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High Pressure Processing of Chicken Meat: Change in Total Aerobic Counts after Pressure Treatment and During Chilled Storage

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SB and NC designed and conducted the experimental work. Author SB performed the statistical analysis and wrote the first draft of the manuscript. Authors ML and ALB supervised the study and contributed to writing up the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To investigate the effect of pressure, pressurization time, pressurization temperature and their interaction on inactivation and recovery of *Listeria innocua* inoculated in minced chicken meat.
Study Design: Effect of the parameters of high pressure processing (HPP) on the inactivation of *L. innocua* were studied by response surface methodology using Box-Behnken design.
Place and Duration of Study: Study conducted during an 11 months postdoctoral study at Agriculture, Agronomy and Food Sciences Department at LUNAM Université, Oniris, Nantes, France.
Methodology: Minced chicken meat inoculated with *Listeria innocua* strain ATCC 33090 to give a total aerobic count (TAC) of 10^8 cfu/g and samples were subjected to high pressures of 200, 300, 400 MPa, temperatures of 0ºC, 20ºC, 40ºC and holding times of 5, 10 and 15 minutes. Survival of *L. innocua* was determined by TAC immediately after pressure treatment and during 35 days of storage at 3ºC.

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Results: Survival of *L. innocua* decreased with increasing pressure and pressurization time. Effect of pressurization temperature on survival of bacteria was not linear, giving higher reduction at 0ºC and 40ºC compared to treatments at 20ºC. Analysis of variance (ANOVA) showed that pressure (P<.001), time (P<.001), temperature (P=.05) and interaction of pressure and temperature (P=.05) were significant parameters. After a 10 min treatment at 400 MPa and 0ºC, no survival of microorganisms was detected immediately after pressure treatment. However, TAC increased during storage and reached to about the initial level of microbial load (10^8cfu/g) in all samples after 35 days of storage at 3ºC.
Conclusion: Undetected survival of microorganisms in a nutrient rich food immediately after a pressure treatment does not necessarily mean total inactivation of the microorganisms. Injured microbial cells could recover during the refrigerated storage and compromise the safety of pressure treated foods. Therefore, care must be taken to ensure the safety of high pressure processed foods.

Keywords: High pressure processing; Listeria innocua; microbial inactivation; microbial recovery.

1. INTRODUCTION

High pressure processing (HPP) at refrigeration, ambient or moderate temperature allows inactivation of pathogenic and spoilage microorganisms in foods with fewer changes in texture, colour and flavour as compared to conventional technologies [1]. There has been a great interest in applications of high hydrostatic pressure (HPP) on food after 1980's and as a result there is now a range of pressure-treated food products such as fruit preparations, fruit juices, jams, sauces, rice cakes, raw squid, guacamole, oysters and ready to eat meat products on the market in Japan, USA and Europe. The major advantage of these products over their pasteurised counterparts is that the pressure treatment causes no change in the colour, flavour and nutritive quality since the relatively small molecules responsible for these properties are largely unaffected by the pressure treatment [2].

Most of the published reports on HPP-induced microbial inactivation have analysed the number of surviving cells immediately after pressure treatment, which does not take into account the recovery of injured cells [3]. Micro-organisms surviving the lethal action of preservation agents may be sublethally injured and could repair the damage and outgrow only when the environmental conditions are suitable [4]. The number of surviving microorganisms after HPP treatment is usually determined by plating the bacterial suspension on solid media before and after treatment [5]. In most of the studies, a selective media is used in order to differentiate and enumerate a specific target microorganism [6]. Many of the accepted methods used for isolation and enumeration of microorganisms in foods by using a selective media do not allow for the repair of injured microorganisms and thus fail to detect them [7]. This in turn increases the risk of overestimating the efficacy of pressure treatment which could lead to an increased risk of food poisoning or spoilage and might be critical for the safety of high-pressure-processed food [8].

Studies showed that HPP injured cells could repair themselves, indicating the potential for bacterial recovery during storage [9,10]. Implications of recovery of microorganisms after a HPP treatment could compromise safety of pressure treated food products during shelf life and therefore, more work is required to determine the effect of high pressure on microorganism in various foods and the recovery of microorganisms during storage.

The purpose of this study is to investigate the effect of pressure, pressurization time and pressurization temperature on inactivation and recovery of *Listeria innocua*, a non-pathogenic surrogate for *Listeria monocytogenes* [11], inoculated in minced chicken meat. A statistical approach was employed in order to understand the effect of each parameter and their interaction. The change in total number of surviving microorganisms after HPP treatment during 35 days of storage at 3ºC was followed by using non-selective media.

2. MATERIALS AND METHODS

2.1 Bacterial Strain and Growth Conditions

Pure culture of *Listeria innocua* strain ATCC 33090 were obtained from Ecole National Vétérinaire de Nantes (France) and maintained on Tryptic Soy Agar (Oxoid, Basingstoke, UK) slants at +4ºC. The inoculum was prepared by transferring isolated colonies from TSA slants into 200 ml of sterile Brain Heart Infusion broth (Biokar, Beauvais, France) in 1 liter sterile flasks and incubated at 37°C for 24 h to give approximately 10^9 cfu/ml at stationary phase. The cells were then harvested by centrifugation (7000 x g for 10 min) washed in sterile phosphate buffer (pH 7.0) composed of Na_2HPO_4 (0.2 mol/L) and NaH_2PO_4 (0.2 mol/L) (Merck, Darmstadt, Germany) and resuspended in phosphate buffer at pH 6.2 (measured pH of minced chicken meat) to give a concentration of approximately 10^9 cfu/ml stock suspension.

2.2 Preparation of Minced Chicken Meat Samples and Enumeration of Microbial Load before Inoculation

Chicken breasts obtained from a local market (Carrefour, Nantes, France) were minced with a food processor (Moulinette, Moulinex, France). Three samples from minced chicken were taken in sterile stomacher bags and total number of aerobic microorganisms in these samples was determined by plating serial dilutions on plate count agar (PCA, Biokar, Beauvais, France) to determine the initial microbial load of minced chicken meat.

About 40 ml of *L. innocua* suspension in PBS (pH, 6.2) were sprayed slowly on meat while mixing by a Kenwood mixer to give about 10^8 cfu/g. Samples of inoculated minced chicken meat and uninoculated control samples were vacuum packed in sterile plastic films impermeable to air and water (AES, Combourg, France). The size of vacuum packed samples were 10g and the whole sample (10g) were used to prepare the serial dilutions (10g meat sample + 90 ml PBS) during microbiological analysis. Samples were kept at 3 ± 1ºC overnight to allow the cells to recondition to the new environment before pressure treatment. After pressurization, control and pressurized samples were kept in a fridge operating at 3 ± 1ºC. Microbiological tests were carried out on day 0, 4, 21 and 35 on triplicate samples and the average of counts were used for calculations.

2.3 High Pressure Treatment

The samples were placed in the pressure chamber (3.5-liter volume) of a high hydrostatic pressure vessel (ACB Pressure Systems, Nantes, France) equipped with temperature and pressure regulator devices. The medium used for pressure transmission in the system was ethanol/water solution (50%, v/v). Pressure was increased to experimental pressure at a rate of 3 MPa/s and the pressure was released in about 1 s after the experiment. Temperature of the pressure transmitting medium inside the pressure chamber was controlled with cooling

jacket surrounding the pressure vessel. A thermocouple K-type (0.3 mm diameter, Omega, Stamford USA) positioned close to the sample used to monitor temperature during treatment.

2.4 Storage of the Samples

After the pressure treatments the samples were immediately transferred to a refrigerator and the temperature was kept at 3 ± 1ºC for up to 35 days. Microbiological analyses were done at day 0, 4, 21 and 35 during chilled storage.

2.5 Enumeration of Surviving Cells

Control and pressure treated samples (10 g) were mixed with 90 ml of 0.1% peptone water, pH 7.0 (Oxoid, Basingstoke, UK) and homogenized in a Stomacher (Seward Medical, London, England) for 2 min. A 1 ml sample of suspension from the stomacher bag was taken into a sterile Eppendorf and serial dilutions were prepared by transferring 0.1 ml of this suspension into 0.9 ml of 0.1% peptone water in sterile Eppendorf tubes. A vortex mixer (Stuart, UK) was used at each step of serial dilution to assure a homogenous mix. Drop plate method [12] was used in triplicate to enumerate the surviving cells on PCA. The plates were incubated at 37ºC for 48 h before counting of colonies. The averages of three counts from each dilution were used for calculation of surviving cells.

In the present study, TAC was defined as all cells detected on a non-selective medium (PCA) without distinguishing reproduction of cells after recovery from injury. Microbial reduction was expressed in terms of logarithmic reduction corresponding to the logarithmic difference between the initial number of microorganism before pressure treatment and the number of microorganisms survived after pressure treatment and during storage.

2.6 Statistical Analysis

The effect of three main variables (pressure from 200 to 400 MPa, holding time from 5 to 15 min and temperature from 0ºC to 40ºC) and their interactions on the inactivation of *L. innocua* were studied by the surface response methodology employing Design-Expert V8 Software (Stat-Ease, Inc. Minneapolis, USA) using Box-Behnken design. Assessment of error was derived from three times repetition of the centre point of experimental design, which was set as 300 MPa at 20ºC for 10 minute. Level of significance was set for P=0.05 and the significance of each response variable were assessed by *F-test* statistical analysis.

3. RESULTS AND DISCUSSION

3.1 Microbial Load of Chicken Meat

The initial number of microorganisms in minced chicken meat before inoculation of *L. innocua* determined by total aerobic count was 6.0×10^4 cfu/g. After inoculation with *L. innocua* strain ATCC 33090, the average TAC from 3 control samples was 9.9×10^7 cfu/g. Considering the microbial load of minced chicken meat before and after inoculation with *L. innocua*, it could be stated that 99.94% ((9.9×10^7 cfu/g - 6.0×10^4 cfu/g)/ 9.9×10^7 cfu/g) of the microorganisms in inoculated minced chicken meat were *L. innocua*. Therefore, it could be considered that TAC grossly represented the number of *L. innocua* in the inoculated

samples. Inoculation of *L. innocua* into minced chicken at this level could be considered as extreme for contamination of *L. innocua* to minced chicken meat.

3.2 Effect of Process Parameters on Inactivation of *L. innocua* Inoculated in Chicken Meat

Numbers of surviving cells determined by TAC immediately after HPP treatment were statistically analysed by Design-Expert software. A third order polynomial equation were chosen for analysis of variance (ANOVA) and the results showed that pressure (P<.001), time (P<.001), temperature (P=.05) and interaction of pressure and temperature (P=.05) were significant parameters. The model P-value (P<.001) and R-squared value (0.99) showed that the model was representing the data well. Response surface model produced the following equation for prediction of survival of microorganisms (mainly *L. innocua*) in logarithmic unit immediately after pressure treatment:

$$Log\ reduction\ (cfu/g) = 1.50+2.27A+0.15B+0.80C-0.12AB+0.08AC-0.05BC+0.45A2+0.73B2 +0.47C^2-0.27A^2B-0.68A^2C+ 0.39AB^2$$

Where, A: Pressure in MPa, B: Temperature in ºC and C: Time in min.

3.3 Effect of Pressure

Reduction in number of *L. innocua* was minimal at 200 MPa, where only 0.1-0.3 log cycle reductions in TAC were observed immediately after pressure treatment. Previous studies also showed that pressures in the range of 200 MPa resulted in minimal reduction in number of microorganisms [13-16].

Increased pressure resulted in higher reduction in number of survivals immediately after pressure treatment. After 10 min of treatment at 400 MPa and 0ºC, there was no detection of survival which corresponds to a minimum 5.8 log reduction based on a 1.7×10^2 cfu/g minimum detection level.

Fig. 1 shows the surface response for log reduction in number of *L. innocua* as a function of pressure and treatment time. In general, the positive effect of pressure and pressurization time on inactivation of vegetative cells is well documented in the literature [13,14,17].

3.4 Effect of Treatment Temperature

Effect of temperature on reductions in number of *L. innocua* was not straightforward. As shown in Fig. 2, a curvature was observed at the mid and upper end of the pressure scale (300- 400 MPa), where log reductions were higher at 0ºC and 40ºC and minimal at 20ºC. This finding is in agreement with the fact that the resistance of most microorganisms to high pressure is believed to be greatest at optimal growth temperatures [14,18-20]. Inactivation under high pressure is higher at temperatures outside the optimum growth temperatures, as membrane fluidity can be more easily disrupted at temperatures beyond optimal growth temperatures [21]. terSteeg et al. [22] observed an increased efficacy of HPP treatment if the pressurization temperature was reduced under conditions where the liquid crystalline state of the cytoplasmic membrane during growth of the organisms is altered to a more rigid, semicrystalline state during pressurization.

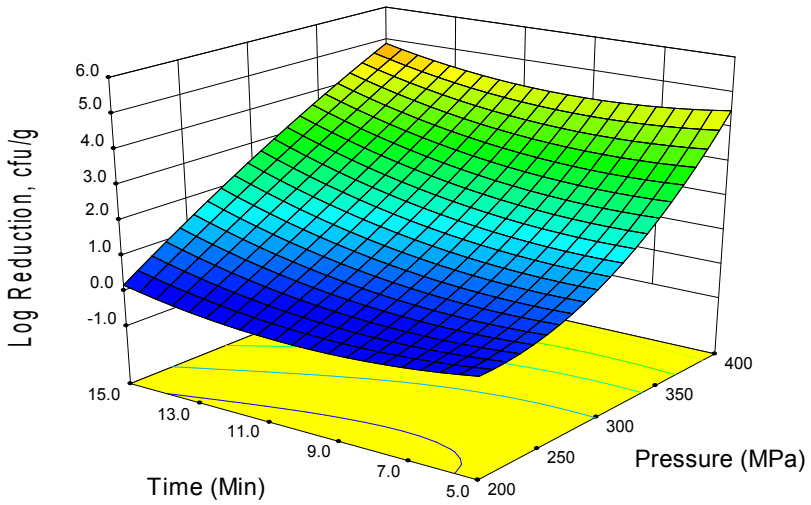

Fig. 1. Response surface for log reduction in number of *L. innocua* as a function of pressure and time immediately after pressure treatment

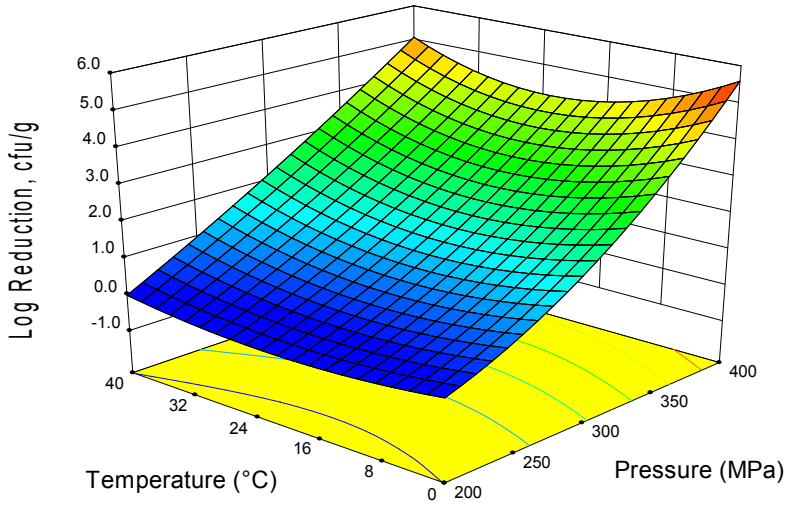

Fig. 2. Response surface for log reduction in number of *L. innocua* as a function of pressure and temperature immediately after pressure treatment

Compared to pressure treatment at 20ºC, higher reductions were observed in number of *L. innocua* after pressure treatments at 40ºC. It is known that when HPP is combined with mild heat treatments, the inactivation of microorganisms is greater. It has been suggested that HPP combined with mild heat could be an effective way to pasteurize and extend shelf-life of various foods such as milk and poultry [23].

3.5 Effect of Treatment Time

As seen in Fig. 1, at low treatment times (5 min), the log reduction increased exponentially as a function of pressure whereas, at higher treatment times (15 min) the rate of increase in

log reduction as a function of pressure was more or less linear. This implicates that a given degree of sterility (log reduction) could be achieved by a short treatment at a high pressure or by a longer treatment at a relatively lower pressure.

3.6 Changes in Total Aerobic Counts during Chilled Storage

In pressure treated samples, there was no significant difference between the number of survival detected at day zero and day 4, however, the number of surviving *L. innocua* significantly increased at day 21 and at day 35. As shown in Table 1, there was no growth (min detection level, 1.7×10^2 cfu/g) after a 10 min treatment at 400 MPa and 0ºC. However, all the samples showed almost a full recovery after 35 days of storage at 3ºC.

Regardless of the number of surviving cells observed at day zero, the numbers reached to approximately the same initial level of microbial load (~10^8 cfu/g) for almost all the experimental conditions after 35 days of storage at 3ºC. On the contrary, the number of cells in control samples did not change significantly (8.2±0.2) during the whole storage (Table 1), which implies that the increased number of survival during chilled storage in pressure treated samples was not due to microbial growth, but rather was mainly due to recovery of injured cells. As the study was not initially designed to assess the injury as a result of pressure treatment, we did not use selective media to directly assess the extent of injury immediately after pressure treatment. However, assessing the change in TAC in pressure treated samples against the change in TAC in control samples during the 35 days of storage reveals that the injured microorganisms (mainly *Listeria spp.*) could recover during the storage at refrigerated conditions.

Pressure is known to inflict sublethal injury in vegetative cells of bacteria [5,8,9,19,24-28] and sublethally injured bacteria could recover in nutrient rich medium. Bozoglu et al. [9] stated that primary and secondary injuries could occur after HPP treatment and injured cells may not form visible colonies on selective or non-selective agar, however colonies could first form on non-selective agar and later on selective agar during prolonged storage. They showed that there was no detection of *L. monocytogenes* CA in milk (10^7 cfu/ml initial inoculation level) after a 550 MPa treatment at 45ºC but colony formation was observed on non-selective agar after 6 days of storage at 4ºC determined by streak plate method.

Other authors [29] also emphasized the importance of microbial injury during storage of pressure-treated foods. Kalchayanand et al. [13] used selective and non-selective medium to study the injury of some pathogenic bacteria. They reported that all the survivors of *Staphylococcus aureus* and *Salmonella Typhimurium* were injured after 5 min of treatment at 345 MPa and 25ºC. Some of the studies that supports the recovery of sublethally injured cells during storage after a pressure treatment of various types of foods are minced beef [30], sliced cooked ham [31] and poultry meat [18].

In the literature, there are variations in degree of recovery of microorganisms in pressure treated food products and model food systems during storage. However, existence of some degree of recovery depending on the parameters of pressure treatment, type of food and temperature of storage is evident in the literature. Our results contributing to the existing data and providing insights into the recovery of microorganisms (mainly *Listeria spp*.) in a nutrient rich medium such as, chicken meat during chilled storage.

Table 1. Experimental parameters for HPP treatment and TAC in minced chicken meat immediately after HPP treatment (day 0) and during chilled storage at 3ºC for up to 35 days. Results are from average of 3 counts and the standard deviations are derived from these 3 counts for each experiment

HPP Treatment Conditions			TAC during chilled storage			
Pressure (MPa)	**Temperature (ºC)**	**Time (Min)**	**Day 0 Log cfu/g**	**Day 4 Log cfu/g**	**Day 21 Log cfu/g**	**Day 35 Log cfu/g**
Control	-	-	7.8±0.22	8.2±0.06	8.3±0.03	8.3±0.07
200	20	15	7.6±0.07	8.0±0.04	8.1±0.04	8.0±0.03
200	40	10	7.8±0.06	7.9±0.11	8.1±0.02	7.9±0.03
200	0	10	7.9±0.02	7.9±0.13	8.0±0.13	8.0±0.06
200	20	5	7.9±0.04	8.1±0.06	8.1±0.03	8.0±0.06
300	0	5	6.1±0.04	5.9±0.07	7.0±0.15	7.3±0.05
300	20	10	6.4±0.10	6.4±0.03	7.3±0.02	7.4±0.13
300	0	15	4.4±0.07	3.4±0.12	6.5±0.04	7.0±0.05
300	20	10	6.3±0.03	6.4±0.03	7.5±0.01	7.7±0.06
300	40	15	4.2±0.04	4.7±0.09	6.8±0.08	8.1±0.01
300	40	5	5.7±0.09	7.5±0.04	8.0±0.13	7.9±0.08
300	20	10	6.3±0.05	5.7±0.13	7.4±0.06	7.4±0.04
400	40	10	2.7±0.01	2.7±0.01	6.8±0.03	8.3±0.10
400	20	5	3.3±0.13	2.9±0.17	2.9±0.17	8.0±0.04
400	20	15	2.9±0.17	2.7±0.30	6.0±0.10	8.3±0.03
400	0	10	NG	2.2±0.20	7.1±0.07	7.9±0.05

NG – No growth

4. CONCLUSION

Our results showed that survival of *L. innocua* decreased with increasing pressure and pressurization time. Compared to treatments at 20°C, reduction in number of microorganisms was higher when samples were pressure treated at 0°C and 40°C. Number of survival detected immediately after pressure treatment decreased with increasing pressure. After 10 min of treatment at 400 MPa and 0°C, there was no detection of survival. However, in all the experimental conditions examined, number of surviving microorganisms in almost all the samples approximately reached to the initial level of microbial load (10^8 cfu/g) after 35 days of storage at 3°C. Despite the absence of growth immediately after a pressure treatment, recovery of the microorganisms during storage might have important implications on the safety of high pressure treated foods. In nutrient rich environments such as, minced chicken meat, a total recovery could be possible during storage even if no viability is detected after the pressure treatment. Therefore stringent controls that takes into account the recovery of injured cells during chilled storage needs to be put in place to assure the safety of high pressure processed foods.

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COMPETING INTERESTS

Authors declare that there are no competing interests exist.

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