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Inactivation of *Salmonella* Enteritidis in Chicken Breast Fillets by Single-Cycle and Multiple-Cycle High Pressure Treatments

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Abstract

The effect of single-cycle and multiple-cycle high hydrostatic pressure (HHP) treatments on the survival of three *Salmonella* Enteritidis strains in chicken breast fillets was investigated. The surface of fillets was inoculated with a cocktail of three *Salmonella* strains at approximately 10^7 colony-forming units (CFU)/g, and held at 4°C for 20 hours before HHP treatments. Reduction of *Salmonella* counts on tryptic soy agar (TSA) by single-cycle treatments at 300 MPa and 12°C ranged from 0.58 log CFU/g for a 0-minute (no dwell time) cycle to 3.35 log CFU/g for a 20-minute cycle, whereas with 400 MPa treatments the decline ranged from 0.93 log CFU/g to more than 5 log CFU/g, respectively. The 4.8 log unit reduction in *Salmonella* counts on TSA achieved by a 15-minute treatment at 400 MPa should suffice to eliminate the pathogen naturally present in contaminated chicken meat. When plated on Salmonella Shigella agar (SSA), the reduction of *Salmonella* counts by single-cycle treatments at 300 MPa and 12°C ranged from 0.69 log CFU/g for a 0-minute cycle to 4.21 log CFU/g for a 20-minute cycle, and with 400 MPa treatments from 1.25 log CFU/g to more than 5 log CFU/g, respectively. From the comparison of *Salmonella* counts on SSA and TSA it was concluded that not only the lethality but also the proportion of injured *Salmonella* cells increased with the length of HHP treatments. The use of multiple-cycle treatments instead of single-cycle treatments of the same HHP time for the inactivation of *Salmonella* Enteritidis inoculated on chicken breast fillets showed to be more advantageous at 400 MPa than at 300 MPa. No recovery of injured *Salmonella* cells was observed when fillets treated at 300 or 400 MPa for 5 minutes were held for 72 hours at 4°C.

Introduction

Salmonellosis is a major foodborne illness in many countries (D'Aoust, 1997). Although *Salmonella* is ubiquitous, its primary reservoir is the intestinal tract of animals. The prevalence of this microorganism in poultry products at the retail level may reach up to 60%, as shown by surveys carried out in different countries (Antunes *et al.*, 2003; Capita *et al.*, 2003; Soultos *et al.*, 2003; Bohaychuck *et al.*, 2006). Poultry products are a significant vehicle for *Salmonella* transmission to humans and have been incriminated in numerous *Salmonella* outbreaks (Baeumler *et al.*, 2000). Although the U.S. Food Safety and Inspection Service published the "Pathogen reduction; hazard analysis and critical control point (HACCP) systems; final rule" in 1996 with the goal of reducing the prevalence of *Salmonella* and other pathogens in meat and poultry products, 16% sets of broiler chicken carcasses and 40% sets of ground chicken still failed to pass the pathogen reduction (PR)-HACCP *Salmonella* testing program in 2003 (Naugle *et al.*, 2006).

The microbiological safety of foods can be improved by decontamination during processing or at the end of the production line. High hydrostatic pressure (HHP) treatment is an adequate procedure to reduce microbial contamination and growth in foods that might be altered by heat treatment. For example, it took 7 days for raw ground chicken stored at 4°C with an initial microbial load of 5.25 log colony-forming units (CFU)/g to reach 7 log CFU/g in the absence of HHP treatment, and more than 70 days when treated at 408 MPa for 10 minutes (O'Brien and Marshall, 1996). The inactivation of *Salmonella* by HHP treatments has been investigated in food substrates such as liquid whole egg, minced chicken, milk, cheese, cooked ham, and low-acid fermented sausages (Ponce *et al.*, 1999; Yuste *et al.*, 2003; Guan *et al.*, 2005; Marcos *et al.*, 2005; De Lamo-Castellví *et al.*, 2007; Jofre *et al.*, 2008a,b).

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Multiple-cycle HHP treatments were shown to be more lethal than continuous pressurization against bacterial spores suspended in saline solutions (Hayakawa *et al.*, 1994). Also, multiple-cycle treatments were more effective than continuous treatments of the same total time against *Salmonella* in liquid whole egg (Ponce *et al.*, 1999; Huang *et al.*, 2006; Bari *et al.*, 2008) and against *Escherichia coli* O157:H7 in ground beef (Morales *et al.*, 2007). However, multiple-cycle treatments performed only slightly better against psychrotrophs in mechanically recovered poultry meat than continuous pressurization (Yuste *et al.*, 2001).

The objective of the present study was to investigate the effect of different single-cycle and multiple-cycle HP treatments at 300 and 400 MPa on the inactivation of three *Salmonella* Enteritidis strains inoculated on the surface of chicken breast fillets.

Materials and Methods

Microorganisms

Salmonella Enteritidis strains CECT 4155, CECT 4300, and CECT 4396 from the Spanish Type Culture Collection (Valencia, Spain) were kept frozen at −80°C in tryptic soy broth (Biolife, Milano, Italy) with 15% glycerol added. Stationary phase cultures of *Salmonella* strains, separately grown in tryptic soy broth for 18 hours at 37°C, were centrifuged at 5000 *g* for 5 minutes at 4°C, and cell pellets were suspended in 0.1% peptone water. For HHP experiments with a cocktail of strains, the three cell suspensions were mixed and held at 4°C until the inoculation of chicken breast fillets, which was performed within 2 hours.

Sample preparation and HHP treatments

Chicken breasts were sliced into 5-mm-thick fillets and distributed in 15-g samples. Fillets were inoculated by spreading a fixed amount of the cocktail of *Salmonella* strains on the surface in order to achieve a final population of approximately 10^7 CFU/g. Inoculated fillets were individually vacuum-packed in double bags of CN300 (Cryovac Grace S. A., Barcelona, Spain) and held at 4°C for 20 hours prior to HHP treatments. For the comparison of the barotolerance of the three *Salmonella* strains, fillets individually inoculated with each of the cell suspensions were prepared as described above. Noninoculated fillets were also prepared.

HHP treatments were performed in duplicate experiments carried out on different days in a high-pressure batch apparatus (model ACIP 6000; ACB, Nantes, France) of 3.5-L capacity and 600 MPa maximum working pressure. Single-cycle treatments were carried out at 300 and 400 MPa for 0 (come-up, no dwell time, come-down), 1, 3, 5, 10, 15, and 20 minutes, at 12°C. Multiple-cycle treatments consisted in two, three, or four 1-minute cycles, two or three 3-minute cycles, and two 5-minute cycles, at 300 or 400 MPa and 12°C. Initial and final temperature of the water used as pressure-transmitting fluid was 8° and 13°C, respectively, without exceeding 18°C any time during treatment. Come-up times to reach 300 and 400 MPa were 1.6 and 2.2 minutes, respectively, and come-down times were 0.27 and 0.32 minutes, respectively. Pressurized and control (nonpressurized) fillets were held at 4°C until analysis, which was carried out within 2 hours of HHP treatments. In order to ascertain if some recovery of injured *Salmonella* cells occurred, inoculated fillets treated (in triplicate) at 300 and 400 MPa for 5 minutes, as well as inoculated untreated fillets, were analyzed before and after storage for 72 hours at 4°C.

Microbiological analysis

Samples (15 g) of chicken breast fillets were homogenized in 135 mL of sterile 0.1% peptone + 0.85% NaCl aqueous solution using a homogenizer (IUL, Barcelona, Spain). Decimal dilutions of the homogenate were prepared in the same sterile solution. *Salmonella* population in inoculated control and HHP-treated fillets was determined in duplicate on plates of Salmonella Shigella agar (SSA; Biolife) and tryptic soy agar (TSA; Biolife) incubated at 37°C for 24 and 48 hours, respectively. Initial counts of noninoculated fillets on TSA averaged 4.02 log CFU/g at the time of vacuum-packing, and 4.45 log CFU/g after 20 hours at 4°C, prior to HHP treatments. Only atypical colonies were observed when samples of noninoculated chicken breast fillets were plated on SSA.

Statistical treatment of data

Data were subjected to analysis of variance by means of SPSS program Win 9.0 software (SPSS Inc., Chicago, IL), with type of HHP treatment and experiment as main effects. Significant differences between means were assessed by Tukey's test with $p < 0.05$.

Results and Discussion

Single-cycle HHP treatments

The lethality of *Salmonella* strains in chicken breast fillets subjected to single-cycle treatments at 300 MPa is shown in

Table 1. Counts of *Salmonella* Enteritidis in Chicken Breast Fillets After Single-Cycle and Multiple-Cycle High Hydrostatic Pressure (HPP) Treatments at 300 MPa and 12°C on Tryptic Soy Agar (TSA) and Salmonella Shigella Agar (SSA)[a]

HHP Treatment		*TSA counts (log CFU/g)*	*SSA counts (log CFU/g)*
Single-cycle	1×0 min	6.32 ± 0.11 J	5.68 ± 0.22 I
	1×1 min	6.08 ± 0.07 I	5.27 ± 0.12 H
	1×3 min	5.43 ± 0.20 G	4.81 ± 0.07 G
	1×5 min	5.20 ± 0.31 F	4.43 ± 0.20 F
	1×10 min	4.47 ± 0.23 C	3.53 ± 0.08 D
	1×15 min	4.21 ± 0.55 B	2.78 ± 0.55 B
	1×20 min	3.55 ± 0.48 A	2.16 ± 0.55 A
Multiple-cycle	2×1 min	5.64 ± 0.51 H	5.03 ± 0.06 GH
	3×1 min	5.39 ± 0.12 G	4.80 ± 0.40 G
	4×1 min	4.95 ± 0.62 E	4.15 ± 0.59 EF
	2×3 min	4.75 ± 0.06 D	3.86 ± 0.02 E
	3×3 min	4.56 ± 0.06 C	3.52 ± 0.12 D
	2×5 min	4.43 ± 0.33 C	3.15 ± 0.41 C

[a]Mean values of duplicate determinations on duplicate experiments. *Salmonella* counts in inoculated untreated fillets were 6.90 log colony-forming units (CFU)/g on TSA and 6.37 log CFU/g on SSA. Single-cycle treatment for 0 minutes consisted in come-up, no dwell time, and come-down. The limit of detection was 1.40 log CFU/g. Counts within the same column followed by the same letter do not differ significantly ($p < 0.05$).

Table 1. According to the analysis of variance, the type of HHP treatment at 300 MPa was statistically significant. The reduction in TSA counts ranged from 0.58 log CFU/g for the 0-minute cycle to 3.35 log CFU/g for the 20-minute cycle. Individual declines in TSA counts of the three *Salmonella* strains pressurized separately in fillets treated at 300 MPa and 12°C for 10 minutes did not differ by more than 0.7 log units (data not shown), a result that indicates a similar barotolerance. This scarce variability is in agreement with the data reported for 40 *Salmonella* Enteritidis serovars, the most resistant of which only showed 0.5 log unit higher counts than the average count of the 40 serovars after treatment at 350 MPa for 10 min at 20°C (Sherry *et al.*, 2004).

After plotting the data of single-cycle treatments ranging in time from 0 (no dwell time) to 20 min, and taking into account the similar barotolerance of the three *Salmonella* strains used in the present work, regression equations of TSA log counts on length (minutes) of treatment were calculated for various lethality models. The regression equation for a linear model of single-cycle treatments at 300 MPa was $y = -0.1302\,x + 6.0406$ ($r^2 = 0.899$). From this regression equation, a D_{300MPa} value of 7.68 minutes may be cautiously estimated for the cocktail of *Salmonella* strains. This is a lower value than the 10.14 and 8.37 minutes, respectively, obtained for *S. typhimurium* 7136 and *S. senftenberg* 75W in strained chicken treated at 300 MPa (Metrick *et al.*, 1989) and, presumably, than the D value of *S. typhimurium* DT104 in UHT whole milk, in which the reduction was only 3 log CFU/mL after 120 minutes at 350 MPa (Guan *et al.*, 2005). On the other hand, the viability loss reported for cell suspensions of *Salmonella* Enteritidis strains FDA and VL in 1% peptone solution after 5 minutes at 345 MPa was as high as 5.45 and 7.48 CFU/mL, respectively (Alpas *et al.*, 1999), from which D values below 1 minute may be assumed. The fact that different *Salmonella* strains and substrates were used in the above quoted works may account for the differences in D values observed when comparing our results with those from other authors.

An exponential lethality model achieved a slightly better fit for the regression of TSA log counts on the length of single-cycle treatments at 300 MPa, from 0 to 20 minutes, according to the equation $y = 6.0097e^{-0.0274x}$ ($r^2 = 0.903$). A second-order polynomial lethality model attained an even slightly better fit than the linear and exponential models, according to the regression equation $y = 0.0042x^2 - 0.2123x + 6.2148$ ($r^2 = 0.925$).

The declines recorded for SSA counts after 300-MPa treatments, ranging from 0.69 log CFU/g for the 0-minute cycle to 4.21 log CFU/g for the 20-minute cycle (Table 1), were more pronounced than for TSA counts. Differences between TSA counts and SSA counts increased with HHP treatment length, as previously reported for *Salmonella* counts in liquid whole egg on TSA and SSA (Ponce *et al.*, 1999). However, no significant differences were found between *Salmonella* counts on TSA and bismuth sulfite agar after treatment of liquid whole egg at 350 or 400 MPa for up to 40 minutes (Bari *et al.*, 2008). When comparing mild heat and various nonthermal food preservation treatments, the sublethal injury of *Salmonella* was more pronounced for HHP pressure and mild heat treatments than for high pressure homogenization, pulsed electric field, and pulsed white light (Wuytack *et al.*, 2003). In the present work, after one 20-minute cycle at 300 MPa, only 4.1% of the cells forming colonies on TSA were able to form colonies on SSA (i.e., 95.9% were injured cells).

Table 2. Counts of *Salmonella* Enteritidis in Chicken Breast Fillets After Single-Cycle and Multiple-Cycle High Hydrostatic Pressure (HPP) Treatments at 400 MPa and 12°C on Tryptic Soy Agar (TSA) and Salmonella Shigella Agar (SSA)[a]

HHP Treatment		*TSA counts (log CFU/g)*	*SSA counts (log CFU/g)*
Single-cycle	1×0 min	5.94 ± 0.26 H	5.03 ± 0.18 F
	1×1 min	5.51 ± 0.54 G	4.52 ± 0.13 E
	1×3 min	4.52 ± 0.54 E	3.02 ± 0.25 C
	1×5 min	3.97 ± 0.55 D	2.39 ± 0.64 B
	1×10 min	2.90 ± 0.79 B	<1.4
	1×15 min	2.08 ± 0.15 A	<1.4
	1×20 min	<1.4	<1.4
Multiple-cycle	2×1 min	5.12 ± 0.22 F	3.53 ± 0.08 D
	3×1 min	4.03 ± 0.35 D	2.39 ± 0.10 B
	4×1 min	3.35 ± 0.21 C	1.83 ± 0.57 A
	2×3 min	3.62 ± 0.51 C	2.60 ± 0.02 B
	3×3 min	2.23 ± 0.15 A	<1.4
	2×5 min	2.68 ± 0.44 B	<1.4

[a]Mean values of duplicate determinations on duplicate experiments. *Salmonella* counts in inoculated untreated fillets were 6.87 log colony-forming units (CFU)/g on TSA and 6.28 log CFU/g on SSA. Single-cycle treatment for 0 min consisted in come-up, no dwell time, and come-down. The limit of detection was 1.4 log CFU/g. Counts within the same column followed by the same letter do not differ significantly ($p < 0.05$).

The effect of single-cycle treatments at 400 MPa on the cocktail of *Salmonella* strains is shown in Table 2. According to the analysis of variance, the type of HHP treatment at 400 MPa was statistically significant. Reductions in TSA counts ranged from 0.93 log CFU/g for the 0-minute cycle to 4.79 log CFU/g for the 15-minute cycle, with counts after one 20-minute cycle under the limit of detection. The regression equation for a linear lethality model of single-cycle treatments at 400 MPa was $y = -0.2518x + 5.5812$ ($r^2 = 0.892$). From this equation, a D_{400MPa} value of 3.97 minutes may cautiously be estimated for the cocktail of *Salmonella* strains. The D value of *S. typhimurium* 7136 in strained chicken declined from 10.14 to 7.63 minutes when the pressure was increased from 300 to 340 MPa, and that of *S. senftenberg* 75W from 8.37 to 7.13 minutes (Alpas *et al.*, 1999). Similarly, TSA counts of a *Salmonella* Enteritidis strain, which suffered a 1.44 log CFU/mL reduction when treated at 350 MPa for 5 minutes in liquid whole egg, declined by 4.04 log CFU/mL when treated at 450 MPa (Ponce *et al.*, 1999).

As observed for treatments at 300 MPa, the exponential lethality model achieved a slightly better fit for the regression of TSA log counts on the length of single-cycle treatments at 400 MPa, according to the equation $y = 5.7595e^{-0.0695x}$ ($r^2 = 0.923$). A second-order polynomial model attained a slightly better fit than both linear and exponential models, according to the equation $y = 0.0125x^2 - 0.4372x + 5.8837$ ($r^2 = 0.928$).

Decreases in SSA counts at 400 MPa ranged from 1.25 log CFU/g for the 0-minute cycle to 3.89 log CFU/g for the 5-minute cycle (Table 2). Counts on SSA were under the detection limit for 10-minute and longer cycles. Both the lethality and the proportion of injured *Salmonella* cells were higher at 400 MPa than at 300 MPa. Thus, the percentage of cells forming colonies on TSA able to grow on SSA after one

5-minute cycle at 300 MPa was 17.0%, whereas only 2.6% cells were able to grow on SSA after one 5-minute cycle at 400 MPa.

Multiple-cycle HHP treatments

The population of *Salmonella* in chicken breast fillets subjected to multiple 1-minute cycles at 300 MPa suffered reductions that ranged from 0.82 log CFU/g for one cycle to 1.95 log CFU/g for four cycles, if determined on TSA, and from 1.10 to 2.22 log CFU/g, respectively, if determined on SSA (Table 1). Multiple-cycle treatments at 300 MPa were not more lethal than single-cycle treatments of the same HHP time, with the only exception of two 5-minute cycles, a significantly more effective treatment than one 10-minute cycle.

Reductions in the population of *Salmonella* in chicken breast fillets subjected to multiple 1-minute cycles at 400 MPa ranged from 1.36 log CFU/g for one cycle to 3.52 log CFU/g for four cycles, if determined on TSA, and from 1.76 log CFU/g to 4.45 log CFU/g if determined on SSA (Table 2). Three 1-minute cycles at 400 MPa were significantly more effective than one 3-minute cycle, and four 1-minute cycles more than one 5-minute cycle.

Multiple-cycle HHP treatments have been reported by some authors to be more effective than single-cycle treatments for the destruction of bacterial and mold spores (Hayakawa *et al.*, 1994; Palou *et al.*, 1998), although variable results have been obtained for vegetative cells. In the case of *Salmonella*, multiple-cycle treatments of liquid whole egg at 350 or 450 MPa (Ponce *et al.*, 1999) or at 138 MPa (Huang *et al.*, 2006) were more lethal than single-cycle treatments. Similarly, decreases in *Salmonella* counts in liquid whole egg of nearly 7 log CFU/mL after four 2-minute cycles at 350 MPa, and of less than 2.5 log CFU/ml after one 10-minute cycle, have been reported (Bari *et al.*, 2008).

When multiple-cycle treatments were applied to mechanically recovered poultry meat, they performed slightly better than continuous pressurization against psychrotrophs, but not against mesophiles (Yuste *et al.*, 2001). In the case of *E. coli* O157:H7 inoculated into ground beef and treated at 400 MPa, the reduction after four 1-minute cycles was 1 log unit higher than after one 15-minute cycle (Morales *et al.*, 2007). According to the results obtained in the present work on the inactivation of *Salmonella* Enteritidis in chicken breast fillets, using multiple-cycle treatments instead of single-cycle treatments of the same HHP time was more beneficial at 400 MPa than at 300 MPa.

In inoculated fillets HHP treated for 5 minutes and held for 72 hours at 4°C, SSA counts increased during storage by only 0.17 log CFU/g if treated at 300 MPa and by 0.12 log CFU/g if treated at 400 MPa (data not shown), indicating that no significant recovery of injured *Salmonella* cells occurred during refrigerated storage. TSA counts of these fillets increased by only 0.10 log CFU/g if treated at 300 MPa, whereas they declined by 0.36 log CFU/g if treated at 400 MPa (data not shown). It had been proven that *Salmonella* inoculated on cooked ham was not able to grow after treatment at 400 MPa during storage at 6°C for 90 days (Jofre *et al.*, 2008a). In the present work, *Salmonella* counts of inoculated untreated fillets on TSA increased by only 0.08 log CFU/g during storage for 72 hours at 4°C, whereas SSA counts declined by 0.21 log CFU/g (data not shown), in agreement with the absence of growth on chicken meat at 4°C recorded for *Salmonella* (Pintar *et al.*, 2007).

Conclusions

A considerably shorter D-value was obtained at 400 MPa (3.97 minutes) than at 300 MPa (7.68 minutes) when a linear lethality model was applied to the inactivation of a cocktail of three *Salmonella* Enteritidis strains of similar barotolerance, inoculated on chicken breast fillets, by single-cycle HHP treatments. Also, the proportion of injured *Salmonella* cells was higher after 400 MPa treatments than after 300 MPa treatments of the same length. The 4.8 log CFU/g reduction in *Salmonella* counts on TSA achieved by a 15-minute treatment at 400 MPa should suffice to eliminate the pathogen naturally present in contaminated chicken meat. The use of multiple-cycle treatments instead of single-cycle treatments of the same HHP time for the inactivation of *Salmonella* Enteritidis inoculated on chicken breast fillets showed to be more advantageous at 400 MPa than at 300 MPa. No recovery of injured *Salmonella* cells was observed when fillets treated at 300 or 400 MPa for 5 minutes were stored for 72 hours at 4°C.

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Disclosure Statement

No competing financial interests exist.

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