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REVIEW

Effects of high hydrostatic pressure on *Salmonella typhimurium* and aerobic bacteria in milk and fruit juices

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Abstract

Effects of high hydrostatic pressure (HHP) on Salmonella typhimurium inoculated in broth and foods were studied from 200 to 400 MPa. No growth occurred in broth after 50, 30 and 25 min of pressurization at 300, 350 and 400 MPa respectively. S. typhimurium was completely inactivated within 45 min at 400 MPa in raw milk, while aerobic bacteria (AB) were reduced by 6.51 log cfu/ml at the same condition. In orange juices, S. typhimurium and AB were completely (7.04 log cfu/ml) inactivated at 400 MPa pressure after 10 and 15 min respectively. The injury of cells in broth at 300 MPa ranged from 42.5 to 74.3 % depending on magnitude of pressure and treated time. The pH, temperature, types of media and age of cell significantly ($P<0.05$) affected the response of bacteria to pressure.

Keywords: high hydrostatic pressure, *Salmonella typhimurium*, injury, milk, fruit juice

Introduction

Since the 1950s, foodborne salmonellosis has been the major cause of foodborne diseases. Estimates of human foodborne salmonellosis in the United States vary from 740,000 to 5,300,000 cases annually[1]. The salmonellosis may be due to the presence of large number of *Salmonella* species in animals, birds, pets, insects and humans, increasing farming of food animals and birds, processing and marketing of foods[2,3].

A major advantage of nonthermal methods of food preservation is that they inactivate microorganisms without the need of severe heating and therefore cause minimum damage to the flavor, color, texture or nutritional value of the food[4]. Pressure treatment (from 200 to 600 MPa) at ambient temperature sufficiently inactivates vegetative bacteria for the purpose of food pasteurization[5-8]. High hydrostatic pressure (HHP) technology (200-1000 MPa) permits microbial inactivation at low or moderate temperatures and has been investigated to destroy foodborne microorganisms[9-11]. The system is also energy efficient, economical and producing consumer acceptance foods[12,13]. Some species of foodborne pathogens contain strains that are relatively resistant to pressure[14-16]. This could be the reason for the variation in results obtained by researchers using different strains of the same species. In the present study, survival of *Salmonella typhimurium* in tryptone soy broth, raw milk, orange juice and peach juice was investigated with exposure to HHP from 200 to 400 MPa at different temperatures (from 15 to 45^{o}C) and pHs (from. 5.0 to 7.0). Injury of *S. typhimurium* in broth and behavior of *S. typhimurium* depending on age were also studied at 300 MPa. In previous reports[12,17], some of the results obtained from this research were evaluated with kinetic and modelling analysis.

Materials and Methods

Bacterial culture preparation

Salmonella typhimurium KUEN 1357 was obtained from University of İstanbul, Faculty of Medicine, Microorganism's Culture Collection Center, İstanbul, Turkey. The cultures were maintained on tryptone soy agar (TSA; Difco, Detroit) slants and stored at 4°C. The cultures for experiments were subcultured twice from slant culture by inoculating into 10 ml of tryptone soy broth (TSB; Difco, Detroit), and incubated at 35°C for 18 h.

Preparation of cell suspensions for pressurization

Sub-cultured *S. typhimurium* was inoculated into TSB (pH=7.0) in duplicate and one of them was incubated at 35°C for 18 h (young culture) and the other was incubated at 35°C for 5 days (old culture). The young and old cultures were diluted using sterile TSB to obtain a cell number about $5.75x10^7$ cfu/ml.

The young culture (about 250 ml) was centrifuged at 4000 g for 30 min under aseptic conditions. The cell pellets were resuspended in 10 ml of sterile physiological saline (PS; 0.85 % NaCl solution, pH=7.0) and recentrifuged, cell pellets were again resuspended in 10 ml of PS. About 1 % (v/v) of suspended PS cell culture was added into 100 ml of raw milk (pH=6.65), fresh peach juice (pH=5.21) and fresh orange juice (pH=3.55). The final number of *S. typhimurium* was $2.29x10^7$, $2.95x10^7$ and $1.10x10^7$ cfu ml^{-1} of raw milk, peach juice and orange juice respectively. Initial aerobic bacteria (AB) counts in raw milk, fresh peach juice and fresh orange juice were 7.30, 7.28 and 7.28 respectively. Raw milk without *S. t*yphimurium addition (with $3.02x10^5$ cfu ml^{-1} natural flora) was also used in the pressure treatment. Peaches and oranges were purchased from a local market and processed at commercial maturity. The fruits were cleaned, cored and juiced with a juice extractor (Frutto, Arzum, Arzum Dış Ticaret ve Pazarlama A.Ş., İstanbul, Turkey). Then the juices were filtered through a 4-layer cheese cloth and freshly used in the HHP treatment.

Equipment

A hydrostatic pressure vessel (internal diameter; 4 cm, length, 12 cm; maximum pressure tolerance level=1500 MPa) with internal volume of 150 cm^3 and a hydraulic unit (Kon hidroliksan; hydraulic pressure and manufacturing industry, Inc., Konya, Turkey) were used for high hydrostatic pressurization[18]. The pressure vessel and piston were made of steel (type 45WCRV7) which was processed into the required sizes at the Mechanical Engineering Department, Faculty of Engineering, University of Gaziantep, Gaziantep, Turkey.

High pressure treatment of S. *typhimurium*

Ten ml from each of stock culture was placed into sterile polyethylene bags (sterilized by 0.1 % H_2O_2; 5.5 cmX4.0 cm). The bags were sealed after eliminating air inside. In pressurization study, the following high pressure (HP) experiments were performed: (i) bags containing 18 h culture (young culture) suspensions in TSB (pH=7.0) were exposed to 200, 250, 300, 350 and 400 MPa pressure from 5 to 60 min at 25°C, (ii) bags containing 18 h culture (young culture) suspensions in TSB (pH=7.0) were exposed to 15, 25, 35 and 45°C from 5 to 60 min at 300 MPa pressure, (iii) bags containing 18 h culture (young culture) suspensions in TSB adjusted to four different pH (7.0, 6.0, 5.5 and 5.0) were exposed to 300 MPa pressure from 5 to 60 min at 25°C, (iv) bags containing 5 days culture (old culture) suspensions in TSB (pH=7.0) were exposed to 300 MPa at 25°C, (v) bags containing raw milk, peach juice and orange juice contaminated with *S. typhimurium* (in PS) added were exposed to 400 MPa pressure from 5 to 60 min at 25°C and (vi) bags containing raw milk without *S. typhimurium* addition were exposed to 400 MPa pressure from 5 to 25 min at 25°C.

The vessel (containing deionized water) has been held in water bath (equipped with a temperature controller at 50°C) to heat up to required target temperature, then the vessel was

transferred to another water bath for target temperature and held in this water bath during pressurization. Samples in pressure vessel were precooled at appropriate temperatures to reach the target temperature after pressure build up, taking into consideration the monitored adiabatic heating (approximately 3°C/100 MPa). Pressure and temperature in the chamber were constantly monitored and recorded (in 1 s intervals) during the process.
When the temperature was equilibrated, the bags were placed into vessel. HHP levels were generated using deionized water. The rates of pressure increase and release times were about 100 MPa 5 sec^{-1} and 200 MPa 5 sec^{-1}, respectively, in HP apparatus. Pressurization time reported in this study did not include the pressure increase and release times.

Immediately after pressurization the bags were removed, cooled in an ice bath and used for enumeration (within about 3 min after pressurization) of viable cells (cfu ml^{-1}). When the counts are not detected on plates that were inoculated with 1 ml of treated samples, the result was recorded as zero.

Enumeration of viable and injured cells

Pressurized and control cell suspensions in bags were serially diluted in 0.1 % (wt/vol) sterile peptone (Difco, Detroit) water[19]. Selective agar medium bismuth sulfide agar (BSA; Difco, Detroit) was used in the enumeration of viable *S. typhimurium* cells from pressure treated TSB. Samples from pressure treated TSB cultures of young cells (18 h culture) and old cells (5 days culture) were spread plated in duplicate on BSA and incubated at 35°C for 24 h. Brown, gray, or black characteristic colonies of *S. typhimurium* were counted. The nonselective brain heard infusion agar (BHIA, Difco, Detroit) medium was also used to count *S. typhimurium* from pressure treated TSB at 300 MPa and 25°C to detect injured cells. Samples of milk, peach juice and orange juice before and after treatments were spread-plated in duplicate on BHIA and BSA for aerobic bacteria (AB) and *S. typhimurium* counts respectively. Plates were incubated overnight at 35°C and the colonies were counted manually[19].
Each experiment, with duplicate bags, was performed three times on separated days, and the average results are presented. The difference between the viable counts (at 300 MPa) of young cells in BHIA and BSA was used to estimate injured survivors[19]. The difference between the viable counts on BHIB from pressure treated old culture (N_{old}) and young culture (N_{young}) were used to estimate resistance of *S. typhimurium* cells to high-pressure (HP) treatment.

Statistical analysis

The results were compared by one-way analysis of variance (one-way ANOVA, Microsoft Office Excel 2003, Microsoft Corp., USA) to test the significant differences at α=0.05 level.

Results

The inactivations of the young culture (18 h culture) of *S. typhimurium* in TSB (pH= 7.0) from 200 to 400 MPa pressure in TSB at 25°C are given in Figure 1. At 200 MPa, the reduction in the number of *S. typhimurium* was about 1.18 log cfu/ml after 25 min of pressurization while it is about 3.76, 5.40 and 7.23 log cfu/ml at 250, 300 and 350 MPa respectively. After 25 min pressurization at 400 MPa, no surviving cells were obtained while this was attained after 30 min at 350 MPa. Increasing the pressure decreased the time require to inactivate the same number of cells. S. *typhimurium* was reduced by 3.89 log cfu/ml at 300 MPa after 10 min while increasing the time from 10 to 50 min resulted in a 3.87 log cfu/ml reduction. On the other hand, increasing the pressure from 300 to 400 MPa resulted in a 4.71 log cfu/ml reduction in 10 min. High levels of inactivation were observed at HP treatments. Inactivation was about 3.56 and 3.60 log cfu/ml at 300 and 400 MPa, respectively, after 5 min of pressurization. It appears that pressurization for a longer time at a lower pressure may not be of great advantage for microbial inactivation.

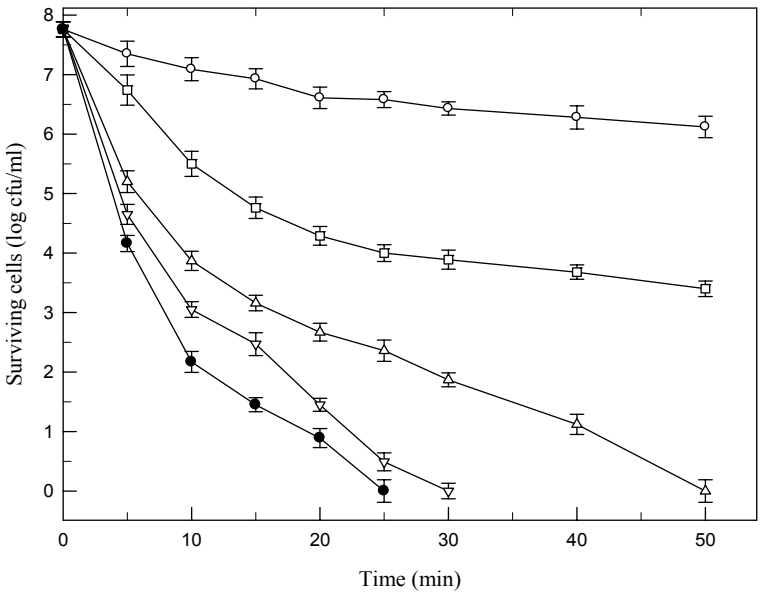

Figure 1. The effect of high hydrostatic pressure on *S. typhimurium* in TSB, at 25°C and 200 MPa (open circle); 250 MPa (open square); 300 MP (open triangle up); 350 MPa (open triangle down); and 400 MPa (closed circle) as a function of time.

The inactivations of young culture of *S. typhimurium* when treated in the temperature range from 15 to 45°C at 300 MPa in TSB (pH=7.0) are given in Figure 2 (the 25°C data in this figure is same with the data reported in Figure 1 for 300 MPa). Increasing the pressurization temperature from 15 to 45°C had a significant ($p<0.05$) effect on the viability loss of *S. typhimurium*. The reduction in *S. typhimurium* was about 4.53, 4.40 and 5.75 log cfu/ml after 25 min of pressurization at 15, 25 and 35°C, respectively, while all added *S. typhimurium* cells (7.76 log cfu/ml) were inactivated at 45°C after 25 min.

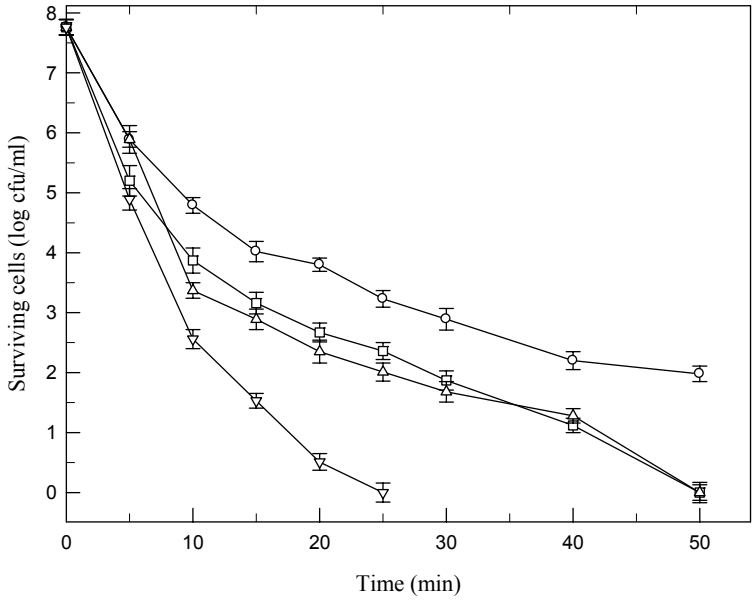

Figure 2. The effect of high hydrostatic pressure on *S. typhimurium* in TSB at 300 MPa and 15°C (open circle), 25°C (open square), 35°C (open triangle up) and 45°C (open triangle down) as a function of time.

The inactivations of the young culture of *S. typhimurium* in pH from 5.0 to 7.0 at 25°C and 300 MPa in TSB are given in Figure 3 (the pH 7.0 data in this Figure is same with the data

reported in Figure 1 at 300 MPa and Figure 2 at 25°C). The lethality of *S. typhimurium* increased as the pH decreased from 7.0 to 5.0 ($p<0.05$). The reduction in the number of *S. typhimurium* was about 3.89 and 4.64 log cfu/ml after 10 min of pressurization at pH 7.0 and 6.0, respectively, while all added *S. typhimurium* cells (7.76 log cfu/ml) were inactivated after 15 and 10 min at pH 5.5 and 5.0 respectively. The increasing of pH to neutral did not increase cell death to a great extend inactivation of *S. typhimurium*, while reducing pH ≤ 6.0 greatly extend inactivation.

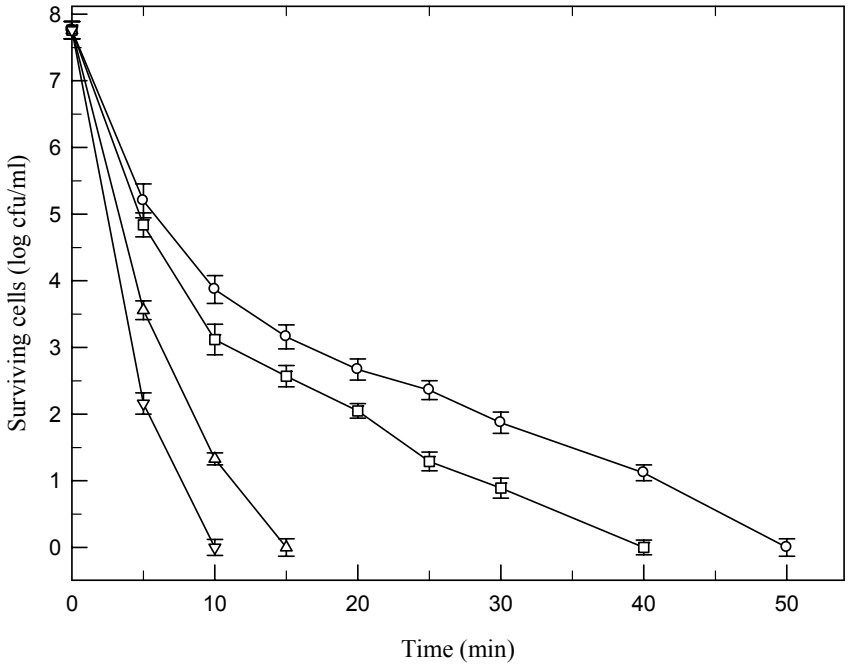

Figure 3. The effect of high hydrostatic pressure on *S. typhimurium* in TSB with pH 7.0 (open circle), 6.0 (open square), 5.5 (open triangle up) and 5.0 (open triangle down) at 25°C and at 300 MPa as a function of time.

The effects of 300 MPa pressure at 25°C on old cells (5 days culture) and young cells (18 h culture) of *S. typhimurium*, cell injury and cell resistance in TSB (pH=7.0) are given in Table 1. About 2.91 log cfu/ml of old cells were inactivated with 20 min pressure treatment at 300 MPa while 3.87 log cfu/ml of young cells were inactivated. The HHP was more effective on young cells than the old cells ($p<0.05$). Data from old cell pressure treatment is applicable to the food industry as it considers the use of hydrostatic pressure as an industrial method of food processing.

Table 1. Inactivation of young culture (18 h) and old culture (5 days) of *S. typhimurium* (log cfu) in TSB at 300 MPa at 25°c pressure treatment and cell injury

Time	Young culture		Injury[b]	Old culture	Resistance[c]
(min)	NSC[a]	SC	(%)	(NSC)	(%)
5	5.79	5.20	74.3	5.89	20.6
10	4.11	3.82	48.7	4.48	57.3
15	3.45	3.13	52.1	3.38	25.9
20	2.92	2.67	43.8	2.98	12.9
30	2.15	1.87	47.5	2.46	25.5
40	1.36	1.12	42.5	1.45	18.7
50	0.85	0.36	67.6	1.10	43.8
60	NG[d]	NG	-	NG	-

[a]NSC = Nonselective count (intial about 5.89×10^7 cfu/ml), SC = Selective count (also plotted on Fig. 1). [b]Percent injury = (number of count from NSC - number of count from SC)x100/NSC).
[c]Percent resistance (from NSC) = $[(N_{old} - N_{young})/N_{old}]x100$. [d]NG = No growth.

Survival curves for a young culture of *S. typhimurium* and AB in raw milk, and peach and orange juices at 400 MPa pressure treatments at 25°C are shown in Figure 4. All initially added *S. typhimurium* (7.36 log cfu/ml) into raw milk were inactivated after 45 min while 0.85 log cfu/ml of AB was survived in raw milk. No surviving AB were detected in milk after 60 min of pressurization at 400 MPa. AB in raw milk (natural flora: without added *S. typhimurium*) were completely inactivated after 20 min. All added *S. typhimurium* (7.36 log cfu/ml) were inactivated in peach and orange juices after 15 and 10 min respectively. The inactivation effect of HHP on AB and *S. typhimurium* was higher in orange juice than peach juice or milk. This would be due to the low pH value (3.55) of orange juice. Low pH would show a synergistic effect with pressure on the inactivation of AB and *S. typhimurium*. A reduction in the pH of surrounding medium causes a progressive increase in cell sensitivity to pressure[7].

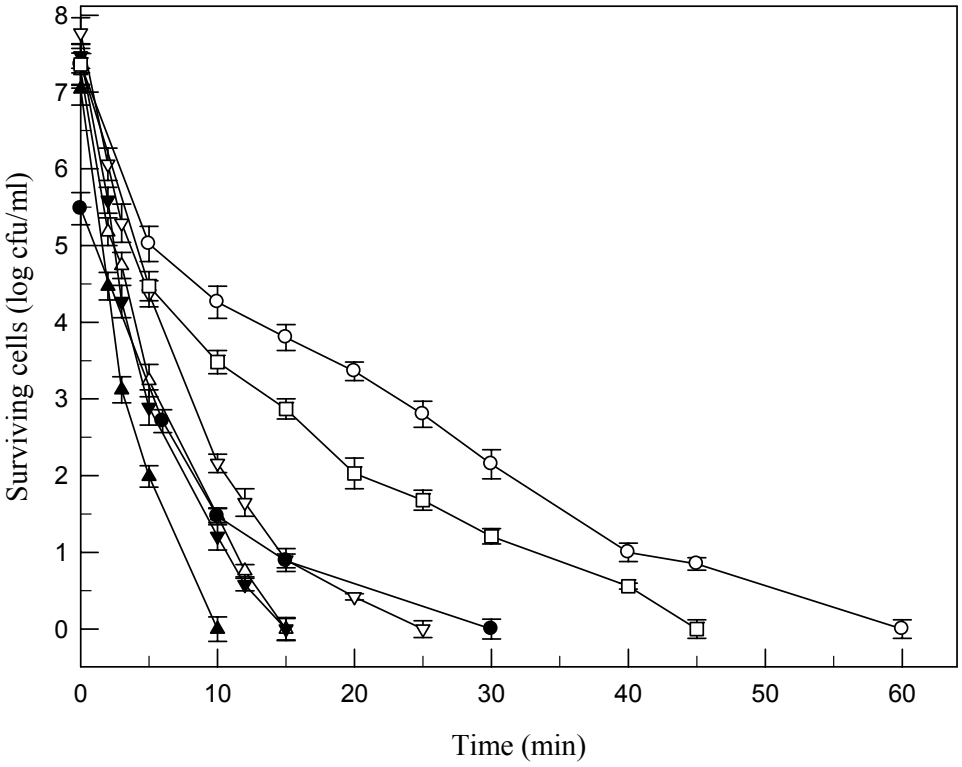

Figure 4. The effect of high hydrostatic pressure on aerobic bacteria (AB) and *S. typhimurium* at 400 MPa and 25°C in raw milk (AB, open circle; *S. typhimurium*, open square; natural flora, closed circle), peach juice (AB, open triangle down and *S. typhimurium*, open triangle up) and orange juice (AB, closed triangle down and *S. typhimurium*, closed triangle up) as a function of time.

Discussion

Resistance of bacteria to pressure treatment was higher in milk than peach juice and in peach juice than orange juices. This would be due to the composition of milk other than high pH. Certain food constituents, like proteins, carbohydrates, lipids, amino acids and vitamins, can have a protective effect on microbial inactivation[5,6,20]. It is therefore important to evaluate the process conditions in the food of interest rather than extrapolating data from other substrates.

The relationships between the reduction (in log cfu/ml) and treatment time are not linear (Figures 1-3). Generally, inactivation curves tend to be exponential (except treatment at 200 MPa at 25°C), with a rapid initial decrease in cell numbers during early time of treatment (especially within 10 min) followed by low levels of inactivation. "Tails" in the inactivation

curves were observed which suggest that small fractions of the population were more pressure-resistant. HP treatments are considered to be isostatic (i.e., equal pressure at every point of the treatment vessel). This study shows that cellular damage is not equally withstood by all the cells, suggesting that more resistant cells are present in pressurized cellular population. The effects of the growth phase of *S. typhimurium* (young and old culture) on the pressure resistance is in agreement with previous researches on *Listeria monocytogenes*[21]. They reported that *L. monocytogenes* cells at stationary phase were more pressure resistant than exponential phase cells.

The results indicated that the injured cells are able to grow within 18 h in non-selective medium after pressure treatment, but not grow in selective medium. There was significant ($p<0.05$) difference between non-injured and injured cell counts. The *S. typhimurium* cell injury was detected from 42.5 to 74.3. Wuytack *et al.*[22], Erkmen and Dogan[5] and Ukuku *et al.*[8] observed the injury on bacteria at HHP treatment. Injury data are applicable to the food industry as it considers the use of HHP as an industrial method of food processing.

There were significant ($p<0.05$) differences in the inactivation of *S. typhimurium* among pressure treatments. The effect of pressure was very positive since treatment efficiency improved with pressure increase. There were multiple target sites in/on cells for pressure effect and cell death occurs only after inactivation of a certain number of these targets. HHP has been reported to interfere with various cellular structures of functions, such as cytoplasmic membrane[23,24,], ribosome[25] and specific enzymes[26,27].

In general, pressure treatments at 400 MPa for 60, 25 and 15 min are sufficient to produce milk, peach juice and orange juice, respectively, free from AB (including natural flora and initially added *S. typhimurium*). HHP can be used to inactivate pathogens in foods. Many food constituents appear to protect microorganisms from the effects of HHP. Using this method, reversible damage may occur and cellular repair can take place under favorable conditions. This would be considered in food processing by HHP. This study indicated that HHP applications at room temperature would be applicable for the effective control of microorganisms in high and low pH foods.

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Disclosure Statement

No competing financial interests exist.

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