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High Pressure Inactivation Kinetics of *Salmonella enterica* and *Listeria monocytogenes* in Milk, Orange Juice, and Tomato Juice

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Abstract Effects of pressure come-up and holding times on the inactivation of *Salmonella enterica* and *Listeria monocytogenes* were evaluated in deionized water, milk, orange juice, and tomato juice with pH 6.76, 6.85, 3.46, and 4.11, respectively. The inoculated samples were subjected to high pressure treatments at 300, 400, and 500 MPa for less than10 min at 30°C. At 500 MPa, the numbers of *S. enterica* and *L. monocytogenes* in deionized water, orange juice, and tomato juice were reduced by more than 6 log CFU/mL during the come-up time. Compared to orange and tomato juices, milk showed a considerable baroprotective effect against *S. enterica* and *L. monocytogenes*. At 300 MPa, the *D* values for *S. enterica* in milk, orange juice, and tomato juice were 0.94, 0.41, and 0.45 min, while those for *L. monocytogenes* were 9.56, 1.11, and 0.94 min, respectively. Low pH resulted in a noticeable synergistic effect on the inactivation of *S. enterica* and *L. monocytogenes* in orange and tomato juices. Therefore, these results might provide more useful information for designing the entire high hydrostatic pressure (HHP) conditions, taking the come-up time reduction, and food system.

Keywords: pressure come-up time, pressure holding time, baroprotective effect, *Salmonella enterica*, *Listeria monocytogenes*

Introduction

High hydrostatic pressure (HHP) treatment of food provides not only process-technological advantages such as homogeneous temperature distribution and non-thermal process, but also a significant improvement in terms of microbiological safety and quality which fulfill the current consumer expectations such as minimal adverse effects in the taste, texture, color, and nutritional value (1-4). In recent years, HHP has received much attention due to its potential application in the food industry as an alternative to conventional thermal processing. In particular, HHP processing of low-acid foods is of great interest because it can lead to effective inactivation of harmful foodborne pathogens at low temperature with less adverse impact on food quality (1).

During HHP processing, the food product is subjected to pressure, which converts mechanical energy into internal energy, through 3 phases, including the come-up time (cell volume decrease), pressure holding time (irreversible mass transfer), and decompression time (cell membrane rupture) (5,6). HHP processing between 300 and 800 MPa can eliminate most vegetative microorganisms such as *Campylobacter jejuni*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* (7-10). For commercial-scale application, microbiological challenge studies are necessary to understand HHP inactivation characteristics of foodborne pathogens in different food systems. Establishing a safe and efficient HHP process requires proper knowledge related to the inactivation behaviors of various target foodborne pathogens in different food systems.

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Food constituents such as fats, carbohydrates, and proteins may also provide a protective effect during high pressure processing (7,10-12). Pressure denaturation of proteins causes the disruption of hydrophobic and electrostatic interactions and the solubility of water in fats (13,14). Carbohydrates such as glycerol, glucose, fructose, sucrose, and trehalose show the protective effects on the reduction of microorganisms during pressure processing (15). Lower pH can increase the efficacy of high pressure processing (11,16). During the HHP processing, the ionization of weak acids as function of pressure and temperature is resulted in pH drop (11). Pressure directly influences the dissociation of water and buffers and eventually lowers pH (11). Therefore, the application of HHP with low pH and other preservatives such as nisin, lactoperoxidase, argon, and carbon dioxide could enhance the bacterial inactivation (17-20).

There have been many studies with regard to the HHP inactivation in various food systems (21-28). However, relatively little attention has been paid to the efficacy of pressure come-up time on reduction of microorganisms in different food systems. HHP inactivation characteristics determined under well-defined process conditions would be beneficial for food processors. Therefore, the objective of this study was to examine the pressure come-up reductions and inactivation kinetics of Gram-negative *S. enterica* and Gram-positive *L. monocytogenes* in milk, orange juice, and tomato juice.

Materials and Methods

Bacterial strains and culture conditions Strains of *Listeria monocytogenes* (KACC 12671) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis (KACC 10763) were provided by the Korean Agricultural Culture Collection (KACC; Suwon, Korea). The strains were cultivated

Table 1. Typical pressure and temperature conditions during high hydrostatic pressure (HHP) treatment

Target pressure (MPa)	Preheating time (min)	Conditions within the high pressure processor		
		Jacket temperature (°C)	Initial temperature[1)] (°C)	Target temperature[2)] (°C)
300	10	30.0±1.0	22.4±0.9	30.2±1.0
400	10	30.0±1.0	20.8±1.0	30.8±1.2
500	10	30.0±1.0	18.1±0.9	29.9±1.1

[1)]Sample temperature before compression.
[2)]Temperature just before depressurization.

aerobically in trypticase soy broth supplemented with 0.1% yeast extract (TSBYE; BD, Becton, Dickinson and Co., Sparks, MD, USA) at 37°C for 22 hr. The cultures were harvested at 3,000×g for 20 min at 4°C and resuspended to original volume in 0.1% sterile peptone water (PW) for inoculation.

Sample preparation Deionized water was used as a control. Milk (pH 6.85, Aw 0.993, fat 3.2%, carbohydrate 3.7%) was selected as representative of low-acid food. Orange juice (pH 3.46, Aw 0.995, fat≈0, carbohydrate 4.1%) and tomato juice (pH 4.11, Aw 0.992, fat≈0, carbohydrate 3.3%) were selected as representatives of acid foods with different pH values. The samples were inoculated separately with approximately 2.0×10^7 CFU/g of *L. monocytogenes* or *S. enterica*. The inoculated samples (2 mL each) were packaged in sterile bag (Fisher Scientific, Pittsburgh, PA, USA) and then heat-sealed (Impulse Bag Sealer, American International Electric, Whittier, CA, USA).

High pressure treatment In order to achieve the desired final process temperature, the initial temperatures of test samples were adjusted as a function of final target pressure (Table 1). The water jacket temperature was adjusted to a temperature close to the samples temperature, taking the heat of compression of water into account. The inoculated samples were subjected to combinations of pressure (300, 400, or 500 MPa) and heat (30°C) for different hold time intervals (0, 1, 2, 3, 5, 7, and 10 min) using a high hydrostatic pressure (HHP) tester (Ilshin Autoclave Co., Deajeon, Korea). The pressure come-up times were approximately 1.50, 2.50, and 3.83 min at 300, 400, and 500 MPa, respectively. The depressurization time (<2 sec) was not included in the process hold-time. After depressurization, the samples were cooled immediately in an ice bath to avoid further inactivation.

Microbial analysis Pouches containing the HHP-treated samples were opened aseptically, and viable counts were determined. The samples (1 mL each) were mixed with 10 mL of 0.1% sterile PW. Dilutions of the HHP-treated samples were serially (1:10) prepared with 0.1% sterile PW. The sample dilutions (0.1 mL) were plated on the surface of trypticase soy agar (TSA). The agar plates were incubated to determine the populations of *L. monocytogenes* or *S. enterica* at 37°C for 24 to 48 hr.

Inactivation kinetics The kinetic parameters were analyzed using first order reaction kinetics. *D* values, the time required for 90% reduction in the initial bacterial population, were calculated at the initial linear portion of inactivation curves, assuming the logarithmic number of bacteria is a linear function of treatment time (29,30):

$$\mathrm{Log}\frac{N}{N_i}=-\frac{t}{D} \quad (1)$$

where N_i and N are the inoculum of bacterial cells and the number of bacterial cells at time t.

The data were also fitted using the biphasic model, which describe the biphasic inactivation kinetics of microorganisms (31,32):

$$\mathrm{Log}(N_0/N_i)=\log[2f/(1+e^{k_1 t})]+\log[2(1-f)/(1+e^{k_2 t})] \quad (2)$$

where N_i is the inoculum level, N_0 is the bacterial population immediately after process come-up time, N is the bacterial population at time t, k_1 is the inactivation rate at the first fraction of the survival curve, k_2 is the inactivation rate at the second fraction of the survival curve, and f is the initial resistant proportion at the first fraction of the survival curve.

Statistical analysis Three independent experiments were conducted and each experiment was replicated in duplicate. Data were analyzed using the Statistical Analysis System software (SAS 8.2; SAS Institute Inc, Cary, NC, USA). The general linear model (GLM) and least significant difference (LSD) procedures were used to compare means. Significant mean differences were estimated by Fisher's least significant difference (LSD) at $p<0.05$.

Results and Discussion

Inactivation of *S. enterica* during the pressure come-up time The come-up time reductions of *S. enterica* inoculated in various food systems treated with 300, 400, and 500 MPa at the target temperature of 30°C are shown in Fig. 1. Considerable reductions were observed during the come-up time for all treatments. The magnitude of the log reduction was most obvious in orange juice and tomato juice during the come-up time, followed by deionized water. The results suggest that the come-up time reductions ($\log N_0/N_i$) needs to be taken into account in kinetic models for determining the HHP resistance of microorganisms (5,33). Therefore, this finding highlights the importance of documenting the pressure come-up time and corresponding log-reduction during HHP inactivation studies. The reductions of *S. enterica* cells increased with increasing pressure level from 300 to 500 MPa. The least amount of inactivation of *S. enterica* was observed in milk treated at 300 MPa during the come-up time. *S. enterica* in milk had approximately 3 log CFU/mL survivors after 10 min pressure holding time

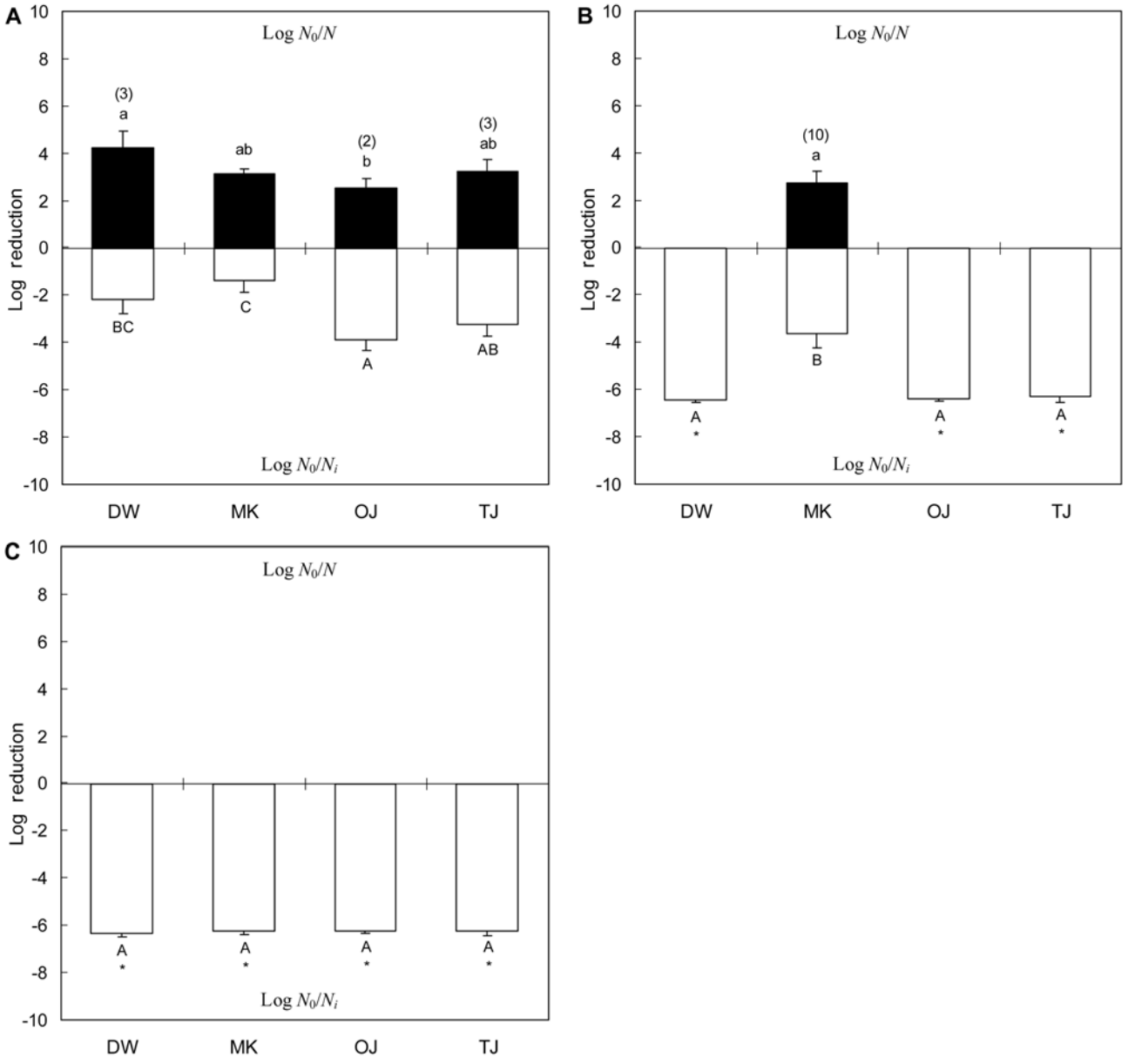

Fig. 1. Process come-up time reduction (open bar, □; log N_0/N_i) and pressure holding time reduction (closed bar, ■; log N_0/N) of *S. enterica* in deionized water (DW), milk (MK), orange juice (OJ), and tomato juice (TJ) treated at 30°C under 300 (A), 400 (B), and 500 MPa (C). Log reductions with different letters on the bar are significantly different at $p<0.05$. N_i and N_0 represent the inoculum level and the bacterial population immediately after process come-up time, respectively. The initial population (N_i) was approximately 7.35 log CFU/mL. (*) indicates that N_i was reduced to below the detection limit (<1 log CFU/mL) during the come-up time. Value in parenthesis indicates the pressure holding time (min) that N_i was reduced to below the detection limit.

(Fig. 1A). The numbers of *S. enterica* in all samples treated at 400 and 500 MPa were reduced to below the detection limit of 1 log CFU/mL (>6 log reduction) during the come-up time, except those in milk treated at 400 MPa that the bacterial population was reduced by 3.64 during the come-up time (Fig. 1B and 1C). The results suggest that food protective effect on the HHP inactivation of bacterial cells is associated with food components (34). The pressure-resistance of bacterial cells in milk might be attributed to the composition of milk (35-37). Thus, in particular, the higher fat content might have contributed to the HHP resistance of *S. enterica* inoculated in milk when compared to orange and tomato juices.

Inactivation of *L. monocytogenes* during the pressure come-up time The reductions of *L. monocytogenes* in different food systems treated at 300, 400, and 500 MPa are shown in Fig. 2. Similar to *S. enterica*, HHP effect on the reduction of *L. monocytogenes* was noticeable during the come-up time with increasing pressure level. The numbers of *L. monocytogenes* in deionized water, orange juice, and tomato juice treated at 300 MPa were reduced by approximately 1 log CFU/mL during the come-up time, whereas those at 500 MPa were reduced by more than 6 log CFU/mL (Fig. 2A and 2C). However, the number of *L. monocytogenes* cells in milk treated at 500 MPa still remained at the detectable level of approximately 1.51 log

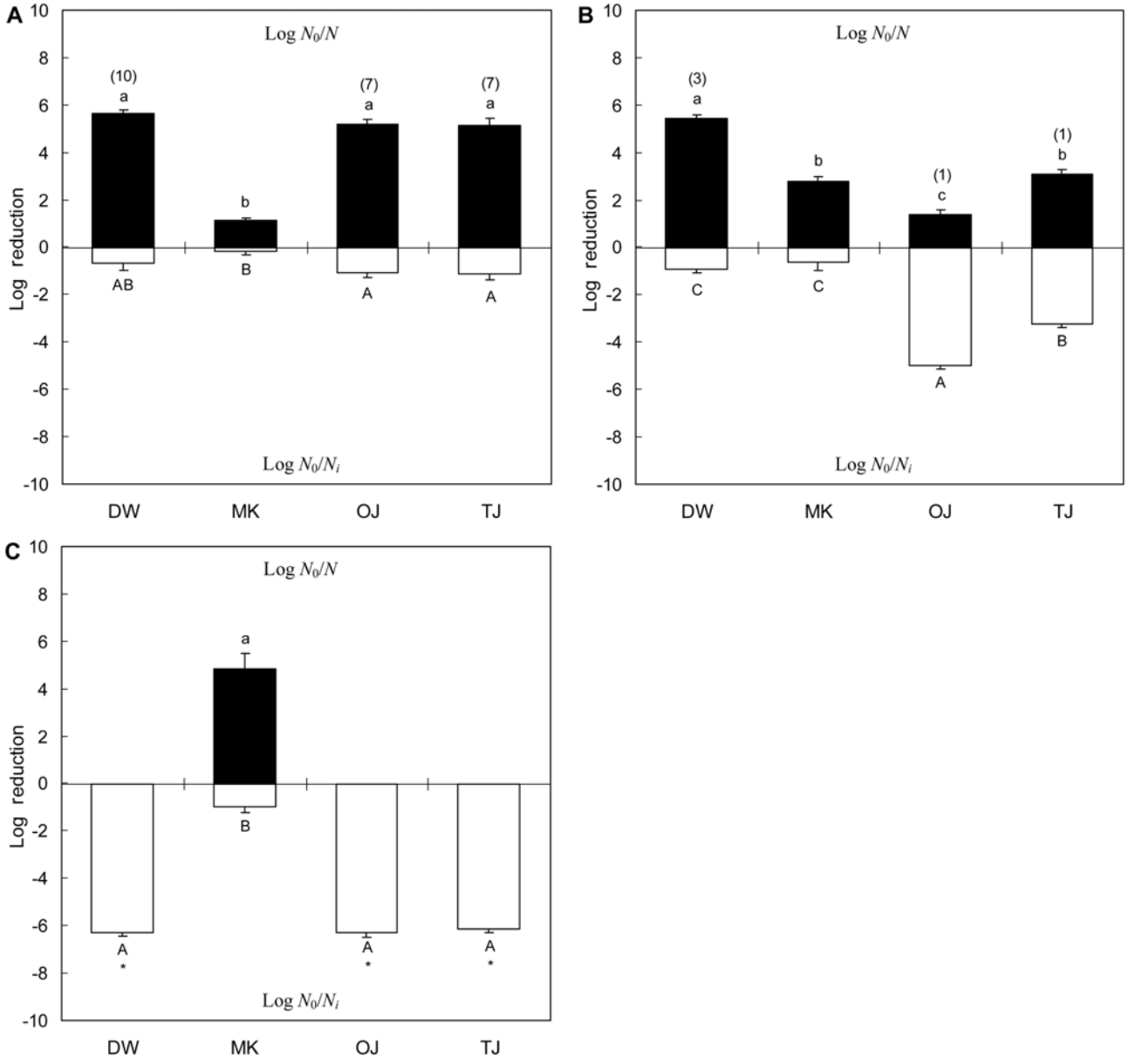

Fig. 2. Process come-up time reduction (open bar, □; log N_0/N_i) and pressure holding time reduction (closed bar, ■; log N_0/N) of *L. monocytogenes* in deionized water (DW), milk (MK), orange juice (OJ), and tomato juice (TJ) treated at 30°C under 300 (A), 400 (B), and 500 MPa (C). The initial population (N_i) was approximately 7.31 log CFU/mL

CFU/mL (Fig. 2C). *L. monocytogenes* cells inoculated in orange juice and tomato juice were more sensitive to HHP than those in milk. Like *S. enterica* (Fig. 1), this might be due to the baroprotective compounds present in foods (34). After the entire pressure process at 300, 400, and 500 MPa, the numbers of *L. monocytogenes* in orange juice (pH 3.46) and tomato juice (pH 4.11) were reduced to below the detection level (Fig. 2). This result suggests that HHP may synergistically act with low pH (orange and tomato juices) to inactivate *L. monocytogenes*. Previous studies showed that bacterial cells are more susceptible to high pressure when exposed to low pH (11,16). To achieve complete bacterial inactivation, it might be necessary to explore synergistic ways of HHP combined with additional barriers such as low pH and antimicrobials.

Pressure holding time reductions of microorganisms in different food systems The inactivation of *S. enterica* and *L. monocytogenes* was accelerated with increasing pressure level. However, *S. enterica* cells in various food systems were more pressure sensitive than *L. monocytogenes*, resulting in shorter HHP processing time to achieve a corresponding reduction. As shown in Fig. 1A and 2A, *S. enterica* cells in deionized water, orange juice, and tomato juice were inactivated by more than 6 log CFU/mL after 3, 2, and 3 min pressure holding time, respectively, at 300 MPa, whereas the corresponding reductions of *L. monocytogenes* in deionized water, orange juice, and tomato juice was achieved after 10, 7, and 7 min pressure holding times. The HHP inactivation patterns of *S. enterica* and *L. monocytogenes* in deionized water, orange juice, and

Table 2. Kinetic parameter of log-linear for *S. enterica* and *L. monocytogenes* in different food samples treated at 30°C under 300, 400, and 500 MPa

Pressure (MPa)	Sample	*D* value (min)[1)]	
		S. enterica	*L. monocytogenes*
300	Deionized water	0.63±0.02[b]	1.40±0.15[b]
	Milk	0.94±0.13[a]	9.56±2.20[a]
	Orange juice	0.41±0.03[c]	1.11±0.08[b]
	Tomato juice	0.45±0.04[bc]	0.94±0.09[b]
400	Deionized water	NC[2)]	1.12±0.03[b]
	Milk	0.79±0.06	3.84±0.53[a]
	Orange juice	NC	0.53±0.01[b]
	Tomato juice	NC	0.61±0.01[b]
500	Deionized water	NC	NC
	Milk	NC	2.45±0.01
	Orange juice	NC	NC
	Tomato juice	NC	NC

[1)]Means with different subscripts within a row (a-c) are significantly different at $p<0.05$.
[2)]Not calculated due to the lack of data points during the come-up time.

tomato juice followed first-order reaction kinetics, which is in good agreement with previous reports that the pressure inactivation of microorganisms exhibited a log-linear behavior (23,38,39). However, the patterns of HHP inactivation of *S. enterica* and *L. monocytogenes* in milk exhibited biphasic phenomenon, including a rapid initial decline followed by a characteristic tailing during the extended pressure holding time. This observation is in agreement with previous reports in which the biphasic model well described the pressure inactivation kinetics of *L. monocytogenes* in milk (30). HHP inactivation showed biphasic behavior because of the presence of 2 different resistant sub-populations to pressure (40). The degree of HHP inactivation of bacterial cells varied, depending on many intrinsic and extrinsic factors such as bacterial strain, pH, Aw, medium composition, pressure level, processing temperature, and food composition (4,9). No typical shouldering was observed in this study, indicating that the HHP may be responsible for lethal effect on the inactivation of *S. enterica* and *L. monocytogenes* rather than cumulative effect causing sublethally injured cells.

Inactivation kinetics of microorganisms in different food systems The *D* values for *S. enterica* and *L. monocytogenes* were well-fitted by the first-order kinetic model (Table 2). The degree of HHP resistance of *S. enterica* and *L. monocytogenes* inoculated in different food systems decreased with increasing pressure from 300 to 500 MPa. The *D* values for *L. monocytogenes* in milk were 9.56, 3.84, and 2.45 min at 300, 400, and 500 MPa, respectively. The *D* values for *L. monocytogenes* were significantly higher than those for *S. enterica* in all food samples, indicating that *L. monocytogenes* is more pressure resistant than *S. enterica*. This observation is in good agreement with previous reports that Gram-positive bacteria are more pressure resistant than Gram-negative bacteria (41,42). The linear model adequately described the HHP inactivation of *S. enterica* and *L. monocytogenes* in various food systems. The regression coefficients (R^2) values were more than 0.936, showing the goodness-of-fit of the linear model. The survival curves of *S. enterica* inoculated in milk treated at 300 and 400 MPa had slope tailing, which was fitted using biphasic model. However, because the other survival curves were close to linearity having level tailing, the log-linear model was fitted to calculate the *D* values (Table 2). The kinetic parameters (k_1, k_2) estimated from biphasic model for *S. enterica* in milk were 1.54 and 0.09 (R^2=0.991) at 300 MPa, respectively, and 1.85 and 0.18 (R^2=0.998) at 500 MPa, indicating upward concavity ($k_1>k_2$ and $f\approx1$). This observation suggests that the remaining survivors are more HHP resistant due to the presence of heterogeneous resistant cells, resulting in extended tailing phenomenon. Therefore, the k_1 and k_2 values could be used as reliable indicators of HHP resistance of foodborne pathogens.

In conclusion, the most significant finding in this study was that *S. enterica* and *L. monocytogenes* were effectively inactivated during the pressure come-up and holding times. Because a significant reduction in the number of *S. enterica* and *L. monocytogenes* in various food systems was observed during the come-up time that would be considered as an important variable for industrial-scale application of HHP. The results suggest that the come-up time and food systems should be taken into consideration in inactivating pathogenic microorganisms under high pressure. Therefore, the results obtained in this study provide practical information to better understand the HHP inactivation characteristics of microorganisms in food systems. It is worthwhile to generate HHP data for modeling microbial inactivation in various food systems and develop an optimized process-based solution for enhancing food safety.

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