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Estimation of the pasteurization treatment of liquid whole egg inoculated with *Salmonella senftenberg* 775W by High Hydrostatic Pressure Treatments

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----------ABSTRACT----------

The inactivation of Salmonella senftenberg 775 W (10^8 cfu ml $^{-1}$) inoculated in liquid whole egg (LWE) under combinations of pressure (200, 300, 400 and 500 MPa) and time (5 min x 1 pulse, 5 min x 2 pulses, 10 min x 1 pulse, 5 min x 3 pulses and 15 min x 1 pulse) at environmental temperature was studied. The inactivation rate was dependent on time and pressure being minimal at 200 MPa and 25 ºC for 20 min (< 1 Log reduction) and reaching total inactivation after a treatment of 500 MPa for 20 min. Treatment in pulses (5 min x 2 pulses and 5 min x 3 pulses) showed greater effectiveness than single-pulsed treatments (5 min x 1 pulse, 10 min x 1 pulse and 15 min x 1 pulse) comprising the same total time. The characteristic tailing inactivation curves were described by a first-order biphasic model; it is suggested that the phase change of LWE during pressure treatment affects the inactivation rate of S. senftenberg 775W. From the results of approximately 5 log reductions of S. senftenberg 775W is regarded to be as effective as conventional thermal pasteurization. The results of this study provide information that can be used by processors to aid in producing safe, pasteurized egg yolk products by high hydrostatic pressure, and for satisfying USDA pasteurization performance standards and developing industry guidance.

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I. INTRODUCTION AND LITERATURE SURVEY

Food safety of egg-products at present time is assured by means of heat treatments (pasteurization and/or ultra-pasteurization). The thermal process of pasteurization tries to reach the eradication of pathogenic micro-organisms that can be present in the whole liquid egg, mainly Salmonella spp. and thus, ensure product safety, as well as the maintenance of the technological and physico–chemical characteristics of the product. Unfortunately, egg products are also responsible for a large number of foodborne illnesses, with Salmonella being responsible in most cases (European Food Safety Authority, 2011). During the last three decades the number of food poisoning outbreaks in which different serotypes of Salmonella have been involved has increased steadily, being eggs and egg products the most frequently involved foods. Traditional thermal treatments used to pasteurize LWE (e.g., 60 ºC for 3.5 minutes in the USA (USDA, 1984), or 64 ºC for 2.5 min in the U.K.) ensure food safety by giving from 5 to 9 $\log_{10}$ reductions of the most frequent Salmonella serotypes (*Salmonella enteritidis* and *Salmonella typhimurium*) (Alvarez et al., 2006; Mañas et al., 2003). However other very heat resistant serotypes, such as Salmonella senftenberg 775 W have been also isolated in foods. Traditional pasteurization treatment is not able to reach more than 4 reduction cycles in the population of *Salmonella senftenberg* 775 W (Mañas et al., 2003), therefore the safety level for pasteurized liquid egg contaminated with *Salmonella senftenberg* 775 W is low. Besides these low levels of inactivation in *Salmonella senftenberg,* pasteurization heat treatments show some disadvantages such as the creation or destruction of covalent bonds which promote flavour changes and qualitative and quantitative losses in functional properties of liquid whole egg and egg white (foam capacity and stability, emulsion capacity, coagulation, texture, colour, nutritional value, etc.). On the other hand, the high thermal sensitivity of liquid egg components limits the temperature at which the product can be heated. However, the use of lower temperatures or shorter times could be an important health risk as the treatment may not be enough to eradicate Salmonellae. Pasteurization of liquid whole egg is limited to low pasteurization temperatures and long holding times due to the coagulation of its proteins at higher temperatures. Subsequently, incomplete pasteurization at lower temperatures has led to outbreaks involving Salmonella spp. (Tauxe 1991; Tood, 1996; Roig-Xagués et al., 2008). To overcome the limitation of conventional heat pasteurization and to extend the refrigerated shelf life of LWE, alternative non-thermal technologies are being explored for LWE pasteurization. Among the non-thermal techniques such as pulsed electric fields (Monfort et al., 2010), UV radiation (Unluturk

et al., 2008), ultrasonic waves (Mañas et al., 2000), irradiation (Alvarez et al., 2006), high hydrostatic pressure has received the most attention in food processing (Mertens and Knorr, 1992; Garriga et al., 2004; Fonberg-Broczek et al., 2005; García-González et al., 2007; Velázquez et al., 2008; Pina-Pérez et al., ,2009; Lee et al., 2010; Koutchma et al., 2014; Naderi et al., 2017). During the last few decades, high hydrostatic pressure (HHP) food processing at refrigeration, ambient or moderate heating temperature has been applied to food products to improve microbiological and nutritional qualities similar to those of fresh unprocessed products (Chawla et al., 2011; Hasnikova et al., 2009; Malicki et al., 2005; Monfort et al., 2012; Neetoo et al., 2011; Ritz et al., 2006; Velázquez-Estrada et al., 2011).

When evaluating the effectiveness of any preservation treatment, one of the primary considerations is its ability to eradicate pathogenic or spoiling microorganisms. Treatments of 200 to 350 MPa for minutes hardly affect microbial lethality, and pressures of 300 to 450 MPa applied for 5-15 minutes, are required to reduce more than 2 $\log_{10}$ the population of microorganisms such as *Listeria innocua*, Escherichia coli and *Salmonella enteritidis* in egg products (Isiker et al., 2003; Lee et al., 2010). Furthermore, the inactivation of microorganisms by heat and other processing methods has been traditionally assumed to follow first-order kinetics. All cells or spores in a population are assumed to have equal resistance to lethal treatments and, therefore, a linear relationship between the decline in the logarithm of the number of survivors over treatment time would be expected (Schaffner and Labuza, 1977). Nevertheless, significant deviations from linearity have frequently been reported (Cerf,1977; Peleg and Cole,1998; Peleg et al., 2005; Xiong et al., 1999, Van Boekel et al., 2002; Erkmen et al., 2009). Recent studies indicate that microbial survival curves under high pressure often do not follow first-order kinetics, because there are resistance gradients in the microbial population. These nonlinear inactivation curves can be described by a biphasic model proposed by Cerf.,1977, 1997, based on the assumption of two populations, a sensitive (fast inactivating) and a resistant (slow inactivating). This leads to a curve with two distinct segments of linearly decreasing populations, the second (more resistant) with a less negative slope. This model can be formulated as follows (Geeraerd et al., 2000):

$$\mathrm{Log}\left(\frac{N}{N_0}\right) = \mathrm{Log}\left(f \cdot e^{-k_{max1} \cdot t} + (1-f) \cdot e^{-k_{max2} t}\right)$$

Where fis the fraction of the initial population in a major subpopulation, (1-f) is the fraction of the initial population in a minor (more heat resistant) subpopulation, and k_{max1} and k_{max2} are the inactivation rates of the two populations respectively.

Despite the microbial inactivation potential of HHP, processing of eggs products by this technology may induce protein conformation changes (denaturation, aggregation and coagulation) (Monfort et al., 2012; Yan et al., 2010) leading to relevant modifications of functional properties (Monfort et al., 2012; Balasubramaniam et al., 2015). This protein-denaturated stage may results in aggregation and gelation depending on a number of factors related to the protein system (i.e. composition and nature of proteins involved), environmental conditions (i.e. pH, ionic strength) and HHP process variables (i.e. high pressure level, processing time and temperature). Nevertheless, these effects can be minimised in a higher extent than in heat treatments due to the use of lower temperature process, so high pressure treatment open a possibility for egg products with improved microbiological quality and only negligible effects on functional properties. Therefore, it is important to know the effect of high pressure treatment on pathogens in each specific product.

The aim of this study was to characterize the inactivation kinetic of *Salmonella senftenberg* 775W in liquid whole egg by HHP in order to calculate the necessary time to reach pasteurization inactivation level (5 log cycles) and to evaluate the influence on the inactivation effectiveness of single pulse and multi-pulse pressure treatments.

II. RESEARCH ELABORATIONS

2.1 High Hydrostatic Pressure System

High pressure treatments were carried out in a discontinuous isostatic equipment model FPG 5740, Stansted Fluid Power LTD, Stansted, UK) with a pressure vessel of 17 mm internal usable diameter and 120 mm internal usable height, 5 L capacity and 900 MPa pressure limit (Figure 1).

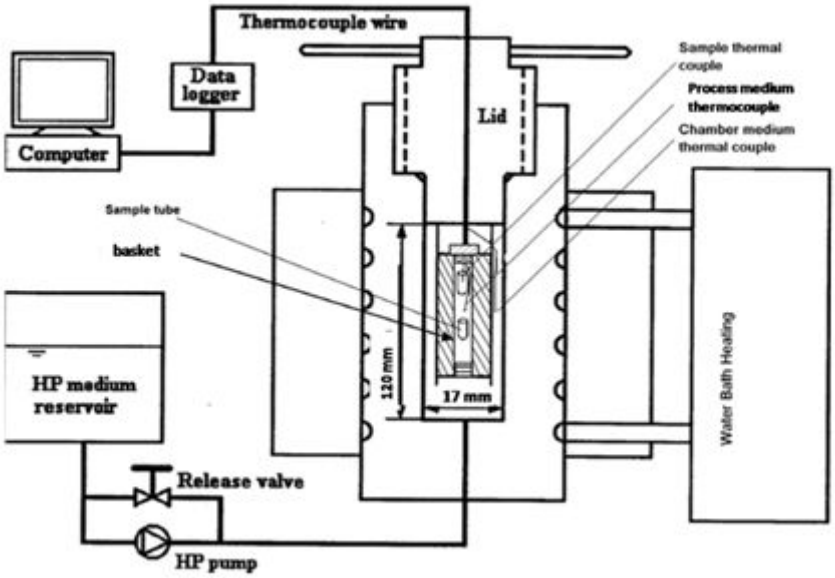

Figure 1. Scheme of the High Hydrostatic Pressure system used in this research

The medium used for pressure transmission in the system was water/propylene-glycol mixture. To maintain a stable temperature during pressure treatments, a refrigerating water bath (1140S, VWR International, USA) was used to circulate temperature controlled water at room temperature (20 ºC) around the pressure vessel in order to regulate the shell temperature. Pressure treatment times reported in this study did not include the pressure build-up and realising times. The rate of pressurization was 240 MPa/min and depressurization time was <20 s. The chamber water temperature was measured with a thermocouple (T-type thermocouple,SFP, UK). The increase and decrease of temperature during pressurization and depressurization was about 2 ºC per 100 MPa as a result of adiabatic heating or cooling. Several pressure (200, 300, 400 and 500 MPa), temperature (25 ºC) and time (5 min x 1 pulse, 5 min x 2 pulses, 10 min x 1 pulse, 15 min x 1 pulse and 5 min x 3 pulses min) combinations were investigated. After the pressure treatments, test samples were kept in an ice water bath prior to enumeration. All samples were analysed by duplicate in at least three independent experiments.

2.2 Raw material

Fresh eggs were purchased from a local supermarket and from a poultry farm and held overnight under refrigeration. Results obtained in eggs from different sources were similar (data not shown). Preparation of the liquid egg was performed according to Huang et al., 2006. The eggshells were sanitised by dipping and rubbing with 70 % ethanol before breaking. The wet shells were allowed to air dry for 10 minutes. The eggs were broken and removed from their shells by hand and mixed during 1 min with a blender (Braun 400, Germany) that had been washed and sterilised using 70 % ethanol. About 3-4 eggs were used to yield approximately 30-40 ml of liquid whole egg. The pH of homogenized liquid whole egg (LWE) used in this study was not modified and ranged from 8.2 to 8.4. 2 ml of LWE were transferred aseptically into polyethylene bags (inoculated with *Salmonella senftenberg* 775W as described below, and heat sealed.

2.3 Preparation and inoculation of samples

A freeze-dried culture of *Salmonella senftenberg* CECT 775W (ATTCC 43845) was obtained from Department of Food Technology (Faculty of Veterinary) of the University of Zaragoza, Spain. Vials were maintained at -80 ºC until use. For revival, the culture was spread-plated on Tryptic Soy Agar supplemented with 0.6 % yeast extract (TSAYE) (Biolife, Milán, Italy) and incubated at 37 ºC overnight. Working cultures were prepared by transferring one colony from TSAYE plate to 10 ml of Tryptic Soy Broth supplemented with 0.6 % yeast extract (TSBYE) (Biolife, Milan, Italy) and grown at 37ºC overnight under agitation. Recovered cells were streaked on 50 ml TSBYE suspension and incubated at 37 ºC overnight to reach approximately 1 x 10^9 cfu $ml^{-1}$and maintained at 4 ºC. Aliquots of 0.2 ml of *S. senftenberg* in TSBYE were inoculated into 2 ml of LWE prepared as described above to obtain approximately 10^8 cfu ml^{-1} and the mix was gently shaken by hand and put aseptically into sterilized polyethylene bags which were heat sealed removing as much air as possible. For pressure treatments, *S. senftenberg* was inoculated in stationary growth phase, since some studies have demonstrated that in the exponential growth phase cells present a lower pressure resistance (Ray and Speck, 1973; Velázquez et al., 2005). All prepared samples were stored on ice to be immediately (maximum 1 h) pressurized. The chamber and the pressurization fluid were pre-equilibrated to 20ºC for at least 1 h before treatment.

2.4. Enumeration of viable S. senftenberg

Untreated (control) and treated samples inoculated with *S. senftenberg* and subjected to pressure treatment were analyzed using appropriate decimal dilutions in Buffer Peptone Water (BPW) (Biolife, Milan, Italy) at 1 % (w/v) and surface-plated onto TSAYE. The plates were incubated at 37 ºC for 48 h. Previous experiments showed that longer incubation times did not influence survivor counts. Reductions were calculated as the logarithm of the ration between the count of colonies in untreated (No) and treated (N) samples (Log No/N).

2.5. Curve fitting and data fit

A mathematical model based on a biphasic inactivation was used to fit the survival curves of *S. senftenberg* 775W. The modified Geeraerd mathematical expression was used (Geeraerd et al., 2000):

$$\mathrm{Log_{10}S} = \mathrm{Log_{10}}\left[(1-\mathrm{P})\mathrm{e}^{-\mathrm{k_1 t}} + \mathrm{Pe}^{-\mathrm{k_2 t}}\right] \quad \text{Equation (1)}$$

Where S is the survival fraction (N_t/N_0), $N_t$is the number of microorganisms at time t, N_0 is the initial number of microorganisms, (1-P) is the fraction of survivors in subpopulation 1, k_2 the specific death rate of subpopulation 1, P is the initial fraction of survivors in subpopulation 2 and $k_2$the specific death rate of subpopulation 2.

Equation were fitted to experimental data by nonlinear regression analysis using the least-squares criterion by the Solver function of the Excel 5.0 package (Microsoft, Seattle, WA, ISA) and the GraphPad PRISM® (GrapthPad Software, Inc., San Diego, CA, USA).

III. RESULTS AND DISCUSSION

3.1. Study of kinetic inactivation of *S. senftenberg* 775W by single-pulse treatments

Survival curves corresponding to the inactivation of *S. senftenberg* 775Win LWE at different pressure intensities are shown in Figure 2. Bactericidal activity of the pressure treatments increased with intensity and with treatment time. Under all the experimental conditions investigated, survival curves of *S. senftenberg* were not linear. The survival curves of *S. senftenberg* 775W were concave-upwards. Inactivation was faster in the first moments of the treatment and then the number of survivors decreased slowly.

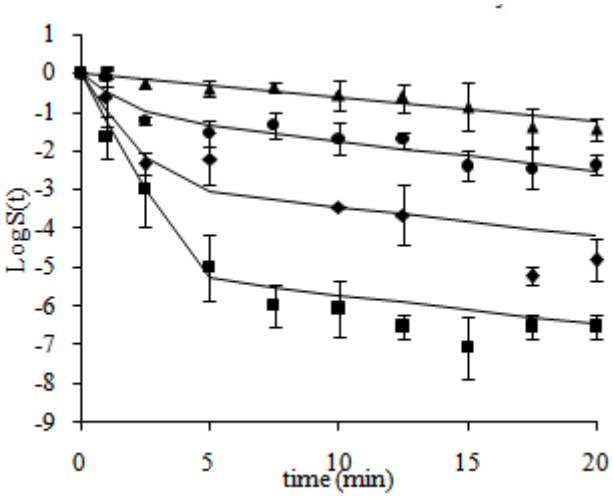

Figure 2. Nonlinear pressure survival curves of *Salmonella senftenberg* 775 W in whole liquid egg. Experimental data: (▲) 200 MPa; (●) 300 MPa; (♦) 400 MPa; (■) 500 MPa; Continuous lines: fit with the biphasic mathematical model.

In order to quantify and compare the bactericidal effectiveness of the HHP treatments at the different pressure intensities, survival curves obtained in this investigation were fitted to equation (1). This model assumes that there are two populations of bacteria that die at different rates. Table 1 shows the estimated parameters of the model with their 95% confidence limits. Determinations coefficients (R^2) and the root mean square errors (RMSE) of the fits that are also included in the Table 1 showed that equation accurately described the survival curves of *S. senftenberg* 775W at all treatment conditions investigated. The P-value decreased with the pressure intensity while the k_2 values increased with this variable.

Table 1: P, k_1, k_2 values estimated from fitting of the equation 1 (biphasic mathematical model) to the survival curves corresponding to the inactivation of *S. senftenberg* inoculated in whole egg by HHP treatments

Pressure (MPa)	P (min) (CL 95%)[a]	k_1(CL 95%)	k_2(CL 95%)	R^{2b}	RMSE [c]
200	13.68	0.23	0.24	0.94	0.200
300	0.093 (-0.046-0.23)	0.17 (0.059-0.277)	1.22 (0.0093-2.425)	0.94	0.210
400	0.022 (-0.033--0.076)	0.399 (-0.217--0.580)	2.279 (-0.017-4.572)	0.97	0.310
500	2.4e-6 (-4.1e6-8.9e5)	0.138 (-0.053--0.329)	2.51 (2.067-2.958)	0.99	0.311

[a] CL 95%: confidence limit
[b]$R^{2:}$ determination limit coefficient
[c]RMSE: Root Mean Square Error

However, correlation coefficients between k_1 values and pressure intensity were very poor. In order to reduce the number of parameters of the equation at each pressure intensity the k_1 values were set at an average value, 0.23. The average k_1 values and the P and k_2 values obtained by fitting the equation to the experimental data with the k_1 value seta at a mean value are shown in table 2. Although the refitted models had one less parameter, the examination of the R^2 and RMSE indicated that the goodness of the fit was comparable to the original model with two parameters (Table 2).

Table 2: P, k_1, k_2 values estimated from the second fitting of the equation 1 (biphasic mathematical model) to the survival curves by keeping constraint the k_1 value corresponding to the inactivation of *S. senftenberg* inoculated in whole egg by HHP treatments

Pressure (MPa)	P (min) (CL 95%)[a]	k_1	k_2(CL 95%)	R^{2b}	RMSE [c]
200	14	0.23	0.24	0.94	0.190
300	0.195 (0.089-0.300)	0.23	1.365 (-0.615-3.344)	0.92	0.241
400	0.0026 (-0.0009-0.0061)	0.23	2.038 (0.626-3.389)	0.93	0.460
500	9.08e-6 (2.09e-6-1.61 e6)	0.23	2.723 (2.082-3.358)	0.98	0.330

[a] CL 95%: confidence limit
[b]$R^{2:}$ determination limit coefficient
[c]RMSE: Root Mean Square Error

The log of the P-value displayed a linear relationship with the pressure intensity investigated (Figure 3).
$\text{LogP} = -0.0204\text{MPa} - 5.356 R^2 = 0.99$ Equation (2)

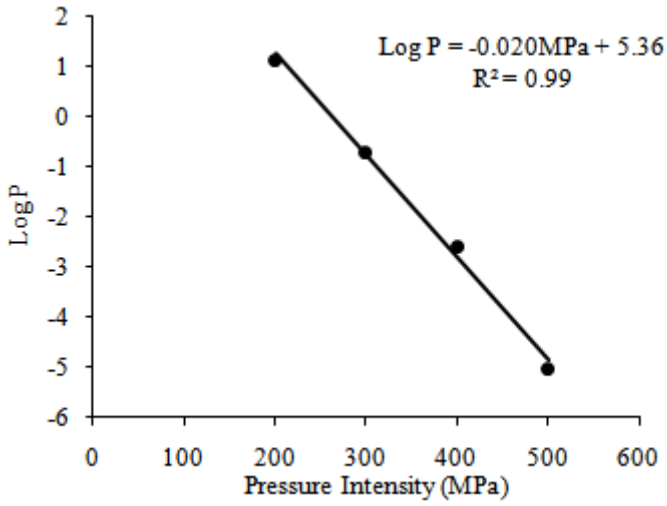

Figure 3: Relationship between the initial fraction of survivors in subpopulation 2 (P-value) and the Pressure intensity (MPa) of the treatment.

And, a linear relationship was observed between the $k_2$values and the pressure intensity (MPa) (Figure 4).

$k_2 = -0.0081\text{MPa} - 1.2049$ $R^2 = 0.98$ Equation (3)

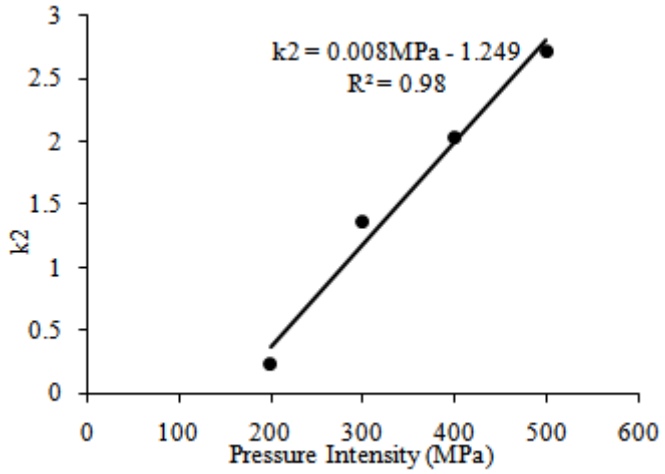

Figure 4: Relationship between the specific death rate of subpopulation 1 (k2 value) and the Pressure intensity (MPa) of the treatment.

Substituting equation (2) and equation (3) into equation (1) we get equation (4) that can predict the survival curves at other pressure levels (MPa) and different treatment times (t):

$$\text{Log}S_t = (\text{Log}1 + (0.0204\text{MPa} + 5.356))e^{-0.23t} + (-0.0204\text{MPa}-5.356)e^{(0.0081\text{MPa}+1.2049)t}$$

Equation (4)

By using this equation, and in order to quantify and predict the antimicrobial effectiveness of high hydrostatic pressure, Figure 5 shows the combinations of treatment time and pressure intensities needed to reach a pasteurization inactivation level of 5 Log_{10} cycles at environmental temperature. Overall, this figure shows that, in the range of pressure intensities studied, the necessary time for reducing 5 Log-cycles the population of *S. senftenberg* from whole egg decreases exponentially. The equation 5 allows to calculate, by a very easy way, the time for obtaining this inactivation level at any pressure intensity into the pressure range studied.From the before equation and considering the condition of getting the pasteurization inactivation level (5 Log-cycles), is possible to obtain the equation 5. This simple mathematical expression allows to calculate the time necessary to reduce 5 Log cycles the population of *S. senftenberg* 775 in whole egg in the range of pressure intensities studied. The R^2 value of the equation is 0.99 (Figure 5)

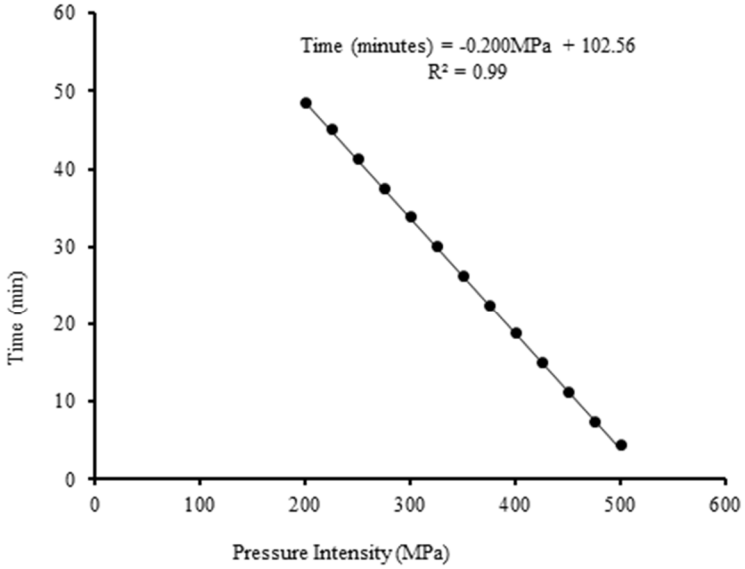

Figure 5: Necessary time to reach the pasteurization level of inactivation (5 Log-cycles) in the population of *S. senftenberg* in whole liquid egg.

Validation of this prediction model (equation 5) was performed by treating 12 samples of whole egg for 9 minutes at different pressure intensities: 250, 350 and 450 MPa (four samples at every pressure intensity). Figure 6 shows the relationship between the inactivation level data estimated by the model and the inactivation log cycles' obtained experimentally. Correlation coefficient was 0.99; therefore, the developed equation could be used successfully to predict the inactivation level at pressure intensities different than those experimentally used.

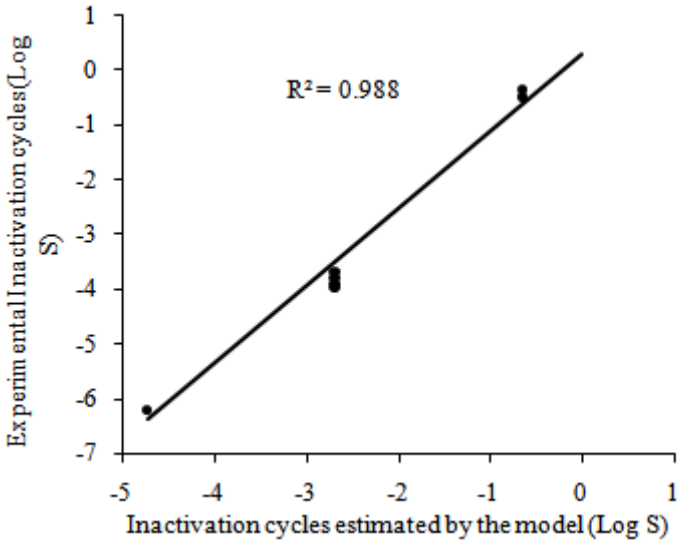

Figure 6: Relationship between the experimental inactivation level (Log cycles) reached at 250, 350 and 450 MPa (4 replicates) for 9 minutes and the estimated inactivation level predicted by the developed model.

3.2. Study of *S. senftenberg* inactivation by pulses pressure treatments

The effect of pulses of pressure in the destruction of *S. senftenberg* at different pressure intensities during multi-pulses of 5 minutes are shown in Figure 7a (two pulses of 5 minutes and a one pulse of 10 minutes) and Figure 7b (three pulses of 5 minutes and one pulse of 15 minutes). All the experiments were carried out by triplicate. Two or three pulses of 5 minutes achieved higher destruction than single-pulse treatments comprising the same total time, 10 and 15 minutes, respectively. A previous similar study has been carried out by Ponce et al., 1999 with *S. enteritidis*. As the same way than this research, that study obtained a higher effectiveness of the multi-pulse treatment in comparison with the single-pulse treatment. In this study treated samples were spread-plated on TSAYE, medium where Ponce et al. got the maximum recovery.

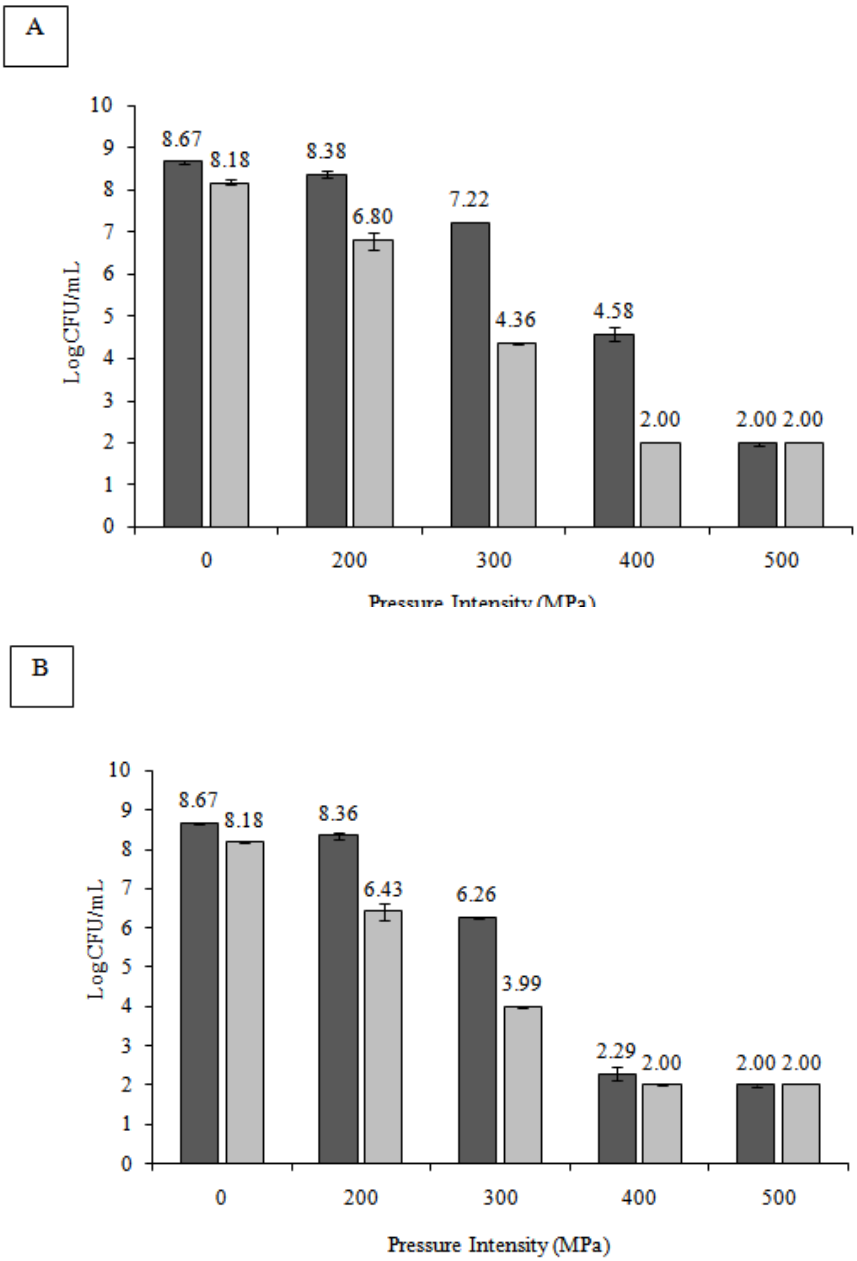

Figure 7. Inactivation of *S. senftenberg* inoculated in whole egg at different pressure intensities and by applying treatments of one pulse (■) of 10 minutes (a) or 15 minutes (b) and/or two pulses (■) of 5 minutes (a) and three pulses of 5 minutes (b). Error bars represent the Confidence Limit 95%

Table 3 shows the inactivation level reached after time-equivalent treatments. The pasteurization inactivation level is nearly reached after a treatment of 3 pulses of 5 minutes at 300 MPa while, a time-equivalent treatment of 15 minutes at the same pressure intensity only reduces the *S. senftenberg* population 2.23 log cycles. The same effect can be observed at 400 MPa with treatments of 10 minutes. 3.6 log and totally inactivation (6.67 log cycles) were reached after a treatment of one pulse of 10 minutes and two pulses of 5 minutes respectively.

Table 3: Comparison of the inactivation level (Log cycles) of *S. senftenberg* after one pulse or multipulse HHP treatments.

HHP treatment	Inactivation level (Log cycles)			
	200 MPa	**300 MPa**	**400 MPa**	**500 MPa**
One pulse (5 min)	0.37	1.21	2.21	5.39
One pulse (10 min)	0.77	1.73	3.60	6.06
Multi-pulse (5 min (x2))	1.87	4.31	6.67	6.67
One pulse (15 min)	1.20	2.23	6.38	6.57
Multi-pulse (5min (x3))	2.24	4.68	6.67	6.67

Limit of the detection level = 6.67

IV. CONCLUSIONS

Determination of the kinetic parameters during microbial inactivation, whether through thermal high pressure, chemical or other processes, is a rather complicated task. So far, the industry has employed the approach of first-order kinetics to determine D and z values for food processing. This approach is based on the assumption that the cells of a population are homogeneous with identical inactivation times. Interestingly, many survival curves do not follow linear trend and exceptions occur in the form of shoulders and/or tailing (Cerf, 1977, 1997), suggesting that this approach is not always correct. Even in the case of a pure culture, biological heterogeneity among the cells results in subpopulation with individual inactivation kinetics. As a result, inactivation curves could be regarded as the cumulative form of underlying distribution of individual inactivation times (van Boekel 2002; Avsaroglu et al., 2005; Buzrul et al., 2004). In our work, it was evident that survival curves deviated from linearity in whole liquid egg and the shapes of the curves changed considerably according to the treatment pressure level (Figure 2). The biphasic nature of the survival curves suggests the existence of two discrete populations that differ in their sensitivity to the applied treatment. However, this reason does not seem to be the cause of the shape of the survival curves observed because the proportion of the more resistant survivors (P-value) was depended on the treatment conditions (Table 2). A protection resulting from dead cells that shield the remaining survivors, cell aggregation during the treatment or an adaptation of the microorganisms to the treatment could be other causes for biphasic curves. However, treated cells observed under microscope formed neither aggregates nor flocs (data not shown). On the contrary, a microbial adaptation is quite improbable considering the rapid inactivation of the most sensitive population. Multi-pulsed treatments significantly promoted the pressure inactivation of *S. senftenberg* in egg whole. The use of multi-pulsed treatments (5 x 2 pulses and 5 x 3 pulses) would allow using lower pressures or shorter treatments times for inactivation of this pathogen. With the role of HHP expanding for the preservation of commercial foodstuffs, reliable estimation of pressure-processing factors will become more critical in order to assure the safety of pressure-treated foods. On the other hand, the development of accurate mathematical models describing the pressure inactivation kinetics of microorganisms should benefit the food industry by optimizing process conditions and helping construct HACCP programs to maintain food safety.

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