

## High Pressure Inactivation of *Enterococcus faecium* – modelling and verification

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### Abstract

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High-pressure inactivation data were obtained for model working suspensions of *Enterococcus faecium* in saline solutions in pH range from 5.5 to 6.8, at water activity 0.99 (corresponding to the saline solution). The data were predicted for the pressure range of 450 MPa to 550 MPa, at the initial model suspension temperature 6–7°C prior to pressurising. The results indicate that *E. faecium* is a highly resistant organism under physiological pH values. With decreasing the substrate pH, the tolerance to the inactivation effects of high pressure decreased. The high-pressure inactivation proved ineffective for the above organism in the range of physiological pH values. The values for high-pressure inactivation model were specified and the calculated parameters of high-pressure inactivation were compared to the experimental data for the working suspensions of *E. faecium* in cow and human milks. As to cow milk, the predicted data showed some deviation from the model experimental results, while with human milk the model failed completely. High-pressure inactivation of *E. faecium* in human milk proved to be significantly more effective than that predicted by a model based on the saline solution.

**Keywords:** high pressure inactivation; *Enterococcus faecium*; model; verification

Milk banks in the Czech Republic continuously monitor the input and output concentrations of microorganisms in human milk. Analyses show occasional presence of the bacterium *E. faecium* in the raw or processed materials. Milk banks employ the critical control point safety system (HACCP) and carry out regular analyses of human milk (MĚŘICKÁ *et al.* 2004a, b). When *E. faecium* is found in a batch of pasteurised milk, the batch is discarded.

REVIRIEGO *et al.* (2005) studied *E. faecium* isolated from human milk of healthy mothers. Microorganisms migrate from the digestive system of the mother to the breast and milk, and then to the digestive system of the infant.

*E. faecium* was found in the Greek feta cheese, with a positive impact on its taste, aroma, colour, structure, and the overall sensorial perception. Physical and chemical properties, such as pH, water content, ashes, salt content in dry matter,

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and fat in dry matter were not affected by the addition of *E. faecium* strains (SARANTINOPOULOS *et al.* 2002).

The effects of *E. faecium* are mostly undesirable, as some strains of the bacterium are resistant to antibiotics, e.g. vancomycin, ampicillin, gentamicin, erythromycin, and streptomycin (PANAGEA *et al.* 1996; BARKHOLT *et al.* 1997; CITAK *et al.* 2005). This causes problems e.g. during the treatment of critically ill patients with burns (fever, increased counts of lymphocytes). BARKHOLT *et al.* (1997) and ROSE *et al.* (2002) found that the use of antibiotics three months before liver transplantation greatly increases the risk of the resistance to ampicillin development in enterococcus.

The available literature on the inactivation of *E. faecium* by high pressure and determination of kinetic model is rare. There are more data on thermal inactivation. KORNACKI *et al.* (1992) studied thermal tolerance of *E. faecium* in ultrafiltered milk. The values of thermal tolerance were established for the temperature of 58°C in relation to the protein content (0.4% to 8.9%) and milk fat content (0% to 11.8%). It was found that the increasing fat content increases the thermal tolerance of the microorganism, while protein concentration has a negligible effect on thermal tolerance.

The prolongation of the shelf life of food products, i.e. decreasing microorganisms counts by a high hydrostatic pressure, is under examination as an alternative to the traditional heat pasteurisation. MOERMAN (2005) studied the inactivation of microorganisms, aerobic and anaerobic spores in pork. A mixture prepared by stewing bits of meat, carrot, and peas (pH neutral) was inoculated with several strains of spore-forming and vegetative microorganisms. The samples were then subjected to a high pressure of 400 MPa for 30 min (under temperatures of 20°C and 50°C). Gram-positive cocci *E. faecalis* were more resistant to the pressure than e.g. *Saccharomyces cerevisiae* or *Escherichia coli*. High pressure processing at 20°C demonstrated that treating food at neutral pH using this procedure only is insufficient.

WUYTACK *et al.* (2003) decontaminated seeds intended for germination (nasturtium, sesame, radish, and mustard) by high pressure treatment under pressures of 250, 300, 350, and 400 MPa for 15 min, at the temperature of 20°C. The germinative capacity of the seeds was then examined. Radish seeds proved to be most sensitive to the treatment while nasturtium seeds were the most tolerant. Nasturtium

seeds were inoculated with a suspension of seven different species of bacteria at  $10^7$  CFU/ml and subjected to the pressure of 300 MPa for 15 minutes. A decrease of 2 to 6 orders of magnitude occurred in six species of bacteria, but *E. faecalis* was not inactivated.

GARCIA-GRAELLS *et al.* (2003) studied the inactivation of eight different species of bacteria in skim milk with the added lactoperoxidase-hydrogen peroxide-thiocyanate (LP) complex. LP produced no effect without the pressure treatment. WUYTACK *et al.* (2002) studied bacterial inactivation under high-pressure homogenisation (100–300 MPa) and the treatment with high hydrostatic pressure (200–400 MPa). They compared the resistance of five species of Gram-positive bacteria (e.g. *E. faecalis*) and six species of Gram-negative bacteria (e.g. *E. coli*). The tolerance to high pressure was similar with both groups.

It is apparent that the amount of the available data on high-pressure inactivation of *E. faecium* is insufficient. Therefore, this work aims to fill partially the gap and determine experimentally the effects of high pressure on the inactivation of *E. faecium* in model solutions. A secondary aim was to incorporate the data into a kinetic model and start to carry out the verification of the model against the data obtained with human and cow milks inoculated with *E. faecium*.

## MATERIAL AND METHODS

**Selection of microorganism, its origin, preparation of the working culture and working suspension, conditions of incubation, obtaining enumerable colonies.** The model microorganism selected was the *Enterococcus faecium* CCM 2308. Another denominations of the strain (as proof of equivalence) are e.g. ATCC 6057, NCIMB 8842 = strain 24, the original taxonomic name (until 1984) was *Streptococcus faecium*. The strain was isolated from cheese.

The *E. faecium* culture was obtained from the Czech Collection of Microorganisms (CCM) in the lyophilised form on gel discs in sterile vials, 20 discs per vial. Each disc was used following the procedure recommended by the CCM to develop a revitalised culture on agar slant. The revitalisation took place at 37°C for the total duration of 24 hours.

The working culture was prepared by inoculation of 3 µl of the revitalised culture from the

agar slant into the standard liquid cultivation medium as recommended by CCM, i.e. BHIB (Brain Heart Infusion Broth) distributed into test tubes in quantities of 9 ml. The culture was uniformly transferred from the agar slant with a full inoculating loop calibrated at 1 µl used three times, always using a different sterile loop. The inoculated BHIB medium was incubated at 37°C for 18 h to obtain the early stationary growth phase commonly recommended and used for experiments studying lethal and sub-lethal effects on bacteria, e.g. SCHLEMMEROVÁ (1987).

The working suspension was prepared by inoculating 1 ml of the working culture grown in BHIB into 99 ml of model saline solution of selected values of pH ranging from 6.8 to 5.5 and  $a_w = 0.99$  (corresponding to NaCl concentration in the saline solution, see ISO 21807:2004). The procedure employed was based on the previous experience with high growth density of *E. faecium* CCM 2308 and was as follows: 1 ml was taken from the mid column of the liquid culture. If high-pressure inactivation process required greater volumes of the working suspension, greater volumes of the working culture grown in BHIB were taken from the individual tubes into the mixed inoculum, which was introduced into correspondingly increased volume of the model solution. The working suspension was transferred into 100 ml brown PET bottles.

The reference sample, marked “witness”, was of the same type, its content being identical to those in the bottles subjected to the pressure treatment, and was subjected to the same time-temperature history during transportation and storage.

The counts of viable cells of *E. faecium* had to be taken before and after the inactivation treatment. This required series of decimal dilutions in saline solution with added peptone (internationally recommended dilution fluid, generally applicable). The next step was the distribution of a specified volume onto the surface of PCA (Plate Count Agar) plates, followed by incubation at 37°C for 24 hours. After incubation, the developed colonies were counted. The numbers of *E. faecium* colonies were determined using the automatic colony counter BIOTRAN III obtained from New Brunswick Scientific Co. Inc., USA.

**Calculation method.** The calculation of the quantity of viable *E. faecium* cells before and after the inactivation treatment ( $N_0$  and the corresponding  $N$ ) conformed to the valid standard ČSN ISO 7218:1998, which includes the modifi-

cation of Amd. 1:2001, and specifies the general formula for the calculation of the weighted average:

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

where:

- $\Sigma C$  – sum of colonies from all plates chosen for the calculation of two consecutive dilutions, where at least one of the plates contains a minimum of 15 colonies
- $V$  – volume in ml inoculated onto each of the plates
- $n_1$  – number of plates chosen for the calculation of the first selected dilution
- $n_2$  – number of plates chosen for the calculation of the second selected dilution
- $d$  – dilution factor corresponding to the first dilution, chosen for the calculation

Each result is the mean of microbial concentrations of the three repetitions. Variability ranges in one logarithm decade. The detection limit of 100 CFU/ml was predicted by preliminary experiments.

**Milk for model verification.** For the verification, we used UHT skim milk from the dairy Kunin, Czech Republic (0.5% fat), purchased in a supermarket. This product was shelf stable.

Another substrate used was human milk supplied by the Human milk bank of the University Hospital in Hradec Kralové. Any human milk delivered from this bank is heat pasteurised and frozen. The human milk was transported and stored frozen and thawed directly one day before the experiment. Prior to use, a separate sample of the same human milk was subjected to a test of spontaneous inhibition of the introduced microorganism *E. faecium* CCM 2308. The treated milk contained  $10^{8.777}$  CFU/ml immediately after introduction of the microorganism, and nearly an identical concentration of  $10^{8.716}$  CFU/ml after 25 min keeping at 25°C. The milk used for the verification demonstrated therefore no spontaneous inhibition of the microorganism.

Both milk types were inoculated with the working culture of *E. faecium* under chilled conditions. The same procedure of inoculation was used as in the case of the preparation of the working suspension. The inoculated milk samples were treated with high pressure using the same procedure and under the same conditions as the working suspension samples.

**Containers and procedure of pressure inactivation.** For the high-pressure treatment of the samples, pet brown bottles (100 ml) were used with screw-on caps.

The pressure treatment of the samples was conducted in an isostatic press CYX 6/0103 (Zdas Company, Ždár nad Sázavou, Czech Republic); the volume of the high-pressure chamber was 2 litres, the pressure medium was potable water. The rate of the pressure increase was approximately 8.3 MPa per second. The temperature of the pressure vessel in its top section was about 21.5°C. The pressure medium was replaced prior to every test. The temperature of the samples was assumed to be identical to that of the medium; the test room temperature was 18–20°C. The samples were transported into the thermal chamber and adjusted to 6–7°C in water bath (40 min) prior to the test. The water in the test chamber as well as the water in which the samples were kept prior to and after the test had the same initial temperature of 6–7°C. After the pressure treatment, the bottles containing the samples were dried and shipped back for microbiological examination. Maximum temperature increase of water or water solutions was approximately 15°C when compressed by 500 MPa. The effects of compression heating on inactivation of the microorganism were considered negligible with respect to the low initial temperature. The thermal exchange with the environment during the holding time was limited by equal temperatures of the samples and the pressure medium in the chamber and the chamber itself.

## RESULTS AND DISCUSSION

The acquired data on the effects of pressure and pressure holding time on the inactivation of *Enterococcus faecium* CCM 2308 are given in Figure 1.

An example of numerical values received with saline solution of pH = 6.8 is given in Table 1. The analyses of the reference “witness/control” sample demonstrated that the microorganism counts remained the same under the conditions of transport between laboratories (the samples were stored in all cases at temperatures up to 16°C for a time shorter than one hour).

The initial results demonstrated that the strain *E. faecium* examined was highly resistant to the pressure under physiological pH = 6.8. Therefore, the effects of decreasing pH value were subsequently studied.

The influence of pH and the initial temperature is apparent from the data given in Table 2. The results indicate that a decrease of pH from 6.8 to 6.4 caused negligible changes in the numbers of the microorganisms present after the high-pressure treatment, despite the use of rather harsh parameters, i.e. 550 MPa for 15 minutes. The working suspension temperature had no effect on the decrease of the microorganism counts at this pH. At pH = 6.0, a pronounced decrease in the microorganism counts was recorded. The influence of the initial temperature is apparent at this pH value. The initial temperatures of 15°C and 10°C provided higher inactivation rates than that obtained at the initial temperature of 20°C.

Table 1. Example of numerical results of high pressure inactivation of *E. faecium* in saline solution of pH = 6.8, initial temperature prior to pressurising 5–7°C

Pressure (MPa)	Pressure holding time (min)	log N
Without pressure treatment*	without pressure treatment	9.130 (log N <sub>0</sub> )
300	5	8.497
400	5	8.434
500	5	8.550
Without pressure treatment*	without pressure treatment	8.905 (log N <sub>0</sub> )
Without pressure treatment reference sample, “witness”**	without pressure treatment reference sample, “witness”	8.884 (log N <sub>s</sub> )
300	10	8.332
400	10	7.644
500	10	7.502

\*these samples were kept chilled in laboratory, \*\* this sample was transported with other samples

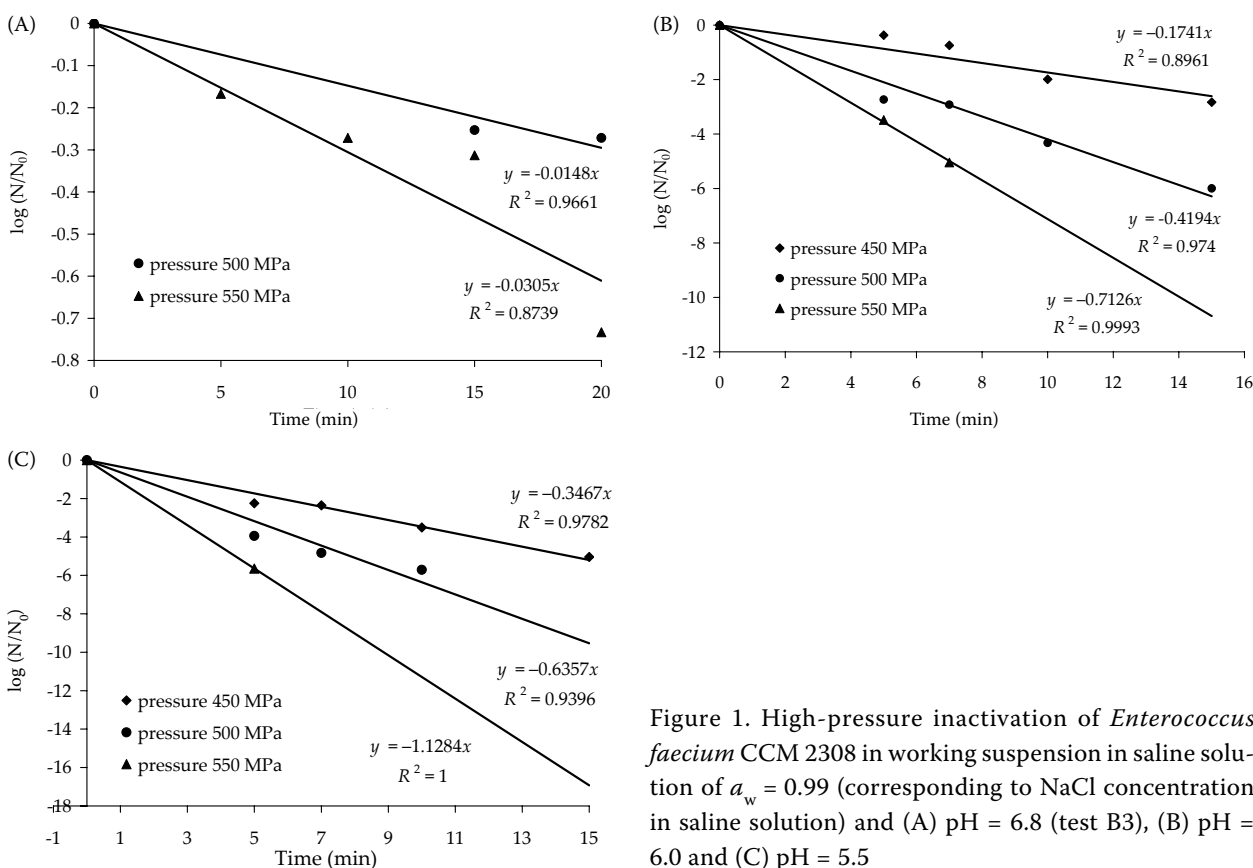


Figure 1. High-pressure inactivation of *Enterococcus faecium* CCM 2308 in working suspension in saline solution of  $a_w = 0.99$  (corresponding to NaCl concentration in saline solution) and (A) pH = 6.8 (test B3), (B) pH = 6.0 and (C) pH = 5.5

Therefore, all experiments were carried out at initial temperatures below 10°C.

The results of the detailed investigation of the pressure and pressure holding time effects on the model working suspension in saline solution at pH = 6.0 are given in Figure 1B. The data listed show that the increasing pressure and duration of the treatment tend to increase the effects of the treatment. Under the harshest conditions (550 MPa for 10–15 min), no detectable num-

bers of micro-organism were found in the treated suspension despite the immense initial counts. It can be assumed that this procedure can decrease the density of *E. faecium* CMM 2308 by up to 9 orders of magnitude.

In order to acquire data for this manner of inactivation over the broadest possible range of parameters, another experiment was conducted with a model suspension of pH = 5.5. The results of this experiment are given in Figure 1C. The data

Table 2. Comparison of the results for the working suspension in saline solution of pH = 6.0 and 6.4 and possible effects of different temperatures prior to pressurising

Pressure 550 MPa for 15 min	Count log N <i>E. faecium</i> in suspension (CFU/ml)	
	pH = 6.0	pH = 6.4
Without pressure treatment	8.544 (log N <sub>0</sub> )	8.422 (log N <sub>0</sub> )
Without pressure treatment reference sample, “witness”	8.306 (log N <sub>S</sub> )	8.452 (log N <sub>S</sub> )
Temperature prior to pressurising (°C)		
10	5.139	7.163
15	5.778	7.236
20	7.232	7.113

Table 3. Values of  $D_p$  as function of pressure and pH

Pressure (MPa)	450	500	550	450	500	550	400	500	550
pH (-)	5.5	5.5	5.5	6.0	6.0	6.0	6.8	6.8	6.8
Tangent	-0.3467	-0.6357	-1.1284	-0.1741	-0.4194	-0.7126	-0.0143	-0.0148	-0.0305
$D_p$ (min)	2.9	1.6	0.9	5.7	2.4	1.4	69.9	67.6	32.8

show that the microorganism investigated is more sensitive at pH = 5.5 as compared to pH = 6.0.

### Evaluation of pressure inactivation data from saline solutions and development of a model

Similar to thermal inactivation, it was assumed that microorganisms die in accordance with the first order kinetics model, the integration of which gives the following equation

$$N = N_0 \exp(-k t) \quad (1)$$

where:

$k$  – inactivation rate (number per second)

$t$  – time in seconds

$N, N_0$  – current and initial numbers of microorganisms per ml

The conversion of the time into minutes and of the logarithm to decimal logarithm gives  $D_p$

$$D_p = 1/(60 \cdot \log ek) = 0.0384/k$$

yielding

$$\log N/N_0 = -t/D_p \quad (2)$$

where:

$D_p$  – decimal reduction time, i.e. time in minutes required for reducing the microorganism counts by one order of magnitude under constant pressure

The evaluation was based on the data from Figure 1. As the initial concentration value  $N_0$ , the numerical value predicted by the experiment with the untreated sample at each pH value was used. Each point in the figures represents three replications of the enumeration.

Linear regression method of least-squares was used to plot the lines passing through the origin. The tangents of the lines and the corresponding decimal reduction times are given in Table 3. It is apparent that with increasing pressure the value of  $D_p$  decreased.

For a given pH, the linear regression was applied and parameter  $Z_p$  predicted using the assumed linear dependence of  $\log D_p$  on pressure  $p$ .

$$\log D_p = A - B p \quad (3)$$

where:

$p$  – pressure (MPa)

$D_p$  – time (min)

Introducing  $B = 1/Z_p$ , where  $Z_p$  is the pressure increment causing a decrease of the decimal time of inactivation by one order of magnitude, the introduction of reference parameters  $p_{ref}$  and  $D_{p,ref}$  allows the conversion of the Eq. (3) into the formulae

$$\log D_p = \log D_{p,ref} + (p_{ref} - p)/Z_p \quad (4)$$

$$D_p = D_{p,ref} \cdot 10^{(p_{ref} - p)/Z_p} \quad (5)$$

Table 4 shows the resulting values of  $Z_p$ . Decreasing values of pH cause a slight increase in the  $Z_p$  values.

Subsequently, the correlation of the high-pressure inactivation model parameters (5) was estimated using regression to determine the relations of  $Z_p$  and of  $D_{p,ref}$  to pH.

The relationship between  $D_{p,ref}$  and pH was determined by non-linear regression using a computer software Datafit (Oakdale Engineering, Oakdale, USA) as follows:

$$D_{p,ref} = \text{pH}/(18.287 - 2.674 \text{ pH}) \quad (6)$$

Table 4.  $Z_p$  and  $D_{p,ref}$  values as a function of pH

pH (-)	6.8	6.0	5.5
$Z_p$ (MPa)	159.2	163.9	194.9
$D_{p,ref}$ (min)	67.5	2.7	1.6

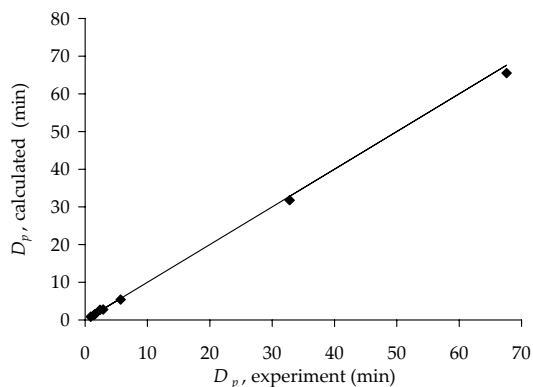


Figure 4. Comparison of calculated and experimental values of decimal reduction time  $D_p$

The relationship between  $Z_p$  and pH was determined by linear regression using parabolic function as follows:

$$Z_p = 44.423 \cdot \text{pH}^2 - 573.87 \cdot \text{pH} + 2007.4 \quad (7)$$

An overall model for high-pressure inactivation valid for the pressure range of 450–550 MPa, pH = 5.5 to 6.8, and  $a_w = 0.99$  was obtained by the substitution of Eqs. (6) and (7) to Eq. (5).

The comparison of the calculated and experimental values of decimal reduction time  $D_p$  is given in Figure 2. The figure shows a good agreement between the experimental and calculated values valid for saline solutions only. This shows only the data from which the model was built. Therefore, further model verification is necessary.

### Model verification

The above model, comprising Eqs. (5), (6), and (7), was used for the prediction of the decrease in microorganism counts in cow and human milks. The comparison is given in Figure 3. It is apparent that the predictions for cow milk at pH = 6.4 do not agree with the experimental data very well. The experiments showed virtually no inactivation of *E. faecium*.

On the other hand, in the case of human milk at pH = 6.8, the model predicts negligible levels of inactivation, while the experimental data demonstrate a decrease in microorganism counts by 6.4 orders of magnitude. A similar result was obtained for thermal inactivation of the microorganism in human milk (ŠPELINA *et al.* 2007). However,

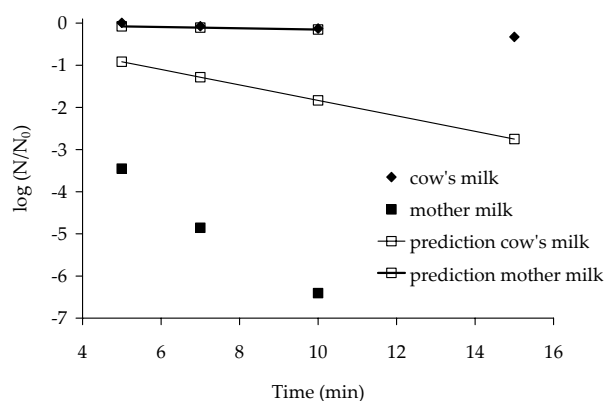


Figure 5. Comparison of experimental and calculated inactivation curves for *E. faecium* in milk

spontaneous inhibition of the microorganism in human milk was not demonstrated. It is assumed that human milk (cautiously pasteurised) may contain immunogenic substances, which may have a synergistic effect with the pressure or whose effects are amplified by the pressure.

### CONCLUSION

The high pressure inactivation model of *Enterococcus faecium* was predicted on experimental data obtained with microorganisms suspended in saline solution. An attempt at the verification of this model was done by comparing the model prediction data with the experimental data obtained with cow and human milks inoculated with the same strain of the microorganism. The model predictions disagree with the data received on real systems.

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