Baroinactivation of *Staphylococcus epidermidis* – Mathematical Model and its Verification Using Human and Cow Milks

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Abstract

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Staphylococcus epidermidis, commonly found on the human skin, may contaminate human milk. High-pressure pasteurisation of human milk under normal temperature preserves the majority of its protective agents. The objective of this study was to acquire baroinactivation data and develop a model for model solutions of pH = 6.4 to 7.2 and water activity a_w = 0.99, in which baroinactivation of *Staphylococcus epidermidis* takes place. Decontamination data manifested exponential kinetics and the resulting model was described by the following equations: $D_p = D_{p,\,\mathrm{ref}} \times 10^{(P_{\mathrm{ref}} - P)/Z}$, $Z = -123.90\ \mathrm{pH}^2 + 1635.54\ \mathrm{pH} - 5210.49$; $D_{p,\,\mathrm{ref}} = -8.89\ \mathrm{pH}^2 + 121.02\ \mathrm{pH} - 408.34$. The developed model was verified using pasteurised human milk and UHT-treated skimmed cow milk. The agreement between the experimental data and model-based prediction was very good for human milk. It was proved that the application of a pressure of 350 MPa for 10 min decreased the concentration of the working suspension of *S. epidermidis* in the model substrate by a minimum of five orders.

Keywords: *Staphylococcus epidermidis*; baroinactivation; human milk

The *Staphylococcus epidermidis* bacteria are naturally present on mucous membranes and on the skin of humans and animals, as well as in the environment. The optimum proliferation temperature of this group of microorganisms is 37°C (Lawley & Halligan 1998, valid for *St. aureus*).

Staphylococci that do not synthesise the enzyme plasmocoagulase have been described as the cause of various contagious diseases. The research has confirmed that these microorganisms should not be overlooked or classified as mere contaminants as they can contribute to the incidence of contagious diseases in adults and infants (Da Cunha *et al.* 2006; Sarkar 2006).

According to Petrelli *et al.* (2006), these microorganisms can create a biofilm on the surface of

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medical implants and contribute to the proliferation of staphylococci colonies on such devices.

The practice of milk banks and human milk collection centres shows that *S. epidermidis* is most frequently encountered in non-pasteurised human milk (Měřička *et al.* 2003). This microorganism, compared to other species, is also found in the highest concentrations. The frequency distribution in non-pasteurised human milk has been recently studied by Landfeld *et al.* (2006). The above given reasons make it imperative to acquire thorough information on the response of the microorganism to the treatment with a high pressure. The use of high-pressure pasteurisation of human milk without heating could result in the future in the preservation of a majority of protective agents (in particular immunoglobulins), which make breastfeeding or at least nutrition with pasteurised human milk indispensable. Despite mild heat pasteurisation, milk loses considerable amounts of these substances in the process.

The existing literature on the high pressure inactivation of *S. epidermidis* is scarce. We have recently found a paper dealing with the high pressure inactivation of *S. aureus* in model ham substrate (Tassou *et al.* 2008).

Therefore, the objective of our work was to acquire reliable baroinactivation data for the substrates of pH from 6.4 to 7.2, and water activity a_w = 0.99, subject to baroinactivation of the selected microorganism. These parameters cover the range of pH and water activity values for cow and human milks, forming the base of infant nutrition.

MATERIAL AND METHODS

Selection of microorganism, its origin, preparation of the working culture and working suspension, incubation conditions, acquiring numerable colonies. The microorganism selected as the model microorganism was the bacterium *Staphylococcus,* in particular the culture *Staphylococcus epidermidis* CCM 4418 (strain also identified as ATCC 12228). It possesses stable reproducible characteristics (often recommended for the verification of the cultivation media quality or as a negative check for plasmocoagulase test).

A culture of *Staphylococcus epidermidis* CCM 4418 was supplied by the Czech Collection of Microorganisms (CCM Masaryk University in Brno, Faculty of Science), freeze-dried on a carrier, in the form of gel discs in sterile vials. Following the procedure recommended by CCM, a revived culture was prepared on the so-called meat-peptone agar slant. The revival of the microorganism cultures took place under 37°C for 24 hours.

The working culture was prepared using the liquid substrate recommended for the cultivation of staphylococci BHIB (Brain Hearth Infusion Broth) filled in 9 ml test tubes. The liquid substrate was inoculated with 3 µl of the revived culture from the agar slant.

The inoculated BHIB medium was incubated under aerobic conditions, at 37°C for 18 h, in order to obtain the early stationary growth stage, commonly recommended for and used in experiments aimed at studying lethal and sub-lethal effects on bacteria, e.g. Schlemmerová (1987).

The working suspension was prepared by inoculating one volume unit of the working culture grown under the above given conditions in the BHIB into nine volume units of saline solution of pH values between 6.4 up 7.2 and water activity of a_w = 0.99 (hereinafter referred to as the model solution). The inoculum was used after stirring the working culture using a vortex type shaker (1000 rpm for 10 s). The pH of the saline solution used for preparing the working suspension was adjusted by adding 0.15M KH_2PO_4 solution and 0.15M Na_2HPO_4 solution. Eventual differences were eliminated by adding NaOH or HCl solutions.

The required volume of the working culture to be used in the experiments, grown on the BHIB (e.g. a corresponding volume of 130 ml) was prepared from separate test tubes with the stirred working culture as a mixed inoculum, introduced into the nine volume units of the model solution under continuous stirring using sterile glass beads. The uniform working suspension thus obtained was put into 100 ml brown PET bottles under aseptic conditions. Each bottle had been labelled with specific baroinactivation parameters in accordance with the experimental protocol (pressure in MPa, time in minutes).

An analogical procedure was employed when working with the real substrate, in particular pasteurised human milk and UHT-treated skimmed cow milk with fat content of 0.5%. The object of microbiological analysis was a working culture of *S. epidermidis* CCM 4418 suspended in pasteurised human milk and in UHT cow milk. Both substrates were investigated simultaneously on the same day

in order to use identical mixed inoculum of the working culture of *S. epidermidis* CCM 4418. Equal volumes of *S. epidermidis* suspensions in human milk and in UHT milk were transferred into 100 ml brown PET bottles under aseptic conditions. Each bottle had been labelled with specific baroinactivation data in accordance with the experimental protocol (pressure in MPa, time in min).

The number of viable cells of *S. epidermidis* CCM 4418 in the working suspensions and human and cow milk suspensions was determined prior to the baroinactivation treatment and after the treatment. Suitable samples were prepared by ten-fold dilution of each suspension with saline solution supplemented with peptone.

In the next step, the solutions were inoculated by smearing of a predefined volume onto PCA (Plate Count Agar) surfaces. The incubation took place under aerobic conditions at 37°C for 48 hours. This procedure was chosen with the aim to recover also sublethally injured cells (Smelt *et al*. 2002).

After the incubation, the number of developed colonies was counted. The approach was based on the generally accepted assumption for the colony counting technique, i.e. that each colony is formed by one viable cell (CFU). The number of *S. epidermidis* colonies was determined using an automated colony counter (model BIOTRAN III, New Brunswick Scientific Co. Inc., USA). In accordance with the experimental protocol, 14 incubated plates were usually examined per each bottle of *S. epidermidis* suspension after each defined baroinactivation treatment, inoculated with the appropriate ten-fold dilutions, always in duplicates. The selection of plates and dilution for viable cells of *S. epidermidis* counting prior to the baroinactivation treatment and after it were conducted in order to satisfy the conditions of the standard CSN ISO 7218:1998 Amd.1:2001 governing the calculation of weighted mean.

Method of calculation of the viable cells quantity. The selection of incubated plates and dilutions was conducted as to satisfy the following conditions. The procedure for calculating the quantity of viable cells of *S. epidermidis* prior to the baroinactivation treatment and after it (N_0 and the corresponding N) complied with the recent edition of ČSN ISO 7218:1998, as amended by Amd. 1:2001, giving the following formula for the calculation of the weighted mean:

$$N = \frac{\Sigma C}{V \times [n_1 + (0.1 \times n_2)] \times d}$$

where:

ΣC – sum of the numbers of colonies from all plates selected for the calculation from two consecutive dilutions, when at least one of the plates contains a minimum of 15 colonies

V – inoculum volume in ml introduced to each of the plates

n_1 – number of plates selected for the calculation from the first selected dilution

n_2 – number of plates selected for the calculation from the second selected dilution

d – dilution factor corresponding to the first dilution selected for the calculation

It should be noted that in such cases where the condition for ΣC minimum of 15 colonies was fulfilled by neither of the plates, the value obtained was interpreted in accordance with the cited ISO standard as a so-called estimated count.

Method of pH and water activity measurements. pH value was measured by Sentron (Model 1001, the Netherland) having electronic ISFET sensor and maximum error 0.1. Rotronic (model AWVD, Switzerland) water activity meter equipped with thermostatic chamber was used to measure water activity of model solutions and milks. The maximum error of water activity was 0.005.

Method of the pressure treatment. The pressure was applied by means of an isostatic press (model CYX 6/0103, Zdas company, Žďár n/S, Czech Republic) with a pressure chamber of 2 l, using drinking water as the pressurising medium. All samples (except the control sample) were transported chilled to 5°C in an insulated box and stabilised in a 15°C water bath for 30 min prior to the pressurising treatment. The temperature of the pressurising medium (drinking water) in the chamber was also adjusted to 15°C. The pressurising medium was replaced prior to each successive pressurising step. The samples were kept at 15°C prior and after pressurising. After the completion of the pressurising of all samples, wiped-off samples were transported in the insulated box back to the microbiological laboratory. The time necessary for the pressure treatment, including transportation, took approximately 5 hours.

As the pressure increases during the treatment, the temperature of the sample rises by approximately 12°C when subjected to a pressure of 500 MPa. Following decompression, the sample temperature decreases again by the same value. It must be therefore considered that the pressu-

rising itself, when the pressure was maintained, took place under a temperature of approximately 27 ± 1°C maximum.

RESULTS AND DISCUSSION

The results on the impact of the pressure and time of application on *Staphylococcus epidermidis* CCM 4418 valid for pH of the model solution of 6.4 are given in Table 1.

The pressures exceeding 350 MPa applied for 10 min proved unsuitable, as the effects were so severe that the eventual number of CFU was below the determination threshold (CFU < 100) – the number of microorganisms therefore dropped by at least five orders. Shorter periods of application were impractical as the time to reach the nominal value of pressure was approximately 1 minute. Higher initial concentrations of the working suspension could not be achieved.

The results on the impact of the pressure and time of application on *Staphylococcus epidermidis* CCM 4418 valid for pH of the model solution of 6.8 are given in Table 2. At this pH value, the threshold pressure was 350 MPa applied for 12 min, at which the number of CFUs decreased dramatically by at least six orders. Table 2 also shows that the results cited were obtained in three independent experiments with different initial concentration of the working suspension.

The results on the impact of the pressure and time of application on *Staphylococcus epidermidis* CCM 4418 valid for pH of the model solution of 7.2 are given in Table 3. At this pH value of the model solution, the threshold pressure was also 350 MPa, applied for 10 minutes. In this case, a decrease by at least five orders was demonstrated (the initial concentration of the order of 10^7, final

Table 1. Results of baroinactivation experiments with *Staphylococcus epidermidis* CCM 4418 in model solution of pH 6.4 and a_w 0.99

Pressure (MPa)	Time (min)	N (CFU/ml)	N/N_0
0	0	N_0 = 5.223E+07	
200	5	7.656E+06	1.47E-01
	10	3.155E+06	6.04E-02
	15	2.046E+05	3.92E-03
250	5	1.545E+06	2.96E-02
	10	8.636E+04	1.65E-03
	15	6.622E+03	1.27E-04
300	3	1.726E+05	3.30E-03
	5	5.767E+03	1.10E-04
	10	5.470E+02	1.05E-05
350	3	7.727E+03	1.48E-04
	5	2.891E+03	5.54E-05
	10	ND	–

ND = non detected colonies – no colonies grown on the most diluted solution

Table 2. Results of baroinactivation experiments with *Staphylococcus epidermidis* CCM 4418 in model solution of pH 6.8 and a_w 0.99

Pressure (MPa)	Time (min)	N (CFU/ml)	N/N_0
0	0	N_0 = 1.43E+08	
450	10	ND	
450	20	ND	
0	0	N_0 = 1.26E+08	
200	5	8.00E+07	6.35E-01
250		1.37E+07	1.09E-01
300		1.89E+06	1.50E-02
350		3.95E+04	3.00E-04
0	0	N_0 = 1.91E+08	
200	8	2.45E+07	1.28E-01
250		1.76E+06	9.21E-03
300		5.29E+05	2.77E-03
350		5.12E+03	2.68E-05
200	12	1.71E+07	8.95E-02
250		1.41E+06	7.38E-03
300		2.79E+05	1.46E-03
350		ND	
200	16	1.04E+07	5.45E-02
250		1.17E+06	6.13E-03
300		1.23E+05	6.44E-04
350		ND	

ND = non detected colonies – no colonies grown on the most diluted solution

Table 3. Results of baroinactivation experiments with *Staphylococcus epidermidis* CCM 4418 in model solution of pH 7.2 and a_w 0.99

Pressure (MPa)	Time (min)	N (CFU/ml)	N/N_0
0	0	N_0 = 6.442E+07	
200	5	1.081E+07	1.68E-01
	10	4.227E+06	6.56E-02
	15	1.273E+06	1.97E-02
250	5	3.296E+06	5.11E-02
	10	1.462E+06	2.27E-02
	15	1.438E+05	2.23E-03
300	3	1.146E+06	1.78E-02
	5	7.516E+04	1.17E-03
	10	6.353E+02	9.85E-06
350	3	1.950E+03	3.02E-05
	5	1.500E+02	2.33E-06
	10	ND	–

ND = Non detected colonies – no colonies grown on the most diluted solution

concentration below the detection limit, i.e. at the worst just below 100).

The highest concentrations of the surviving cultivable microorganisms in the model solutions ranged between the orders of 10^8/ml and 10^2/ml.

The results obtained with the baroinactivation of the working suspension in the model solution were converted to the ratio N/N_0, where the value of N_0 was the value for the particular sample prior to the treatment. This ratio was correlated to the duration of pressurising. Baroinactivation of *S. epidermidis* can use the traditional exponential inactivation model, see the experimental data in Figures 2–4.

Development of the model

The acquired data were processed by linear regression using a Microsoft Excel spreadsheet and values of D_p (duration of pressurising in minutes necessary to decrease the microorganism concentration by one order) were determined as the inversion value of the tangent at the coordinates $\log N/N_0$ vs. time t, as given by:

$$\log N/N_0 = -t/D_p \quad (1)$$

The values of logarithm of D_p acquired from linear regression were correlated to the pressure using the equation below. The relationship between $\log D_p$ and the pressure is linear (see Figure 1). Linear regression yielded the values of parameter Z (the increase in the pressure in MPa, resulting in the decrease of the value of D_p by one order) and $D_{p,\mathrm{ref}}$ for P_{ref} = 300 MPa for various values of pH.

$$D_p = D_{p,\mathrm{ref}} \times 10^{(P\mathrm{ref} - P)/Z} \quad (2)$$

The obtained values of parameters Z and $D_{p,\mathrm{ref}}$ were correlated to the values of pH, while a parabolic function was used. The relationships obtained are as follows:

$$Z = -123.90\ \mathrm{pH}^2 + 1635.54\ \mathrm{pH} - 5210.49 \quad (3)$$

$$D_{p,\mathrm{ref}} = -8.89\ \mathrm{pH}^2 + 121.02\ \mathrm{pH} - 408.34 \quad (4)$$

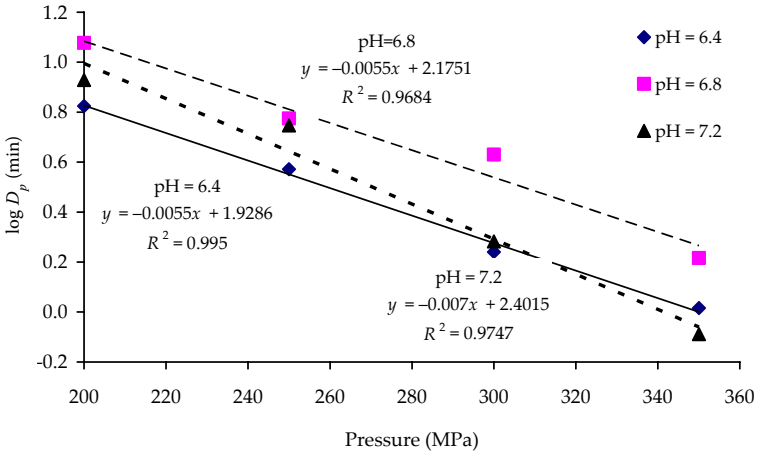

Figure 1. Relationship of log D_p to pressure for pH 6.4 to 7.2

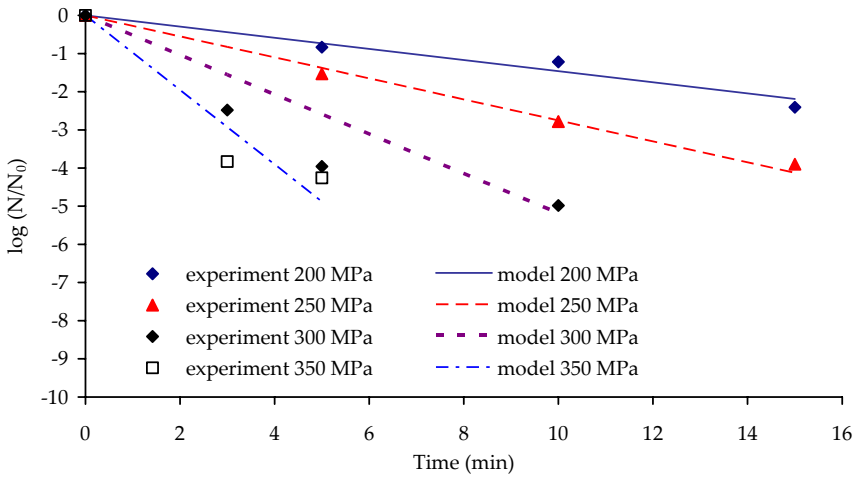

Figure 2. Comparison of measured values and those calculated from model for pH = 6.4

The baroinactivation model, valid for the pressure range of 200 to 350 MPa, pH range from 6.4 to 7.2, and a_w = 0.99, is acquired by substituting Equations (3) and (4) into Equation (2), which then yields the values of D_p for the corresponding model. This value is then used to calculate the value of log N/N_0.

The comparison of log N/N_0 values predicted by the model with the experimental results for different values of pH is shown in Figures 2–4. The figures show an excellent agreement between the experimental data and the results calculated from the mathematical model.

Verrips and Van Rhee (1981) studied thermal inactivation of *S. epidermidis* in a model medium with different water activities. For water activity 0.99 regulated with sucrose and pH 6.5 adjusted with HCl, the decimal reduction time valid for 66°C was 22 seconds. This value is roughly comparable with D_p value valid for high pressure pasteurisation at a pressure of 350 MPa, the same water activity and pH = 6.4, which amounts to 60 seconds.

Walker and Harmon (1996) studied the thermal resistance of *S. aureus* in milk, whey, and phosphate buffer. These authors presented a tailing in the heat inactivation curves. The decimal reduction time valid for skim milk with pH adjusted to 6.65°C and 62°C was 12 s for strain 161-C. Decimal reduction time was detected to be a function of the strain and medium used for the heating at the given heating temperature.

Verification of the model using human and cow milks

The results for the baroinactivation of the working suspension of *Staphylococcus epidermidis* CCM 4418

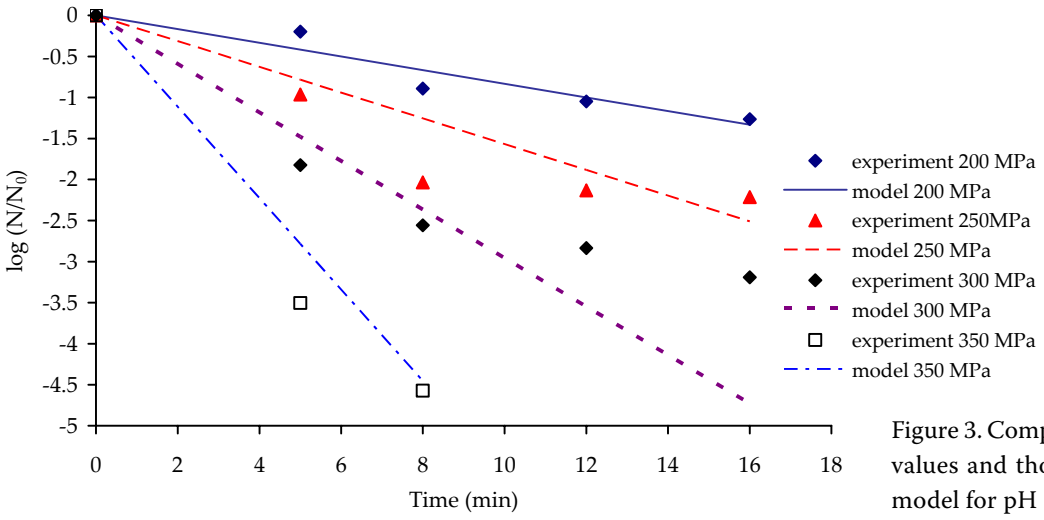

Figure 3. Comparison of measured values and those calculated from model for pH = 6.8

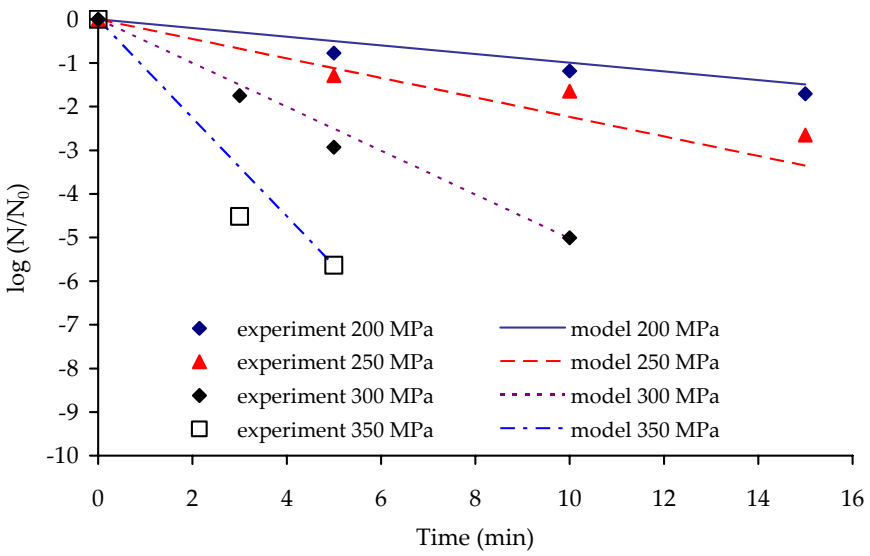

Figure 4. Comparison of measured values and those calculated from model for pH = 7.2

in pasteurised human milk and UHT-treated skimmed cow milk are shown in Figures 5 and 6. The lines in the figures denote the predictions for the given values of pH, pressure, and time of application, yielded by the mathematical model. Water activity in both types of milk is practically identical to the water activity of the model solution (the difference is 0.005, equal to the threshold statistical error value for the water activity determination).

Figure 5 shows that the model and baroinactivation data acquired with human milk are virtually identical. The agreement for the pressure of 300 MPa is nearly perfect; for the pressure of 250 MPa, the experimental and model data differ by half an order, well within the error margin for the log N/N_0 ratio.

In the case of UHT skimmed cow milk, the experimental and model data were comparably less close than in the case of human milk (Figure 6). The baroinactivation of cow milk is somewhat more complicated. In particular, the model predicted a higher impact of pressure in the case of 300 MPa treatment pressure than found experimentally, thus erring on the unsafe side. The differences are in excess of one order and increase with the duration of the application. Experimental data demonstrate higher values of D_p than predicted by the model. The model should therefore be used cautiously and the efficiency of any method based on it should be rigorously verified on the particular food substrate intended for the treatment with high pressure to eliminate living microorganisms. The baroinactivation data on *St. epidermidis* do not exist in the current literature. We can compare our model only with the data generated for *St. aureus* in ham (Tassou *et al.* 2008).

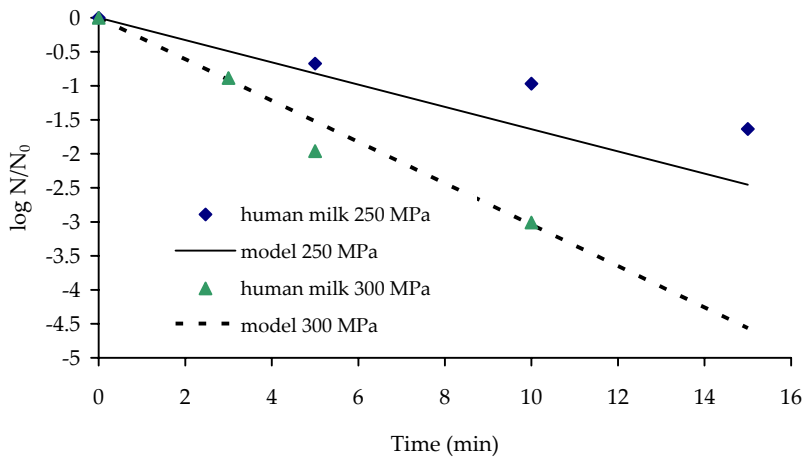

Figure 5. Verification of baroinactivation model for *Staphylococcus epidermidis* CCM 4418 in human milk at pH = 6.7 and a_w = 0.994

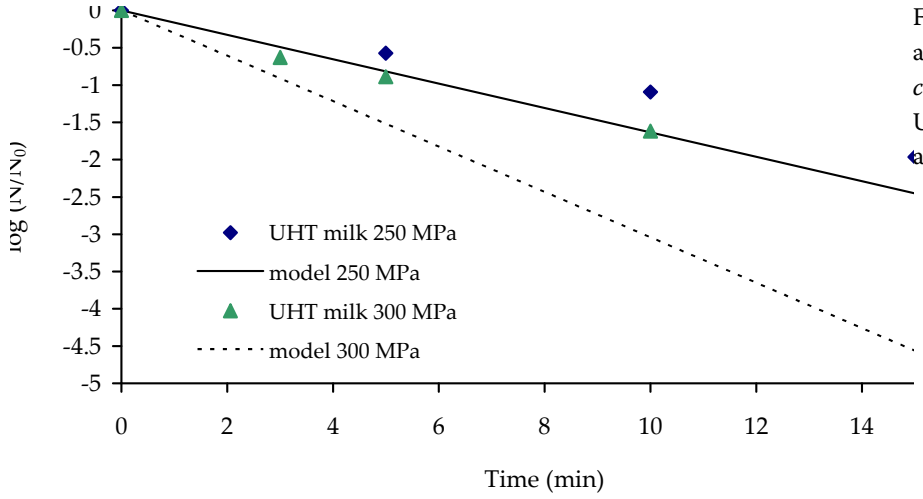

Figure 6. Verification of baroinactivation model for *Staphylococcus epidermidis* CCM 4418 in UHT cow milk (fat content 0.5%), at pH = 6.7 and a_w = 0.997

CONCLUSIONS

Baroinactivation data were acquired for the working suspension of *Staphylococcus epidermidis* CCM 4418 in model solutions of pH 6.4 to 7.2 and water activity a_w = 0.99. The data show an exponential curve of inactivation with a duration under a constant pressure, and therefore can be expressed by a simple baroinactivation model. The parameters for the model were specified: Z, $D_{p,\,ref}$ for the reference pressure of 300 MPa, as a function of pH in the form of parabolic relations. The agreement of the model thus acquired with the experimental data was very good. The resulting concentrations of surviving culturable microorganisms in the model solutions ranged from 10^8/ml to 10^2/ml. The model is valid for a pressure range from 200 MPa to 350 MPa. We have to point out that the model validity is limited by the experimental holding times apparent from Figures 2–4. The pressure treatment was done from the starting temperature of the model solution of 15°C.

The developed model was verified using pasteurised human milk and UHT-treated skimmed cow milk. Value of pH of both substrates was 6.7 and water activity was very near to the water activity of the model solutions. The agreement between the experimental data and the model predictions was near perfect for human milk. In the case of UHT skimmed cow milk, the agreement between the two sets of data was less than perfect, as baroinactivation in this substrate is more complicated. In particular, for the pressure of 300 MPa, the model predicted a better efficiency of the pressure treatment than demonstrated experimentally, therefore erring on the unsafe side. The model should therefore be used cautiously and the efficiency of any method based on it should be rigorously verified on the particular food substrate intended for the treatment with high pressure in order to eliminate live microorganisms.

It was demonstrated that the use of the pressure of 350 MPa for 10 min decreased the concentration of the working suspension of *Staphylococcus epidermidis* CCM 4418 in the model solution by a minimum of five orders.

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