Journal of Food Protection, Vol. 61, No. 1, 1998, Pages 119–122

Research Note

Inactivation of *Listeria innocua* Inoculated in Liquid Whole Egg by High Hydrostatic Pressure

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MS 97-13: Received 31 January 1997/Accepted 20 May 1997

ABSTRACT

The resistance of *Listeria innocua,* as a model microorganism for *Listeria monocytogenes,* to high hydrostatic pressure in liquid whole egg was studied at several pressures (300, 350, 400, and 450 MPa), temperatures (−15, 2, and 20°C), and times (5, 10, and 15 min). *Listeria innocua* was added to liquid whole egg at approximately 10^6 CFU/ml. *Listeria innocua* was not totally inactivated in any of the treatments. In general, reduction was better at 2°C than at room temperature, but the greatest inactivation was obtained at 450 MPa at 20°C for 15 min (over 5 log of reduction). The results indicate that microbial inactivation was increased with prolonged exposure to pressure. *D* values for *Listeria innocua* were obtained at 400 MPa for two temperatures (2 and 20°C), and different times (0 to 20 min). The microbial inactivation followed apparent first-order kinetics, exhibiting a decimal reduction time of 7.35 min at 2°C and 8.23 min at 20°C.

Foods pasteurized by high pressure have been marketed in Japan. There is great interest in this method in Europe and the USA due to consumer demand for high-quality foods that are minimally processed, additive-free, and microbiologically safe. Effects of pressure in combination with temperature are attracting increasing scientific attention *(10, 23),* since temperature and pressure are the essential parameters influencing the physical state of substances.

Egg pasteurization has been designed principally to control salmonellae, and the U.S. minimum pasteurization parameters of 60°C for 3.5 min *(24)* have been reported to be sufficient. The temperature and/or time of pasteurization may be increased only slightly, because of the susceptibility of egg proteins to coagulation or thermal denaturation with the formation or destruction of covalent bonds, which may result in flavor changes and damage to the functional properties of the liquid whole egg.

Presently, it is well known that high pressure can be used to obtain safe foods with identical characteristics to fresh products. With this pressure technology (at low or room temperature) egg producers may be able to improve the microbiological quality of egg products without impairing their functional properties, with only small effects on flavor, color, and vitamins, and may allow reduction or elimination of chemical additives. Since pressure is transmitted instantaneously and homogeneously throughout all food, it does not affect the covalent bonds or produce overtreated zones as may occur in the thermal treatment *(4, 7, 17, 18).*

The resistance of microorganisms to high pressure is variable. Vegetative cells in the growth phase are most sensitive. Gram-positive bacteria are normally more pressure-resistant than gram-negative bacteria *(11).* The effect of pressurization at room temperature or higher has been thoroughly studied. Factors include the medium (pH, ionic strength, presence of added substances) and the strain *(4, 14).* Very little research has been done in egg products and less at low temperature, especially below 0°C. Takahashi *(22)* working with egg found that a treatment at 200 MPa at −20°C for 20 min was enough to eliminate several strains of gram-negative bacteria. This effect was more dramatic at −20 than at 20°C.

Listeria is a genus with more thermoresistant members, some of them pathogenic, than many other nonsporing genera of foodborne pathogens such as *Salmonella* and *Campylobacter.* Listeriae can grow at refrigeration temperatures. Kahn et al. *(12)* observed an increase of 2 log within 2 days in liquid egg held at 4°C. The possible survival in foods receiving inadequate or subpasteurization heat treatments *(6, 15)* may pose a health threat to susceptible humans and have serious international economic consequences.

The aim of this study was to examine the potential for inactivation of *Listeria innocua,* a nonpathogenic indicator for *L. monocytogenes (3, 5, 16),* in liquid whole egg using combinations of high hydrostatic pressure, temperature, and time.

MATERIALS AND METHODS

Raw material. Fresh eggs from the Universitat Autònoma de Barcelona (UAB) poultry farm were utilized throughout the study. Each egg was washed with a brush using soap and water 11°C warmer than the egg and sanitized by immersion in ethanol (70%) for 10 min and flaming *(25).* The egg contents were aseptically removed by cracking into sterile bags and homogenized for 1 min in a Stomacher Lab-Blender 400. The blended eggs were transferred to

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sterile beakers and pasteurized at 60°C for 3.5 min. After pasteurization, pH was adjusted to 8.0 by the addition of 1 N NaOH or 1 N HCl.

Compositional analysis and pH measurement. Total solids, nitrogen, and fat were determined according to standard methods *(1)*. pH was measured using a micro pH 2001 (Crison Instruments S.A., Spain).

Preparation and inoculation of liquid egg samples. A freeze-dried culture of *L. innocua* 910 was obtained from CECT (Spanish Type Culture Collection, Universidad de Valencia, Spain). For revival the culture was inoculated in 10 ml of brain heart infusion broth (BHI, Oxoid Ltd.), followed by incubation at 37°C for 24 h. Revived cells were streaked on BHI agar (BHIA, Oxoid Ltd.), incubated 24 h at 37°C and then maintained at 4°C. Ten milliliters of BHI with 10^8 CFU *L. innocua* per ml was inoculated into 1 liter of liquid whole egg. Working stocks were maintained for 1 month.

After inoculation the liquid whole egg was gently shaken by hand for 5 min, and then 40 ml of inoculated egg was put into disinfected polyester bottles. As much air as possible was expelled from the bottles, and the caps were sealed with Teflon film.

High-pressure treatment. The equipment used was a discontinuous isostatic press from ACB (Nantes, France). The time needed to achieve the treatment pressure was between 3 and 4 min, depending on the required pressure. The decompression time was between 90 and 120 s. The pressure chamber (ca. 2 liters) and 50% water-alcohol solution inside were cooled or heated to treatment temperature with a constant flow of 80% water-alcohol solution, within the walls of the chamber. Several combinations of high pressure (300, 350, 400, and 450 MPa), temperature (−15, 2, and 20°C) and time (5, 10, and 15 min) combinations were used in this study. The kinetic study of destruction for *L. innocua* in liquid whole egg at 2 and 20°C were conducted at 400 MPa and different times (0 to 20 min). All treatments were tested twice.

Determination of resistance to high hydrostatic pressure. Sample dilutions were made with quarter-strength Ringer's solution and 0.1 ml was spread-plated on Palcam agar (base) (Biokar BK111) and incubated at 37°C for 48 h to obtain viable counts of *L. innocua*. Strain was confirmed by the following criteria: gram-positive, nonspore-forming, oxidative-negative, and catalase-positive. Plate count agar (PCA, Oxoid Ltd.) was used to evaluate injured cells and possible contamination of samples.

Statistical treatment of data. Analysis of variance was performed using the general linear models procedure of the Statistical Analysis System (SAS, Cary, NC, USA). Survivor curves were calculated by linear regression analysis. *D* values (*D* = time in minutes to reach a 90% reduction in number of cells) were determined as the reciprocal slope of the survivor regression curve.

RESULTS

The effects of pressure on *L. innocua* 910 in liquid whole egg (dry matter: 22.9%; fat: 9.7%; total nitrogen: 2%) are showed in Figure 1. *L. innocua* was not totally inactivated in any of the treatments. In our study pressure was the most important factor in the inactivation of *L. innocua* in treatments with combinations of pressure, temperature, and time when 450 MPa was the highest pressure examined.

The results indicate that microbial inactivation increased with pressure (Fig. 1). The reduction in plate counts at low pressures (300 and 350 MPa) was greater at 2 and −15°C than at room temperature, but at higher pressures the

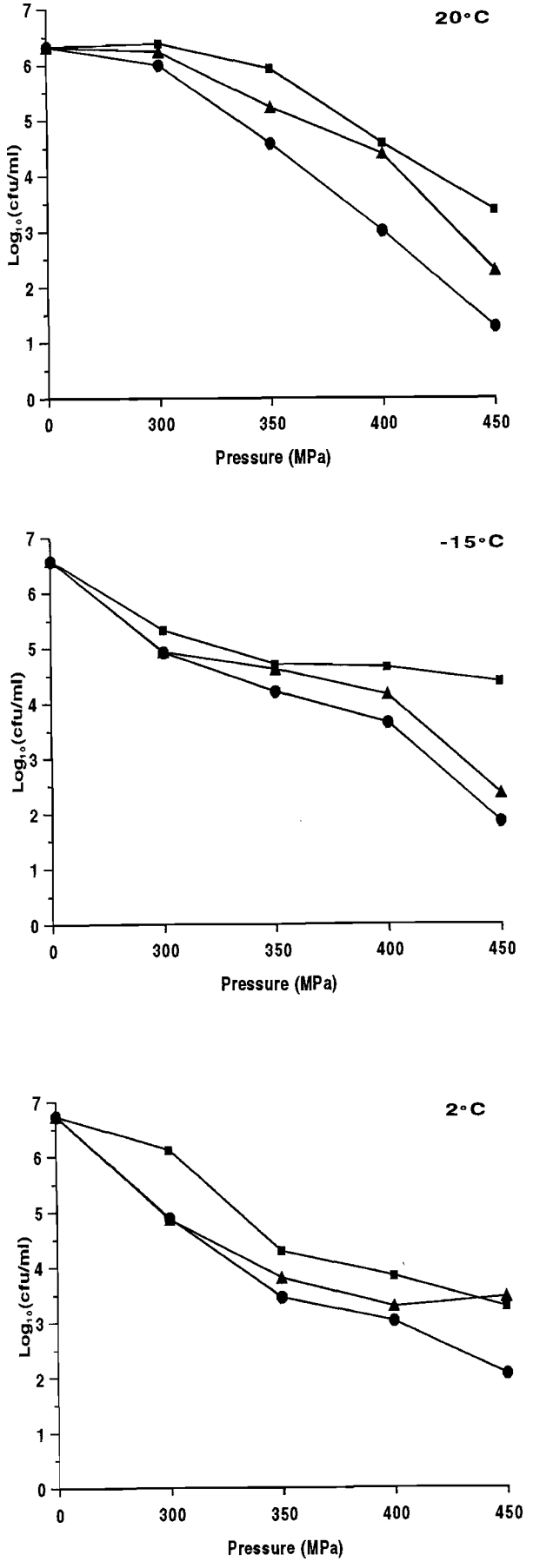

FIGURE 1. *Effect of high hydrostatic pressure (MPa) on* Listeria innocua *910 (CECT), inoculated in liquid whole egg after 5 min (■), 10 min (▲) and 15 min (●) of treatment at 20, −15, and 2°C.*

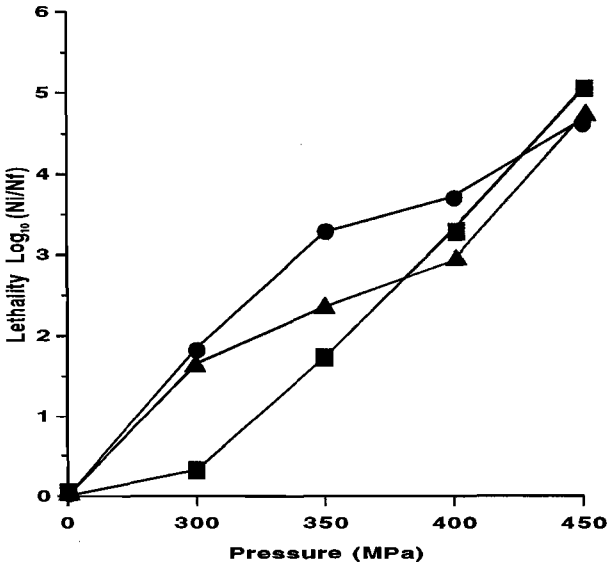

FIGURE 2. *Lethality (log(Ni/Nf)) in liquid whole egg inoculated with* Listeria innocua *910 (CECT) caused by high hydrostatic pressure (MPa) at different temperatures for 15 min at −15°C (▲), 2°C (●), and 20°C (■).*

effect of the temperature was not as important, with higher pressures leading to equalization of the response attributable to the temperature variable (Fig. 2). The greatest inactivation of *L. innocua* was obtained at 450 MPa at 20°C for 15 min (>5 log reduction). The least microbial reduction was found at 300 MPa at 20°C, where destruction was not noticeable at 5 and 10 min and a very slight reduction of 0.3 log was observed after 15 min.

The number of survivors decreased progressively with time and gave linear semilogarithmic plots (Fig. 3). The equation obtained by linear regression was log(CFU/ml) = $6.28 - 0.12t$ with $r^2 = 0.945$ at 20°C and log(CFU/ml) = $6.04 - 0.14t$ with $r^2 = 0.946$ at 2°C. D values were D = 8.23 min at 400 MPa and 20°C and D = 7.35 at 400 MPa and 2°C.

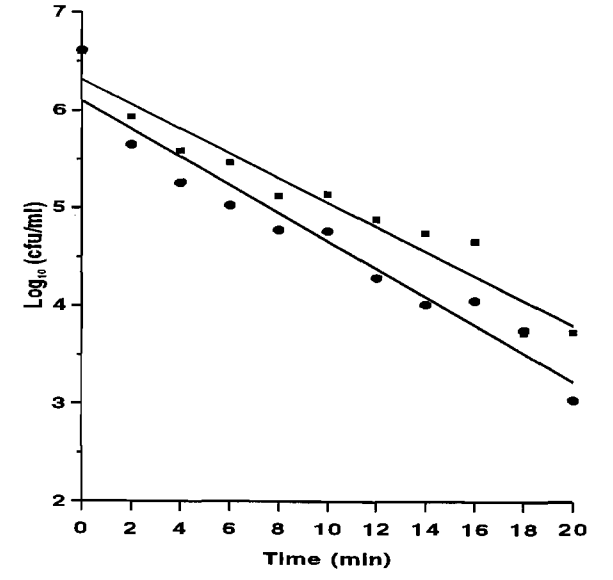

FIGURE 3. *Survival curves of* Listeria innocua *910 (CECT) in liquid whole egg pressurized at 400 MPa at 2°C (●) and 20°C (■).*

DISCUSSION

Very little research has been done at temperatures below 0°C with high pressure. Our results and the literature indicate that bacterial destruction depends on the temperature at which pressure is applied. Our study shows that at 300 and 350 MPa the reduction of *L. innocua* was slightly greater at 2 and −15°C than at room temperature in eggs. These results coincide with those of Carlez et al. *(3)* in inoculated minced beef muscle at 4 and 35°C at 300 MPa. Takahashi *(22)* found that a treatment at 200 MPa at −20°C for 20 min was enough to completely inactivate several strains of gram-negative bacteria in phosphate buffer (pH 7.0), and for certain microorganisms this effect was stronger at −20°C than at 20°C. On the other hand, Hawley *(9)* found that at low temperature some proteins are more susceptible to denaturation by pressure than at room temperature. This fact could explain our observations at low pressure, but not the equalization of the temperature response at higher pressure. Probably at 400 and 450 MPa the pressure effect on *L. innocua* is sufficiently strong to mask the differences caused by changes in temperature.

The greater degree of inactivation at 2°C than at −15°C may be caused by freezing of the sample at −15°C, which could apply a protective effect to the bacteria, although Kalicheusky et al. *(13)* showed that the application of high pressure reduces the freezing and melting points of water. These authors found that water remains in a liquid state at 300 MPa and −15°C, but is solid at 450 MPa and −15°C. More work on the solid-liquid state of liquid whole egg at high pressure and low temperature should be done to better understand the results obtained at temperatures below 0°C.

Previous studies have suggested that the bacterial cell membrane is the primary site of pressure damage, with pressure affecting cell permeability and producing osmotic effects, phase changes in the membrane, crystallization of phospholipids, and denaturation of membrane proteins *(4, 17)*.

At 20°C microbial reductions in inoculated minced meat were greater than those obtained in our study with liquid whole egg. Carlez et al. *(3)* observed that inactivation of *L. innocua* was complete (reduction by over 5 log cycles) at 400 MPa and 20 min. The same level of reduction was obtained in egg by increasing the pressure for a shorter time (450 MPa and 15 min). Possible variation in pressure resistance between different strains of the same species and pH changes in foods under pressure must be considered as suggested by Styles et al. *(21)* and Patterson et al. *(20)*.

Styles et al. *(21)* showed that after 20 min at 300 MPa viable cells of *L. monocytogenes* Scott A were not detectable in 100 mM phosphate-buffered saline (pH 7.0) at 23°C (microbial reduction about 7 log cycles). Carlez et al. *(3)* and Gervilla et al. *(8)* obtained with similar conditions a minimal inactivation (<0.5 log units) of *L. innocua* in minced beef muscle and ewe's milk, respectively. In our study, with a combination of 300 MPa at 20°C for 15 min a 0.3 log reduction was obtained. This suggests that food constituents can have a possible baroprotective effects in liquid whole egg, ewe's milk, and minced beef muscle, allowing microbial survival at higher pressures than in buffer, so the complex nature of foods can itself influence the pressure

resistance of the microorganisms. The variation in inactivation obtained on different foods has been reported elsewhere *(4, 20).*

Comparing the destruction curves from several studies, noticeable differences can be observed. Bartlett and Hawke *(2)* for *L. monocytogenes* Scott A in liquid whole egg obtained a $D = 2.1$ min at 60°C. Gervilla et al. *(8)* obtained destruction kinetics of *L. innocua* in whole ewe's milk (6% fat) of $D = 4$ min at 400 MPa and 25°C. In liquid egg our decimal reduction times were $D = 8.2$ min at 400 MPa and 20°C and $D = 7.4$ min at 400 MPa and 2°C. The pressure caused a microbial inactivation with apparent first-order kinetics, the same as with heat, but the time in minutes to reach a 90% reduction in cell number was greater than for the usual pasteurization. This indicates that pressure is a less aggressive treatment than high temperature with a more gradual microbial reduction. These results could be explained by the baroprotective effect of fat in microorganisms as Styles et al. *(21)* stated. Our liquid whole egg had a fat content of 9.7%, which probably protected *L. innocua.* The protective effect of fat has been well established in thermal processing *(16).*

There is a high incidence of *Listeria* spp. including *L. monocytogenes,* in raw liquid whole egg, but counts are usually low with a mean level of about 1 cell per ml *(19).* Therefore, we can assume that most of the combinations tested in our study would be effective in destroying 1 CFU/ml. We obtained about a 2-log-cycle reduction in *L. innocua* with a combination of 300 MPa for 15 min at 2°C. This should be sufficient to improve the microbiological quality of egg products without applying high temperatures that could impair functional properties.

We can conclude that high-pressure processing has the potential to improve the safety of liquid whole egg without the use of preservatives. The application of pressure at 2 and 20°C is a good method for inactivation of *L. innocua.*

Future research should examine the response of *Salmonella* species inoculated in egg after treatment with high hydrostatic pressure due to their importance and frequent presence in egg products. The effect of these treatments on technological properties of eggs must be also considered.

ACKNOWLEDGMENTS

We would like to thank Spanish Type Culture Collection (CECT) for providing the strain and Maria Rosa Pujol for advice on statistics.

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