Thermal Inactivation of *Enterococcus faecium*

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Abstract

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Data for thermal inactivation of working suspension of *Enterococcus faecium* in model solutions were acquired and used to develop a mathematical model for thermal inactivation of the bacterium. The model is valid within the water activity range 0.97 to 0.99; pH range 6.0 to 7.6; temperature range 60°C to 65°C, and was determined for the microorganism concentration ranges of 10^2 per ml to 10^8 per ml of the model inactivation solution. An Excel procedure was developed in Visual Basic language which enables the calculation of the final concentration of the microorganism from the input data for pH, a_w, $\log N_0$, temperature, and holding time of the treatment. The proposed model was verified in experiments using cow and human milks. With cow milk, the correspondence between the experimental and the predicted data is highly satisfactory. With human milk, the model predicts a smaller effect of heating than is that manifested experimentally.

Keywords: thermal inactivation model; *Enterococcus faecium*; verification

The milk bank of the Teaching Hospital Hradec Králové (Klen 1981; Vavra 1981–1982; Klen *et al*. 1987; Měřička *et al*. 2003) as well as other milk banks in the Czech Republic (Šípek *et al*. 2002) monitor on a long-term basis the incidence and both input and output concentrations of microorganisms in human milk. The analyses show occasional presence of the bacterium *Enterococcus faecium* in the raw or processed material. This microorganism is often capable of surviving mild pasteurisation at 62.5°C for 20 minutes.

Since the milk banks carry out complete checks, using the critical control point preventative safety system (Měřička *et al*. 2004a, b), the health of newborns and infants fed by pasteurised, frozen-preserved milk is not at risk. However, a portion of the material is lost since individual bottles containing the above mentioned bacteria must be discarded. For this reason, it is most desirable to know the inactivation data for the bacterium, and possibly to modify the pasteurisation process in order to prevent the survival of the microorganism. The procedure must remain, at the same time, mild enough to maintain maximum possible amount of milk immunoglobulins.

Why should the presence of *E. faecium* in pasteurised milk be a problem? Numerous papers show that certain strains of the bacteria are a natural component of human milk (Martín *et al*. 2003). Martín *et al*. (2004) even suppose that the

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microorganism diffuses endogenously from the digestive tract of the mother to the breast milk and then colonises the gut of the infant. Kang and Lee (2005) found that a certain strain of *E. faecium* produces a bacteriocin with inhibitive effects on gram-positive and gram-negative bacteria, including pathogens. At the same time, the colonisation of the infant gut with non-pathogenic microflora is a prerequisite for the health of the individual during adolescence and maturity (Lodinova-Zadnikova *et al*. 2003). *E. faecium* is also commonly present in cheese (Morandi *et al*. 2005).

The presence of *E. faecium* is undesirable mainly because the bacterium easily develops resistance to antibiotics (vancomycin, tetracycline), see e.g. Panagea and Chadwick (1996). Novais *et al*. (2005) and Nishimoto *et al*. (2005) described resistant strains isolated in hospital environments. *E. faecium* is capable of growing under certain circumstances even at very low temperatures (Zanoni *et al*. 1993). The above mentioned authors estimated, according to the model, the range of the growth temperature between 0.1°C and 53.4°C, therefore the storage of untreated chilled milk does not provide a sufficient protection against the proliferation of the bacterium.

The data on thermal resistance of *E. faecium* are scarce in the available literature. Martinez *et al*. (2003) determined the data concerning thermal inactivation of *E. faecium* in the dependence on the cultivation temperature corresponding to physiological conditions. They found that the stationary growth phase cells cultivated at lower temperatures are considerably more resistant than the cells collected for thermal inactivation from the exponential growth phase. Sorqvist (2003) collected data on thermal inactivation of numerous microorganisms, including *E. faecium*, from literature. Garzaroli *et al*. (1996a, b) and Zanoni *et al*. (1997) specified thermal inactivation data and kinetic models of thermal inactivation of *E. faecium* in Bologna sausage as the substrate. The main issue is the tailing effect (survival of a portion of cells regardless of the duration of heating). Mulak *et al*. (1995) determined thermal inactivation data and parameters of first-order kinetic model for microorganisms (including *E. faecium*) on various substrates (fish meat, M/15 phosphate buffer). Kornacki and Marth (1992) focused on thermal inactivation of *E. faecium* in ultra filtered milk. The presence of milk fat caused an increase in the thermal resistance of the microorganism while the degree of condensation of low-fat milk had practically no effect on the thermal resistance.

With respect to the above mentioned facts, it is desirable to determine thermal inactivation data for *E. faecium* in model solutions, to develop a kinetic model and carry out the verification of the model using real substrates, namely milk. That has been the objective of this paper.

MATERIAL AND METHODS

Selection of microorganism, its origin, preparation of the working culture and working suspension, conditions of incubation, obtaining of enumerable colonies. The model microorganism selected was *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.

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 in a procedure recommended by the CCM to develop a revitalised culture on agar slant. The revitalisation took place at 37°C for 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 agar slant by full inoculating loop calibrated at 1 µl and used three times, always using a different sterile loop. The inoculated BHIB medium was incubated at 37°C for 18 h in order to obtain early stationary growth phase commonly recommended and used for experiments studying lethal and sub-lethal effects on bacteria, e.g. Schlemmerová (1987).

The working culture grown on BHIB medium was used to develop working suspensions with corresponding conditions under a selected combination of values of pH and a_w. The working suspensions were then heated to 60.0°C, 62.5°C, and 65.0°C for various time periods, as specified in the section Procedure of thermal inactivation.

The basic component of the model solution was the saline solution without added peptone (which dampens lethal and sub-lethal effects, e.g.

Schlemmerová 1987). The volume of buffering substances adjusting the pH value of the inactivation solutions, i.e. $Na_2HPO_4 \cdot 12H_2O$ and KH_2PO_4, was determined according to the desired value of pH (in particular, pH values of 6.8; 6.0; 7.6). The value of water activity a_w was adjusted by the addition of NaCl in accordance with the international standard ISO 21807 (recently introduced in ČSN) as the so-called saline standards. Specific values of a_w used in the experiments were: the highest tolerable value of 0.99 corresponding to NaCl concentration in the saline solution, and a selected lower value of 0.97.

The working suspension was prepared by inoculation of 1 ml of the working culture grown in BHIB substrate into 99 ml of the model solution with the selected values of pH of 6.8 and a_w of 0.99. The procedure used was adjusted according to the previous experience with the high growth density of *E. faecium*: 1 ml of inoculum was taken from the mid column of the liquid culture. When thermal inactivation required greater volumes of the working suspension, BHIB culture was used in the form of a mixed inoculum prepared from individual test tubes and introduced into the correspondingly increased volume of the model solution.

The counts of viable cells of *E. faecium* had to be taken before and after the inactivation treatment. This required a series of decimal dilutions in the 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 (New Brunswick Scientific Co. Inc.).

The calculation was based on incubated plates and dilution ratios in order to meet the conditions of the formula below.

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, which includes the amendment 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:

ΣC – sum of colonies from all plates used for the calculation of two successive dilutions, where at least one of the plates contains 15 and more colonies

V – volume in ml inoculated onto each of the plates

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

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

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

The detection limit of 100/ml was predicted.

Containers and thermal history verification. In the experiments, plastic bags (able to withstand boiling) were used, manufactured by Madapack (Czech Republic), double-ply polyamide/polyethylene film having the thickness 70 μm. The bags were filled with water, heat sealed and autoclaved, and tested for strength. The thermal history of a bag of the size 110 mm × 40 mm filled with 10 ml of water was also determined. Thermocouple sensors were placed in the bags, measuring the water time-temperature history in response to sudden submersion of the bag in a circulation thermostatic bath. Two thermostats were used, one set for the temperature of circulated water of 75°C, and the second for 2°C. The temperature histories of three successive repetitions were measured. We predicted that the duration of the rise to the heating water temperature is 60 seconds. The duration of the decrease to the cooling water temperature is somewhat longer, approximately 140 seconds. That is why the bags were cooled in crushed ice-water mixture after the specified holding time.

Procedure of thermal inactivation. The freshly prepared working suspension was introduced aseptically into special bags (previously marked with the temperature and time durations) and the inlets of the bags were weld-sealed. Water bath HAAKE K 20, controlled by the programming unit HAAKE DC 50, was used for heating the bags. The first experimental temperature was the lowest of the three (60°C): the bags placed in a special holder were removed from the bath at specified time intervals (25 min, 30 min, 35 min, 40 min) and immediately placed in the crushed ice-water bath. The next steps were the preparation of the selected decimal dilutions, inoculation onto the plates of PCA, and incubation at 37°C for 24 hours. The same procedure was followed at the bath temperature of 62.5°C, with time intervals

of 20 min, 25 min, 30 min, and 35 min, and at the bath temperature of 65°C, with time intervals of 15 min, 20 min, 25 min, and 30 min.

RESULTS AND DISCUSSION

Evaluation of thermal inactivation data and development of a model

The data acquired or thermal inactivation are listed in Table 1. The limits of the microbiological test results were calculated at 95% level of confidence. The limits ranged between ±16 and ±52%. The results clearly indicate that heating can considerably decrease the counts of *E. faecium*. It is notable that the least decrease (under otherwise unchanged conditions) was obtained at pH = 6.8.

The acquired data were first analysed using the traditional procedure, and decimal reduction times D_T were determined from the formula:

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

where N_0 represents the values for the sample marked 0/0 (no heating) valid for the corresponding tests.

The data were fitted with a regression line that was forced to pass through the origin, yielding values of D_T valid for the individual temperatures, pH, and water activity values (Table 2).

The effects of temperature on the value of D_T is usually described using the Arrhenius equation, or the simpler semi-logarithmic straight line dependence:

$$\log D_T = A - T/Z \quad (2)$$

where:
T – temperature (°C)
D_T – decimal reduction times (min)
Z – temperature increment causing ten-fold decrease in decimal thermal inactivation time

Introduction of reference parameters T_{ref} and D_{ref} allows to express the formula (2) in the more usual form:

$$\log D_T = \log D_{ref} + (T_{ref} - T)/Z \quad (3)$$

The selected reference temperature was 60°C. Linear data regression was calculated for $\log D_T$ as a function of temperature T (using three values), yielding the values of Z and A. The values of D_{ref} were then calculated using the Eq. (2), with T_{ref} = 60°C and the regression values of the coefficients A and Z from Table 2. The resulting values of D_{ref}

Table 1. Results of thermal inactivation of *Enterococcus faecium* CCM 2308 depending on pH and a_w

Temperature (°C)/heating time (min)	Value of logarithm of the number of CFU/ml of *E. faecium*					
	test H1 pH = 6.8	test H2 pH = 6.0	test H3 pH = 7.6	test H4 H = 6.8	test H5 pH = 6.0	test H6 pH = 7.6
0/0 (no heating)	8.927 (log N_0)	8.707 (log N_0)	8.626 (log N_0)	8.687 (log N_0)	8.549 (log N_0)	8.624 (log N_0)
60/25	8.543	7.613	7.292	8.510	7.453	7.698
60/30	8.364	7.399	6.398	7.956	7.330	7.399
60/35	8.193	7.121	6.190	7.819	6.992	7.227
60/40	7.572	6.787	5.699	7.176	6.931	7.143
62.5/20	7.068	6.599	6.915	7.700	6.413	6.890
62.5/25	6.497	6.350	5.860	7.440	6.238	6.100
62.5/30	6.476	6.100	5.491	7.187	6.100	6.072
62.5/35	5.924	6.810	5.283	6.918	5.773	5.442
65/15	5.726	5.623	5.278	6.100	5.497	5.842
65/20	5.146	4.322	4.716	5.884	4.120	4.965
65/25	4.699	3.120	4.364	5.710	3.176	4.283
65/30	4.067	2.522	3.965	5.431	2.698	3.942

a_w = 0.99 corresponds to NaCl concentration in the saline solution (test H1–H3)
a_w = 0.97 corresponds to NaCl water solution of molarity of 0.9 (test H4–H6)

Table 2. Calculated parameters of thermal inactivation model D_T, Z, A, and D_{ref} as a function of pH and water activity

Test No.	a_w	pH	D_T (min)			Z (°C)	log A	$D_{ref, 60}$ (min)
			60°C	62.5°C	65°C			
H1	0.99	6.8	40.8	11.4	5.7	5.9	11.80	37.0
H2	0.99	6.0	21.9	12.8	4.7	7.5	9.41	23.7
H3	0.99	7.6	14.4	10.0	5.7	12.5	5.96	14.9
H4	0.97	6.8	40.8	20.0	8.1	7.1	10.08	42.1
H5	0.97	6.0	23.8	11.6	4.8	7.2	9.68	24.4
H6	0.97	7.6	25.9	11.0	5.9	7.8	9.10	24.9

valid for the temperature of 60°C are given in the same table.

Now, it is a difficult task to find a correlation between the parameters Z and D_{ref} and the pH and water activity. Using the computer program for non-linear regression curve fitting DataFit (by Oakdale Engineering, USA), we tested several suitable correlations. The values of Z acquired by the above method exhibited a pronounced minimum in the dependence on pH for $a_w = 0.99$, which causes problems in finding the correlations. On the other hand, at the water activity of 0.97, the dependence of Z on pH is very weak. As a compromise, the following equation was found for Z:

$$Z = a + b \times \text{pH} \wedge (c + d\, a_w + e\, a_w^2) \quad (4).$$

The above relationship shows that the power of pH depends on the water activity. The correlation coefficient of this relationship was 0.89, and 79% of variability was explained. The regression parameters of this relationship have the values given in Table 3.

The dependence of D_{ref} on pH and a_w was expressed in the following best correlation:

$$D_{ref} = a + b\, a_w^2 + c\ \text{pH} + d\ \text{pH}^2 + e\, a_w\ \text{pH} \quad (5)$$

where the correlation coefficient was nearly 1. The regression parameters of the relationship have the values given in Table 3.

The above relationships allow the compilation of a mathematical model of thermal inactivation of the microorganism, which basically represents a formula for the calculation of the value of decimal reduction time D_T. The formula was obtained by substituting Eq. (4) and (5) into the exponential version of Eq. (3):

$$D_T = (a + b\, a_w^2 + c\ \text{pH} + d\ \text{pH}^2 + e\, a_w\ \text{pH}) \times 10 \wedge \wedge \left(\frac{60 - T}{f + g\ \text{pH} \wedge (h + i\, a_w + b\, a_w^2)}\right) \quad (6)$$

The above Eq. (6) represents a non-linear dependence of D_T on pH and a_w. This equation was used for non-linear regression. All 18 originally determined values of D_T, as given in Table 2, were used. The preliminary values of the regression parameters were used as the initial set of values for the non-linear regression to yield optimised values of these parameters.

The comparison of the parameter values before and after optimisation shows considerable changes in some of them. The model now more accurately determines the most significant value for the prediction of inactivation, i.e. the value of D_T. The correlation coefficient for the equation (6), after optimisation of the parameter values, is 0.986. That explains approximately 97% data variability. The model is valid for the range of the water activity values of 0.99–0.97, pH values of 6.0–7.6,

Table 3. Coefficients in the various regression equations used in modeling

	a	b	c	d	e
Eq. (4)	6.3309	2.3615×10^{-11}	–1.1204	4.6753	9.5803
Eq. (5)	–2052.0	873.9371	655.6875	–27.4609	–290.6250
Eq. (6)	–2050.9	877.3787	654.0706	–27.3042	–290.7012
	f	g	h	i	j
Eq. (6)	6.6926	2.16×10^{-21}	2.5820	8.3885	13.4754

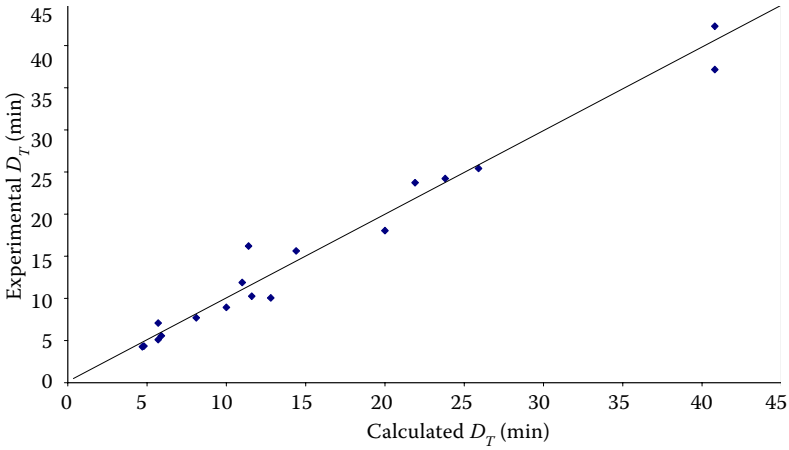

Figure 1. Comparison of experimental and calculated values of D_T

temperatures of 60–65°C, and was specified for microorganism concentrations from 10^2 to 10^8. The final values of the regression parameters are given in Table 3.

The comparison of the values of D_T acquired in the tests and calculated using the model (6) is shown in Figure 1. It is apparent that, with the exception of two values, the model predicts the D_T values with good accuracy. The formula (6), with the values of the optimised regression parameters, was used to write a code (of a procedure) in the Visual Basic programming language, which enables using an Excel spreadsheet to calculate the final concentrations of the microorganism after entering the initial data (log N_0, temperature, holding time, pH, a_w).

Model verification

The model was verified in the manner described above, only the model solution was replaced with two kinds of milk. The first milk was low-fat UHT pasteurised cow milk with the fat content of 0.5%, pH = 6.4, a_w = 0.997, manufactured by the dairy Kunín (Czech Republic). The second was frozen pasteurised human milk from the milk bank of the Teaching Hospital Hradec Králové, the sample designed 61906a, a_w = 0.99, pH = 6.81. The human milk was thawed at 37°C and tested for the absence of microorganisms by the cultivation method with an increased detection limit (volume of 1.0 ml was used as the inoculum, distributed in 0.2 ml volumes to five PCAs, in two parallels); incubated at 37°C for 24 hours.

Before commencing the inactivation experiments, the possible inhibitive effects of milk on the growth of *E. faecium* CCM 2308 were pre-tested. A contaminated sample (log N_0 CFU *E. faecium* CCM 2308 = 8.439/ml) was placed in an incubator with constant temperature of 25°C for 25 minutes. After that time a value of log*N* CFU *E. faecium* CCM 2308 = 8.406/ml was determined, showing that under the conditions of the test mentioned above the difference from the original value was negligible.

Figures 2 and 3 give the results of the experimental verification of the model for thermal inactivation of *E. faecium* in UHT pasteurised cow milk and in frozen pasteurised human milk, respectively. The values of water activity, pH, temperature,

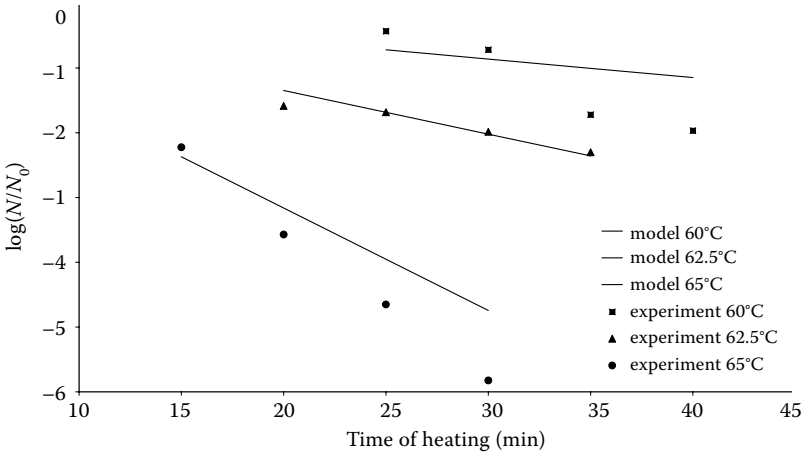

Figure 2. Comparison of experimental and calculated concentrations of *E. faecium* after thermal inactivation in cow milk

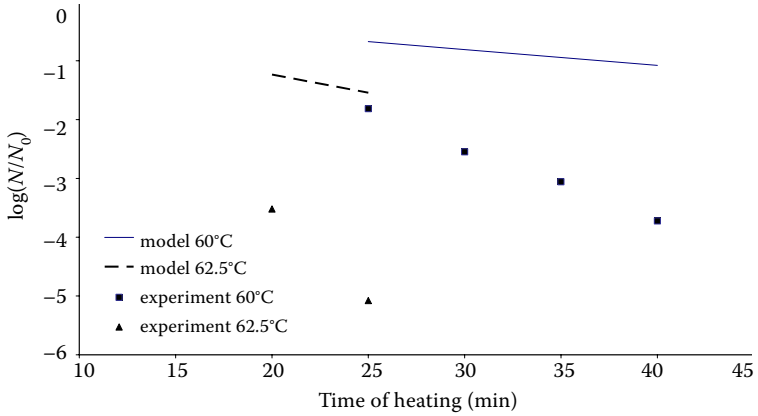

Figure 3. Comparison of experimental and calculated concentrations of *E. faecium* after thermal inactivation in human milk

holding time, and the initial concentrations of microorganisms were entered into the model relationship Eq. (6), yielding theoretical values of *E. faecium* concentrations after the corresponding pasteurisation regime. The lines in Figures 2 and 3 represent the model predictions.

Figure 2 shows a good correspondence between the experimental and the calculated values of the cow milk. The value of the correlation coefficient of the experimental and the calculated values is 0.973, showing a high degree of correlation.

Figure 3, for human milk, shows that the model predicts higher concentrations of the microorganism after thermal inactivation than demonstrated after the real inactivation experiment. Although the value of the correlation coefficient is high (0.988), the factor of the experimentally determined concentration is only 0.3. The model therefore predicts a lower thermal inactivation efficacy than showed in the experiment with human milk.

The reason for the under-prediction by the model may be that *E. faecium* is introduced for the verification into real food substrate that is conducive to a partial repair and/or growth of the cells, making them more vulnerable to the thermal treatment.

This hypothesis is well supported by numerous studies of lethal and sub-lethal injury of microorganisms by physical or physical-chemical effects other than the investigated thermal inactivation. They are based on the uniform methodology of inoculating the microorganism in early stationary phase into a defined substrate containing no nutrients, most often the saline solution without traces of peptone (e.g. Schlemmerová 1987).

Also, the presence of natural antimicrobial substances in mild pasteurised human milk (e.g. lysozyme) can increase the influence of heat on the present *E. faecium* cells.

The results show that the impact of the substrate on the response of *E. faecium* to heat can be considerable. The influence of the substrate was incorporated into a model that can be used in Excel. The acquired model under-predicts the effects of thermal sterilisation of human milk and therefore is on the safe side in predicting the thermal inactivation effects.

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