller MSE indicates better fitting. The MSE between predicted and observed values was calculated from the following equation *(17)*:

$$\text{MSE} = \frac{\sum(\text{predicted} - \text{observed})^2}{n - p} \quad (3)$$

where n and p are the number of observations and the number of parameters, respectively.

Flow cytometric analysis. The cells treated at 200, 300, and 400 MPa for 20 min were analyzed with a flow cytometry system (FACScan, BD). The HPP-treated cells were centrifuged at 5,000 × *g* for 10 min, washed twice in phosphate-buffered saline (PBS, pH 7.4), and resuspended in PBS. LIVE/DEAD *Bac*Light kits (Invitrogen, Eugene, OR) that included SYTO 9 and propidium iodide (PI) were used to stain the collected cells according to the manufacturer's instructions. Live and dead cells (1 ml) were stained with green fluorescence from SYTO 9 (1.5 μl) and red fluorescence from PI (1.5 μl). The excitation and emission maxima were approximately 480 and 500 nm, respectively, for SYTO 9 (FL1 channel) and 490 and 635 nm, respectively, for PI (FL3 channel). The cell size and granularity also were examined by forward scatter and side scatter, respectively. Data were collected from 10,000 events at a flow rate of 1,000 cells per s using a FACstation (BD).

RNA extraction. To inhibit RNA degradation, 0.5-ml volumes of each cell culture treated at 200, 300, and 400 MPa for 0 min were mixed with RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) and centrifuged at 5,000 × *g* for 10 min. The supernatants were discarded, and the pellets were used for RNA isolation according to the RNeasy Mini protocol (Qiagen). The collected cells were homogenized with a buffer containing guanidine isothiocyanate and then incubated in a buffer containing lysozyme. The lysates were mixed with ethanol to provide

TABLE 1. *Nucleotide sequences of primers used in this study*

Strain[a]	Gene	Description	Sequence (5′ to 3′)[b]	Reference
ST	16S rRNA	Reference gene	F: CCAGCAGCCGCGGTAAT R: TTTACGCCCAGTAATTCCGATT	*38*
	invA	Invasion A	F: CAACGTTTCCTGCGGTACTGT R: CCCGAACGTGGCGATAATT	*15*
	stn	Enterotoxin	F: GCCATGCTGTTCGATGAT R: GTTACCGATAGCGGGAAAGG	*24*
LM	16S rRNA	Reference gene	F: CCTACGGGAGGCAGCAG R: GTATTACCGCGGCTGCTG	*3*
	prfA	Positive regulatory factor A	F: AACCAATGGGATCCACAAG R: ATTCTGCTAACAGCTGAGC	*27*
	inlA	Internalin A	F: AATGCTCAGGCAGCTACAMTTACA R: CGTGTCTGTTACRTTCGTTTTTCC	*27*

[a] ST, *Salmonella* Typhimurium; LM, *Listeria monocytogenes*.
[b] F, forward primer; R, reverse primer.

appropriate binding conditions and then loaded onto an RNeasy mini column for isolation of total RNA.

Real-time RT-PCR analysis. cDNA was synthesized according to the QuantiTect reverse transcription procedure (Qiagen). The template RNA (14 μl) was mixed with a master mixture (1 μl of Quantiscript reverse transcriptase, 4 μl of Quantiscript RT buffer, and 1 μl of RT primer mix), incubated at 42°C for 15 min, and then incubated at 95°C for 3 min to inactivate the Quantiscript reverse transcriptase. The real-time PCR amplifications were performed according to instructions in the QuantiTect SYBR Green PCR Handbook (Qiagen). The PCR mixture (20-μl reaction volume) contained 10 μl of 2× QuantiTect SYBR Green PCR master mix, 0.6 μl of primer, 2 μl of cDNA, and 6.8 μl of RNase-free water and was amplified with an iCycler iQ System (Bio-Rad Laboratories, Hemel, Hempstead, UK). The PCR mixture was denatured at 95°C for 15 min and then by 45 cycles of 94°C for 15 s, 59°C for 20 s, and 72°C for 15 s. The melting curve analysis was carried out immediately after amplification in 0.4°C increments from 65°C for 85 cycles of 10 s each. The specific oligonucleotides were used as primers to target the conserved regions of *invA, stn, prfA, inlA,* and the references genes (Table 1). The PCR products were visualized and analyzed using the iQ5 real-time PCR detection system (Bio-Rad). Specific cDNA targets (*stn* and *invA*) were quantified to determine the absolute copy number of mRNA.

Statistical analysis. All the experiments were independently repeated three times with duplicate samples. The curve fitting and the model parameter estimation were done using nonlinear curve fitting software (Microcal Origin 7.5, Microcal Software Inc., Northampton, MA). Data were analyzed using the Statistical Analysis System software (SAS Institute, Cary, NC). Significant mean differences were determined with Fisher's least significant difference test at $P < 0.05$.

RESULTS AND DISCUSSION

This study was conducted to evaluate the combined effect of HPP and nisin on the inactivation of *Salmonella* Typhimurium and *L. monocytogenes* suspended in DIW and nisin solutions. The flow cytometric assessment and real-time RT-PCR were used to improve the understanding of the effect of nisin during HPP.

Effect of HPP and nisin on *Salmonella* Typhimurium and *L. monocytogenes*. The inactivation of *Salmonella* Typhimurium and *L. monocytogenes* suspended in DIW and nisin solutions was evaluated after treatment at three pressure levels (200 to 400 MPa) at 25°C for 20 min (Fig. 1). With increasing pressure, microbial reductions occurred in DIW and nisin solutions. *Salmonella* Typhimurium and *L. monocytogenes* suspended in DIW and nisin solutions remained at more than 3 log CFU/ml (1.4- to 3.8-log reductions) at 200 MPa after 20 min, but populations were below the detection limit (>7-log reductions) at 400 MPa after 5 min. *Salmonella* Typhimurium populations in DIW and nisin solutions were 1.70 log CFU/ml and below the detection limit (<1 log CFU/ml), respectively, at 300 MPa after 20 min. The long processing times were required for DIW samples to achieve reductions corresponding to those in nisin samples. At 300 MPa, *L. monocytogenes* cells in DIW and nisin solutions were below the detection limit after 20 and 10 min, respectively. No shoulder effect was observed in both DIW and nisin solutions treated at 400 MPa, indicating that the high pressure may have direct lethal effect on *Salmonella* Typhimurium and *L. monocytogenes* rather than a cumulative effect, in which cells are sublethally injured. The HPP inactivation of bacterial cells can be accelerated by adding nisin. The HPP-treated *Salmonella* Typhimurium and *L. monocytogenes* cells in nisin solution were more sensitive to the treatment than were those in DIW ($P < 0.05$). Differences in reductions between cultures in DIW solution and those in nisin solution were observed at 200 MPa, indicating that sublethally injured cells were immediately inactivated by nisin. This observation was confirmed by the flow cytometric patterns. A change in permeability resulted from cell wall and cytoplasmic membrane injury caused by HPP, leading to increased sensitivity of the HPP-treated cells to antimicrobials *(16)*.

Inactivation kinetics of *Salmonella* Typhimurium and *L. monocytogenes* treated by combined HPP and nisin. The differences in the resistance of *Salmonella*

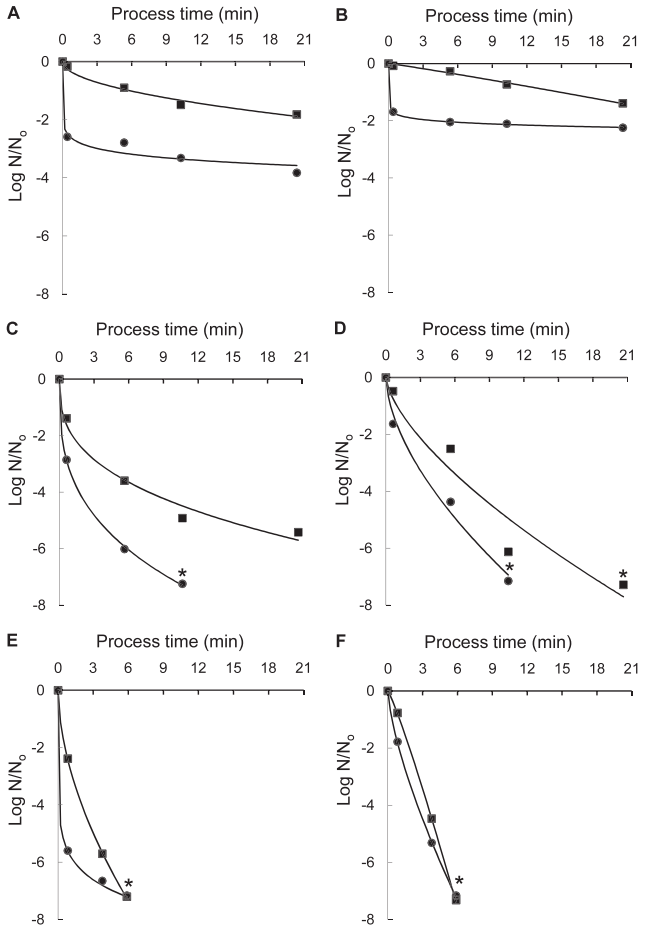

FIGURE 1. *Inactivation curves for* Salmonella *Typhimurium (A, C, E) and* L. monocytogenes *(B, D, F) suspended in deionized water (DIW; ■) and nisin (●) solutions at 200 (A, B), 300 (C, D), and 400 (E, F) MPa for 20 min. Data were fitted with Weibull model. An asterisk indicates a value below the detection limit.*

Typhimurium and *L. monocytogenes* to HPP were confirmed by the values obtained from the Weibull model (Fig. 1 and Table 2). The kinetics parameter values obtained from the Weibull model adequately described the HPP inactivation of *Salmonella* Typhimurium and *L. monocytogenes* in DIW and nisin solutions (Table 2). The degree of HPP resistance of *Salmonella* Typhimurium and *L. monocytogenes* in DIW and nisin solutions decreased as pressure increased, indicating that the *b* values increased. The highest *b* value was 5.75 for *Salmonella* Typhimurium suspended in nisin solution at 400 MPa, followed by 300 MPa (3.35) and 200 MPa (2.70). The inactivation curves of *Salmonella* Typhimurium and *L. monocytogenes* in DIW and nisin solutions were concave upward ($n < 1$). The goodness of fit of the Weibull model was evaluated based on the MSE (Table 2). The smaller MSE values were estimated from the predicted and experimental data. This observation is in agreement with previous reports in which the Weibull model produced a better fit for the HPP-induced inactivation kinetics of *Staphylococcus aureus, Yersinia enterocolitica,* and *L. monocytogenes (5).*

Sensitivity of *Salmonella* Typhimurium and *L. monocytogenes* to HPP and nisin. The pressure coefficient (z_T) and apparent activation volume (ΔV) were calculated as shown in Table 3. The z_T values for *Salmonella* Typhimurium and *L. monocytogenes* suspended in DIW were 243 and 141 MPa, respectively, whereas those in nisin solution were significantly increased to 608 and 871 MPa (Table 3) ($P < 0.05$). These results suggest that *Salmonella* Typhimurium and *L. monocytogenes* cells in DIW were more sensitive to changes in pressure than were the cells in nisin solution. The pressure effect on the inactivation of *Salmonella* Typhimurium and *L. monocytogenes* was more pronounced in DIW samples. In DIW, the z_T value for *Salmonella* Typhimurium was not significantly different from that for *L. monocytogenes* ($P > 0.05$). However, the z_T value for *L. monocytogenes* suspended in nisin solution was higher than that for *Salmonella* Typhimurium.

All ΔV values for *Salmonella* Typhimurium and *L. monocytogenes* in DIW and nisin solutions were negative. The absolute ΔV values for *Salmonella* Typhimurium in DIW (37 cm^3/mol) and nisin (28 cm^3/mol) solutions were significantly larger than those for *L. monocytogenes* in DIW (16 cm^3/mol) and nisin (3 cm^3/mol) solutions ($P < 0.05$). The negative ΔV values for *Salmonella* Typhimurium and *L. monocytogenes* indicate that the decrease in volume was accompanied by an increase in lethal effect *(28)*. According to Le Chatalier's principle, an equilibrium shifts to a decrease in volume for minimizing the effects of chemical

TABLE 2. *Kinetics parameters of Weibull model for* Salmonella *Typhimurium and* L. monocytogenes *in deionized water (DIW) and nisin solutions treated at different pressures*[a]

		b		*n*		MSE	
Strain[b]	Pressure (MPa)	DIW	Nisin	DIW	Nisin	DIW	Nisin
ST	200	0.42 ± 0.30 B b	2.70 ± 0.15 A b	0.54 ± 0.14 A a	0.09 ± 0.02 B b	0.020	0.113
	300	1.93 ± 0.53 B a	3.35 ± 0.16 A b	0.36 ± 0.06 A a	0.33 ± 0.02 A a	0.239	0.034
	400	2.69 ± 0.39 B a	5.75 ± 0.33 A a	0.56 ± 0.07 A a	0.13 ± 0.04 B b	0.001	0.001
LM	200	0.05 ± 0.04 B b	1.82 ± 0.28 A a	0.90 ± 0.11 A a	0.07 ± 0.03 B b	0.063	0.009
	300	1.04 ± 0.41 A a	1.72 ± 0.36 A a	0.67 ± 0.13 A a	0.59 ± 0.10 A a	0.190	0.200
	400	1.36 ± 0.03 A a	2.06 ± 0.26 A a	0.98 ± 0.02 A a	0.71 ± 0.08 B a	0.001	0.001

[a] Values are mean ± standard deviation. Within a row, means with different uppercase letters are significantly different at $P < 0.05$. Within a column, means with different lowercase letters are significantly different at $P < 0.05$.

[b] ST, *Salmonella* Typhimurium; LM, *Listeria monocytogenes*.

TABLE 3. *Pressure coefficients (z_T and ΔV) for* Salmonella *Typhimurium and* L. monocytogenes *suspended in DIW and nisin solutions at different temperatures over a range of pressures (0.1 to 700 MPa)*[a]

Treatment	*Salmonella* Typhimurium		*L. monocytogenes*	
	z_T (MPa)	ΔV (cm^3/mol)	z_T (MPa)	ΔV (cm^3/mol)
DIW	242.93 ± 34.29 B	−37.03 ± 1.72 B a	140.89 ± 34.13 B	−15.93 ± 0.82 B b
Nisin	607.91 ± 2.62 A a	−27.65 ± 1.21 A a	871.21 ± 53.57 A b	−2.95 ± 0.27 A b

[a] Values are mean ± standard deviation. Within a column, means with different uppercase letters are significantly different at $P < 0.05$. Within a row and for the same pressure coefficient, means with different lowercase letters are significantly different at $P < 0.05$.

imbalance with increasing pressure *(10)*. The microbial inactivation for a larger absolute ΔV value is likely to be more pressure dependent, resulting in significant changes in chemical, structural, and molecular interactions *(26)*.

Recovery and viability assessment of *Salmonella* Typhimurium and *L. monocytogenes* cells exposed to HPP and nisin. The HPP-treated samples were stored for 7 days at 4°C to evaluate the recovery of injured *Salmonella* Typhimurium and *L. monocytogenes* cells. Compared with the DIW cultures, the populations of HPP-treated *Salmonella* Typhimurium and *L. monocytogenes* were significantly decreased in nisin solutions, and no cells were recovered after 7 days of storage at 4°C (Table 4) ($P < 0.05$). At 200 MPa, the numbers of *Salmonella* Typhimurium and *L. monocytogenes* cells in DIW were reduced by 1.16 and 1.49 log CFU/ml, respectively, while those in nisin solution were reduced by more than 2 log CFU/ml. At 300 MPa, no growth was obtained in nisin solution after 7 days of storage, whereas surviving *Salmonella* Typhimurium and *L. monocytogenes* cells remained in the DIW solution. No growth in enrichment tests was observed in DIW and nisin solutions treated at 400 MPa after 7 days of storage.

The physiological states of HPP-treated cells were determined with the flow cytometric assay. The subpopulations of *Salmonella* Typhimurium and *L. monocytogenes* cells were more susceptible to the presence of nisin (Figs. 2 and 3). This result was confirmed by the results of the recovery study of HPP-treated *Salmonella* Typhimurium and *L. monocytogenes*. No surviving cells were detected after 7 days of storage at 4°C (Table 4). The heterogeneous subpopulations of *Salmonella* Typhimurium and *L. monocytogenes* in DIW and nisin solutions treated with HPP and nisin combined were illustrated on flow cytometry dual-parameter dot plots (Figs. 2 and 3). The relative percentages of live *Salmonella* Typhimurium cells in the DIW (quadrant 4) were decreased from 70 and 63% to 34% with increasing pressure from 200 MPa (Fig. 2A) and 300 MPa (Fig. 2B) to 400 MPa (Fig. 2C), respectively, while those of dead and injured cells (quadrants 1 and 2) increased to 65% after the 400-MPa treatment for 20 min (Fig. 2C). The relative percentages of injured and dead *Salmonella* Typhimurium cells in nisin solution (quadrants 1 and 2) increased to 98, 96, and 94% after 200-, 300-, and 400-MPa treatments, respectively, for 20 min (Fig. 2D, 2E, and 2F). Similar to *Salmonella* Typhimurium, the relative percentages of injured and dead *L. monocytogenes* cells in nisin solution were increased to more than 90% after 200-, 300-, and 400-MPa treatments for 20 min (Fig. 3D, 3E, and 3F). The increase in the susceptibility of HPP-treated cells may be attributed to structural or functional changes in the cell wall, the cytoplasmic membrane, and nucleic acids *(42)*, leading to membrane permeability and the synthesis of intracellular molecules. The results of the flow cytometric assessment were highly correlated with the results obtained from the inactivation study. Flow cytometric analysis can be used to reliably distinguish viable, injured, and dead cells *(22)*.

Differential gene expression of *Salmonella* Typhimurium and *L. monocytogenes* exposed to HPP and nisin. The differential expression of selected genes (*stn, invA, prfA,* and *inlA*) in DIW and nisin solutions treated at 200, 300, and 400 MPa for 0 min was evaluated with a real-time RT-PCR assay (Figs. 4 and 5). The *stn* transcripts were

TABLE 4. *Recovery behavior of* Salmonella *Typhimurium and* L. monocytogenes *in deionized water (DIW) and nisin solutions treated at different pressure levels during storage at 4°C*[a]

Pressure (MPa)	Treatment	*Salmonella* Typhimurium		*L. monocytogenes*	
		Day 0	Day 7	Day 0	Day 7
200	DIW	5.39 ± 0.49 A a	4.23 ± 0.33 A b	5.86 ± 0.41 A a	4.37 ± 0.52 A b
	Nisin	3.35 ± 0.46 B a	1.21 ± 0.30 B b	4.89 ± 0.19 A a	2.29 ± 0.26 B b
300	DIW	1.70 ± 0.56	+	+	+
	Nisin	+	−	−	−
400	DIW	−	−	−	−
	Nisin	−	−	−	−

[a] Values are mean ± standard deviation ($n = 4$). Within a column, means with different uppercase letters are significantly different at $P < 0.05$. Within a row for each strain, means with different lowercase letters are significantly different at $P < 0.05$. +, visible growth at least once in the three enrichment tests; −, no growth in any of the enrichment tests.

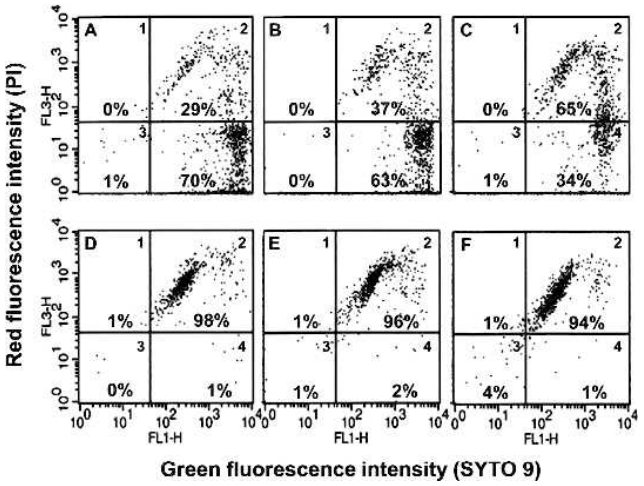

FIGURE 2. *Flow cytometric dot plots of* Salmonella *Typhimurium in DIW (A, B, C) and nisin (D, E, F) solutions treated at 200 (A, D), 300 (B, E), and 400 (C, F) MPa. The treated cells were stained using the LIVE/DEAD BacLight kit containing PI and SYTO 9. The values in quadrants 1, 2, 3, and 4 are the relative percentages of cells positive for red fluorescence only (PI^+, $SYTO\ 9^-$), cells positive for both red and green fluorescence (PI^+, $SYTO\ 9^+$), cells negative for both red and green fluorescence (PI^-, $SYTO\ 9^-$), and cells positive for green fluorescence only (PI^-, $SYTO\ 9^+$), respectively.*

relatively upregulated by 1.52-, 1.20-, and 1.50-fold in DIW treated at 200, 300, and 400 MPa, respectively, for 0 min, and the *stn* transcripts were down-regulated in nisin solution (Fig. 4A). The *invA* transcripts were upregulated by 2.25-, 2.29-, and 1.28-fold in DIW treated at 200, 300, and 400 MPa, respectively, for 0 min. However, *invA* expression relative to the internal control gene (16S rRNA) was

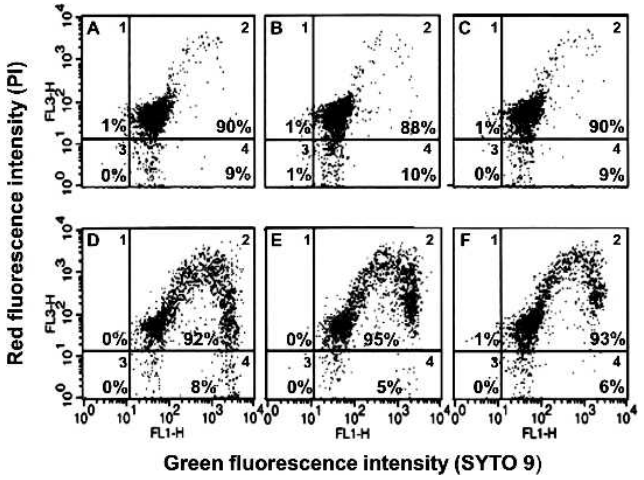

FIGURE 3. *Flow cytometric dot plots of* L. monocytogenes *in DIW (A, B, C) and nisin (D, E, F) solutions treated at 200 (A, D), 300 (B, E), and 400 (C, F) MPa for 20 min. The treated cells were stained using the LIVE/DEAD BacLight kit containing PI and SYTO 9. The values in quadrants 1, 2, 3, and 4 are the relative percentages of cells positive for red fluorescence only (PI^+, $SYTO\ 9^-$), cells positive for both red and green fluorescence (PI^+, $SYTO\ 9^+$), cells negative for both red and green fluorescence (PI^-, $SYTO\ 9^-$), and cells positive for green fluorescence only (PI^-, $SYTO\ 9^+$), respectively.*

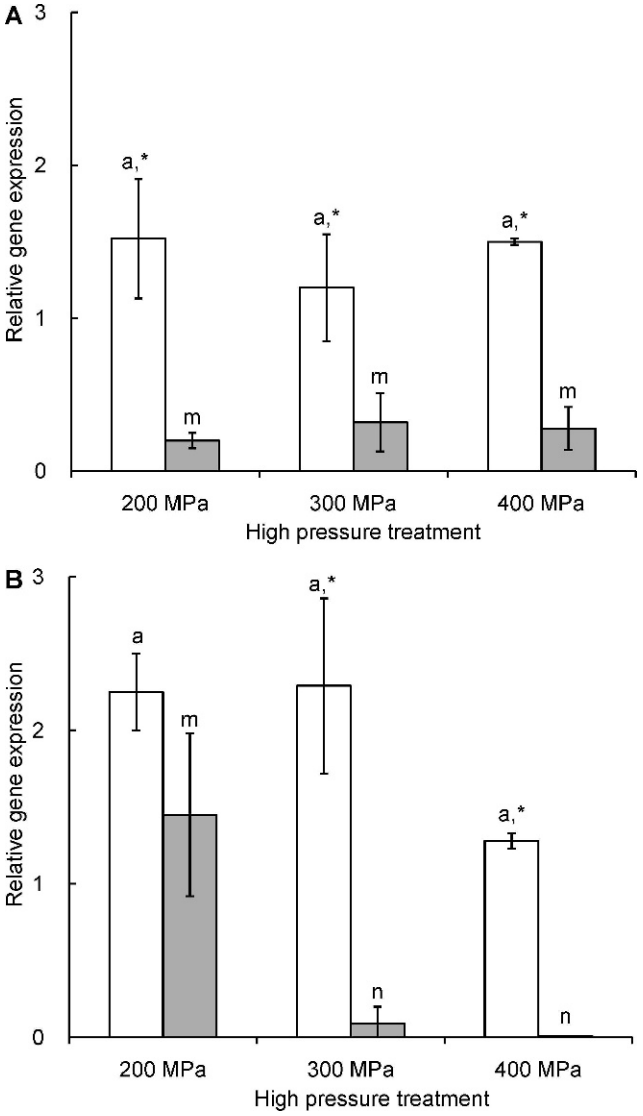

FIGURE 4. *Transcription of* stn *(A) and* invA *(B) relative to the untreated control in* Salmonella *Typhimurium in DIW (□) and nisin (■) solutions treated at 200, 300, and 400 MPa for 0 min. Means with different letters within DIW samples (a and b) and nisin samples (m and n) are significantly different at* $P < 0.05$. *An asterisk indicates a significant difference between DIW and nisin solutions at* $P < 0.05$.

repressed by nisin (Fig. 4B). The relative levels of *invA* expression in nisin samples were significantly decreased from 1.45 to 0.01 with increasing pressures from 200 to 400 MPa, respectively ($P < 0.05$). The enterotoxin gene (*stn*) and the invasion gene (*invA*) in *Salmonella* Typhimurium suspended in DIW were overexpressed in response to high pressure, suggesting that virulence and invasion ability may be enhanced by HPP. In previous studies, enhanced virulence of and invasion by *Salmonella* Typhimurium, *L. monocytogenes, Vibrio chloerae, Pseudomonas aeruginosa,* and *Yersinia* spp. were observed in response to heat, acid, starvation, and osmotic stresses *(1, 8, 21)*. However, *stn* and *invA* were suppressed by the addition of nisin under HPP.

In *L. monocytogenes,* the *inlA* transcripts were relatively down-regulated in DIW and nisin solutions treated at 200, 300, and 400 MPa for 0 min (Fig. 5). The

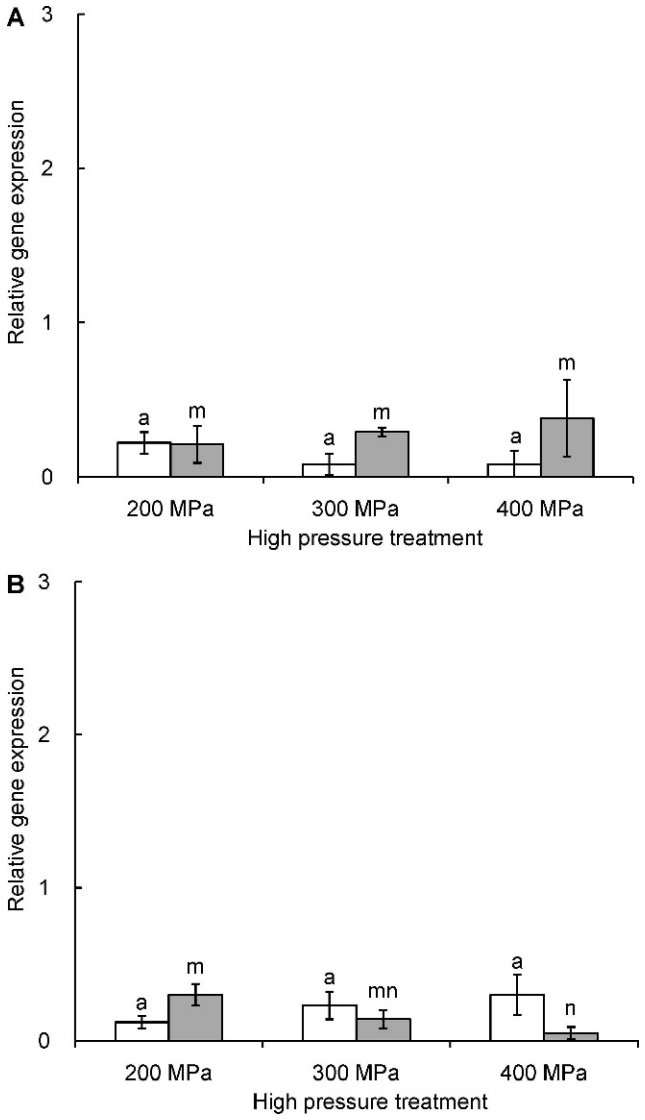

FIGURE 5. *Transcription of* prfA *(A) and* inlA *(B) relative to the untreated control in* L. monocytogenes *in DIW (□) and nisin (■) solutions treated at 200, 300, and 400 MPa for 0 min. Means with different letters within DIW samples (a and b) and nisin samples (m and n) are significantly different at* $P < 0.05$.

expression of *prfA,* the virulence gene regulator in *L. monocytogenes,* also was down-regulated in DIW and nisin solutions after HPP, leading to decreased virulence. This finding is in agreement with those of a previous study, in which the PrfA regulon was suppressed by high-pressure treatment *(3)*. Because *prfA* is involved in the coordinate expression of the attachment, invasion, and virulence genes *(36)*, the expression of *inlA* is likely to be affected by the PrfA regulon. The expression of *inlA* in *L. monocytogenes,* which is as severely invasive foodborne pathogen, was significantly decreased by the HPP treatment ($P < 0.05$).

In conclusion, the most significant findings were (i) the combined treatment with high pressure and nisin synergistically enhanced the inactivation of *Salmonella* Typhimurium and *L. monocytogenes,* (ii) flow cytometric analysis was a reliable method to evaluate the susceptibility of foodborne pathogens to the HPP combined with antimicrobial treatment, and (iii) the expression of virulence-associated genes in *Salmonella* Typhimurium and *L. monocytogenes* was significantly repressed by the combined HPP and nisin treatment. HPP is more effective for inactivating foodborne pathogens when used with antimicrobials, leading to increased food quality and safety. This study also provides important insights into the molecular changes that occur as a result of HPP. This information should be useful for designing an optimized HPP protocol for enhancing the lethality of the process and reducing the pathogenicity of various microorganisms.

ACKNOWLEDGMENTS

This work was supported by a grant (20070401034013) from the BioGreen 21 Program, Rural Development Administration, Republic of Korea. We also thank Ms. Jung-Hee Park in the Central Laboratory of the Kangwon National University (KNU) for the help of her technical assistant in the analytical field using flow cytometry (FCM).

REFERENCES

1. Archer, D. L. 1996. Preservation microbiology and safety: evidence that stress enhances virulence and triggers adaptive mutations. *Trends Food Sci. Technol.* 7:91–95.
2. Black, E. P., A. L. Kelly, and G. F. Fitzgerald. 2005. The combined effect of high pressure and nisin on inactivation of microorganisms in milk. *Innov. Food Sci. Emerg. Technol.* 6:286–292.
3. Bowman, J. P., C. R. Bittencourt, and T. Ross. 2008. Differential gene expression of *Listeria monocytogenes* during high hydrostatic pressure processing. *Microbiology* 154:462–475.
4. Chaturongakul, S., S. Raengpradub, M. Wiedmann, and K. J. Boor. 2008. Modulation of stress and virulence in *Listeria monocytogenes*. *Trends Microbiol.* 16:388–396.
5. Chen, H., and D. G. Hoover. 2004. Use of Weibull model to describe and predict pressure inactivation of *Listeria monocytogenes* Scott A in whole milk. *Innov. Food Sci. Emerg. Technol.* 5:269–276.
6. Chopra, A. K., J. H. Huang, X. Xu, K. Burden, D. W. Niesel, M. W. Rosenbaum, V. L. Popov, and J. W. Peterson. 1999. Role of *Salmonella* enterotoxin in overall virulence of the organism. *Microbial Pathog.* 27:155–171.
7. Chopra, A. K., X. J. Xu, and J. W. Peterson. 1994. *Salmonella typhimurium* enterotoxin epitopes shared among bacteria. *FEMS Microbiol. Lett.* 118:237–242.
8. Chowdhury, R., G. Sahu, and J. Das. 1996. Stress response in pathogenic bacteria. *J. Biosci.* 21:149–160.
9. D'Souza, D. H., F. J. Critzer, and D. A. Golden. 2009. Real-time reverse transcriptase polymerase chain reaction for the rapid detection of *Salmonella* using *invA* primers. *Foodborne Pathog. Dis.* 6:1097–1106.
10. Earnshaw, R. G., J. Appleyard, and R. M. Hurst. 1995. Understanding physical inactivation processes: combined preservation opportunities using heat, ultrasound and pressure. *Int. J. Food Microbiol.* 28: 197–219.
11. Erkan, N., and G. Üretener. 2010. The effect of high hydrostatic pressure on the microbiological, chemical and sensory quality of fresh gilthead sea bream (*Sparus aurata*). *Eur. Food Res. Technol.* 230: 533–542.
12. Farber, J. M., and P. I. Peterkin. 1991. *Listeria monocytogenes*: a foodborne pathogen. *Microbiol. Rev.* 55:476–511.
13. Garcia-Graells, C., I. van Opstal, S. C. M. Vanmuysen, and C. W. Michiels. 2003. The lactoperoxidase system increases efficacy of high-pressure inactivation of foodborne bacteria. *Int. J. Food Microbiol.* 81:211–221.
14. Garriga, M., N. Grebol, M. T. Aymerich, J. M. Monfort, and M. Hugas. 2004. Microbial inactivation after high-pressure processing at 600 MPa in commercial meat products over its shelf life. *Innov. Food Sci. Emerg. Technol.* 5:451–457.
15. González-Escalona, N., T. S. Hammack, M. Russell, A. P. Jacobson, A. J. De Jesús, E. W. Brown, and K. A. Lampel. 2009. Detection of

live *Salmonella* sp. cells in produce by a TaqMan-based quantitative reverse transcriptase real-time PCR targeting *invA* mRNA. *Appl. Environ. Microbiol.* 75:3714–3720.
16. Kalchayanand, N., P. Dunne, A. Sikes, and B. Ray. 2004. Viability loss and morphology change of foodborne pathogens following exposure to hydrostatic pressure in the presence and absence of bacteriocins. *Int. J. Food Microbiol.* 91:91–98.
17. Kingsley, D. H., D. R. Holliman, K. R. Calci, H. Chen, and G. J. Flick. 2007. Inactivation of a norovirus by high-pressure processing. *Appl. Environ. Microbiol.* 73:581–585.
18. Lee, J., and G. Kaletun. 2010. Inactivation of *Salmonella* Enteritidis strains by combination of high hydrostatic pressure and nisin. *Int. J. Food Microbiol.* 140:49–56.
19. Masschalck, B., C. Garcia-Graells, E. Van Haver, and C. W. Michiels. 2000. Inactivation of high pressure resistant *Escherichia coli* by lysozyme and nisin under high pressure. *Innov. Food Sci. Emerg. Technol.* 1:39–47.
20. Matser, A. M., B. Krebbers, R. W. van den Berg, and P. V. Bartels. 2004. Advantages of high pressure sterilisation on quality of food products. *Trends Food Sci. Technol.* 15:79–85.
21. Mekalanos, J. J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.* 174:1–7.
22. Miller, J. S., and J. M. Quarles. 1990. Flow cytometric identification of microorganisms by dual staining with FITC and PI. *Cytometry* 11: 667–675.
23. Moerman, F. 2005. High hydrostatic pressure inactivation of vegetative microorganisms, aerobic and anaerobic spores in pork Marengo, a low acidic particulate food product. *Meat Sci.* 69:225–232.
24. Moore, M. M., and M. D. Feist. 2007. Real-time PCR method for *Salmonella* spp. targeting the *stn* gene. *J. Appl. Microbiol.* 102:516–530.
25. Murchie, L. W., M. Cruz-Romero, J. P. Kerry, M. Linton, M. F. Patterson, M. Smiddy, and A. L. Kelly. 2005. High pressure processing of shellfish: a review of microbiological and other quality aspects. *Innov. Food Sci. Emerg. Technol.* 6:257–270.
26. Mussa, D. M., H. S. Ramaswamy, and J. P. Smith. 1999. High pressure destruction kinetics of *Listeria monocytogenes* on pork. *J. Food Prot.* 62:40–45.
27. Olesen, I., L. Thorsen, and L. Jespersen. 2010. Relative transcription of *Listeria monocytogenes* virulence genes in liver pâtés with varying NaCl content. *Int. J. Food Microbiol.* DOI: 10.1016/j.ijfoodmicro.2010.01.042.
28. Palou, E., A. Lopez-Malo, G. V. Barbosa-Canovas, J. Welti-Chanes, and B. G. Swanson. 1997. Kinetic analysis of *Zygosaccharomyces bailii* inactivation by high hydrostatic pressure. *LWT Food Sci. Technol.* 30:703–708.
29. Pfeifer, C. G., S. L. Marcus, O. Steele-Mortimer, L. A. Knodler, and B. B. Finlay. 1999. *Salmonella typhimurium* virulence genes are induced upon bacterial invasion into phagocytic and nonphagocytic cells. *Infect. Immun.* 67:5690–5698.
30. Ponce, E., R. Pla, E. Sendra, B. Guamis, and M. Mor-Mur. 1998. Combined effect of nisin and high hydrostatic pressure on destruction of *Listeria innocua* and *Escherichia coli* in liquid whole egg. *Int. J. Food Microbiol.* 43:15–19.
31. Ross, A. I. V., M. W. Griffiths, G. S. Mittal, and H. C. Deeth. 2003. Combining nonthermal technologies to control foodborne microorganisms. *Int. J. Food Microbiol.* 89:125–138.
32. San Martin, M. F., G. V. Barbosa-Canovas, and B. G. Swanson. 2002. Food processing by high hydrostatic pressure. *Crit. Rev. Food Sci. Nutr.* 46:627–645.
33. Solomon, E. B., and D. G. Hoover. 2004. Inactivation of *Campylobacter jejuni* by high hydrostatic pressure. *Lett. Appl. Microbiol.* 38:505–509.
34. Stevens, K. A., N. A. Klapes, B. W. Sheldon, and T. R. Klaenhammer. 1992. Antimicrobial action of nisin against *Salmonella typhimurium* liposaccharide mutants. *Appl. Environ. Microbiol.* 58: 1786–1788.
35. Stevens, K. A., B. W. Sheldon, N. A. Klapes, and T. R. Klaenhammer. 1991. Nisin treatment for inactivation of *Salmonella* species and other gram negative bacteria. *Appl. Environ. Microbiol.* 57:3613–3615.
36. Stoll, R., S. Mertins, B. Joseph, S. Muller-Altrock, and W. Goebel. 2008. Modulation of PrfA activity in *Listeria monocytogenes* upon growth in different culture media. *Microbiology* 154:3856–3876.
37. ter Steeg, P. F., J. C. Hellemons, and A. E. Kok. 1999. Synergistic actions of nisin, sublethal ultrahigh pressure, and reduced temperature on bacteria and yeast. *Appl. Environ. Microbiol.* 65:4148–4154.
38. Tolker-Nielsen, T., M. H. Larsen, H. Kyed, and S. Molin. 1997. Effects of stress treatments on the detection of *Salmonella typhimurium* by in situ hybridization. *Int. J. Food Microbiol.* 35: 251–258.
39. Torres, J. A., and G. Velazquez. 2005. Commercial opportunities and research challenges in the high pressure processing of foods. *J. Food Eng.* 67:95–112.
40. van Boekel, M. A. J. S. 2002. On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *Int. J. Food Microbiol.* 74:139–159.
41. Vurma, M., Y. K. Chung, T. H. Shellhammer, E. J. Turek, and A. E. Yousef. 2006. Use of phenolic compounds for sensitizing *Listeria monocytogenes* to high-pressure processing. *Int. J. Food Microbiol.* 106:263–269.
42. Yaldagard, M., S. A. Mortazavi, and F. Tabatabaie. 2008. The principles of ultra high pressure technology and its application in food processing/preservation: a review of microbiological and quality aspects. *Afr. J. Biotechnol.* 7:2739–2767.