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Modelling inactivation of *Staphylococcus aureus* and *Yersinia enterocolitica* by high-pressure homogenisation at different temperatures

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Abstract

A detailed study of the inactivation of *Staphylococcus aureus* and *Yersinia enterocolitica* by high-pressure homogenisation was performed at, respectively, 25 and 35 different combinations of process temperature and process pressure covering a range of 5–50 °C and 100–300 MPa. It appeared that in the entire studied pressure–temperature domain, *S. aureus* was more resistant to high-pressure homogenisation than *Y. enterocolitica*. Furthermore, the effect of the process pressure on the inactivation of *S. aureus* was considerably smaller than on the inactivation of *Y. enterocolitica*. Also, temperature between 5 and 40 °C did not affect inactivation of *S. aureus* by high-pressure homogenisation, while *Y. enterocolitica* inactivation was affected by temperature over a much wider range.

Different mathematical models were compared to describe the inactivation of both bacteria under the experimental conditions applied. Such pressure–temperature inactivation models form the engineering basis for design, evaluation and optimisation of high-pressure homogenisation processes as a new preservation technique.
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1. Introduction

Heat treatment is one of the most commonly used preservation treatments for food and other perishable products. It is an efficient and economical process to inactivate microorganisms, but it cannot be used to treat heat-labile compounds. Therefore, over the last 10 years, considerable research efforts have been directed towards the development of novel nonthermal processes for preservation, such as the use of high hydrostatic pressure, pulsed electric field, ultraviolet light, pulsed light and high-pressure homogenisation.

It is known that high-pressure homogenisation can inactivate microorganisms (Popper and Knorr, 1990; Lanciotti et al., 1994, 1996; Guerzoni et al., 1999). Several studies have indicated that Gram-negative bacteria are more sensitive to high-pressure homogenisation than Gram-positive bacteria (Kelemen and Sharpe, 1979; Vachon et al., 2002; Wuytack et al., 2002). This suggests a correlation between cell wall structure and high-pressure resistance, which indicates

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that high-pressure homogenisation kills vegetative bacteria mainly through mechanical destruction of the cell integrity, caused by the spatial pressure and velocity gradients, turbulence (Doulah et al., 1975), impingement (Engler and Robinson, 1981; Keshavarz-Moore et al., 1990) and/or cavitation (Save et al., 1994; Shirgaonkar et al., 1998), which occur in a liquid during high-pressure homogenisation. Vachon et al. (2002) have made transmission electron micrographs of high-pressure homogenised cells of *L. monocytogenes*, which illustrated that cells are indeed disrupted by high-pressure homogenisation. The finding that high-pressure homogenisation does not cause sublethal injury reflects the 'all or nothing' impact of this technique on bacteria (Wuytack et al., 2002). Bacteria are either disrupted or not.

Furthermore, it has been observed that several successive rounds of high-pressure homogenisation have an additive effect on the viability reduction, which opens perspectives to increase the microbicidal efficiency of the treatment (Wuytack et al., 2002).

Up to date quantitative data to describe inactivation by high-pressure homogenisation are scarce. The objective of this study is therefore to study in more detail the effect of process parameters as pressure and temperature on the inactivation of bacteria by high-pressure homogenisation. *Staphylococcus aureus* and *Yersinia enterocolitica* were chosen as reference bacteria since an earlier study indicated that these were the most resistant of, respectively, five tested Gram-positive bacteria and six tested Gram-negative bacteria (Wuytack et al., 2002). A mathematical model will be proposed to quantify the impact of a high-pressure homogenisation treatment on *S. aureus* and *Y. enterocolitica* as function of homogenisation pressure and temperature.

2. Materials and methods

2.1. Preparation of cell suspensions

S. aureus and *Y. enterocolitica* were used throughout this study. Nutrient broth (CM76 Oxoid, UK) cultures (40 ml) in 100 ml Erlenmeyers were grown to stationary phase by shaking (200 rpm) for 21 h at 30 °C (*Y. enterocolitica*) or at 37 °C (*S. aureus*), centrifuged at $4000 \times g$ for 5 min, and the pellets were

resuspended to a concentration of approximately 10^8 cfu/ml in sterile PBS buffer (10 mM potassium phosphate buffer, pH 7.0; 8.4 g/l NaCl).

2.2. High-pressure homogenisation treatment

Cell suspensions were homogenised at pressures ranging between 100 and 300 MPa and temperatures between 5 and 50 °C. The used temperature–pressure combinations and the number of observations at each combination are shown in Table 1. The high-pressure homogeniser was an Emulsiflex C5 (Avestin, Ottowa, Canada) consisting of an inlet reservoir, an air-driven pump and a ceramic homogenising valve. A heat exchanger was placed immediately after the homogenising valve to withdraw the heat generated during passage of the sample through the homogenising valve. In addition, the entire homogeniser was immersed in a water bath to maintain the desired process temperature and to improve heat dissipation from the homogenising valve block. The outlet temperature of a typical sample homogenised at 300 MPa was without heat exchanger, ± 20 °C higher than the process temperature but was reduced to 18 °C after passage through the heat exchanger whatever the process temperature.

2.3. Determination of the loss of viability

To assess loss of viability caused by a treatment, untreated and treated cell suspensions were serially diluted in PBS and plated on the surface of plate count agar (PCA, CM325 Oxoid) medium. Plates were incubated for 21 h at 30 °C (*Y. enterocolitica*) or at 37 °C (*S. aureus*), and the number of colony forming

Table 1
Number of observations at each temperature–pressure combination for *S. aureus* (S) and *Y. enterocolitica* (Y)

Temperature (°C)	100 MPa		150 MPa		200 MPa		250 MPa		300 MPa	
	S	Y	S	Y	S	Y	S	Y	S	Y
5	2	3	1	3	2	3	2	3	2	2
10	3	5	3	5	2	2	2	3	0	3
15	0	6	0	5	0	6	0	6	0	4
20	0	3	0	3	0	3	0	3	0	2
25	3	5	3	5	3	5	3	3	3	5
35	3	3	3	3	3	5	3	8	1	2
40	0	3	0	3	0	3	0	0	0	0
45	4	3	4	2	4	0	4	0	1	0
50	3	3	3	0	3	0	3	0	1	0

units (CFU) was determined. The logarithm of the reduction factor (log RF) was calculated as

$$logRF = log\left(\frac{CFU \text{ before treatment}}{CFU \text{ after treatment}}\right).$$

2.4. Data analysis and model evaluation

Starting from a second-order polynomial model describing log RF as a function of pressure (P) and temperature (T), a backward regression procedure using the SAS package release 8.01 (SAS online Doc, Version 8) was applied to propose derived models and to determine the parameters of, and evaluate, these models. The backward regression procedure begins with all candidate variables in the model and then systematically removes variables that are not significantly associated ($P>0.1$) with the target, until a model with only significant parameters is obtained.

The regression procedure calculates the set of parameters with the lowest sum of squares of errors (SSE).

$$SSE = \sum(logRF_{experimental} - logRF_{fitted})^2$$

A student t-test was done to evaluate the estimated parameters. Therefore, the t-value and p-value were determined.

$$t = \frac{\text{estimated parameter}}{\text{standard error of the parameter}}$$

p-Value is the corresponding probability for testing the null hypothesis that the parameter is not significantly different from zero.

To evaluate the proposed models, an adjusted regression coefficient r^2 and the root mean square error (RMSE) were determined. In general, r^2 measures how well a linear or a nonlinear model fit the data. Because this statistical parameter is largely dependent on the model structure and on the number of observations (n) and parameters (p), an adjusted r^2 was used to compare different nonlinear models on their ability to fit data sets, making allowance for the number of observations and parameters:

$$\text{Adjusted } r^2 = 1 - \left(\frac{(n-i)(1-r^2)}{(n-p)}\right)$$

i is an indicator variable that is 1 if the model includes an intercept, and 0 otherwise.

The RMSE measures the average deviation between the observed and the fitted values.

$$RMSE = \sqrt{\frac{SSE}{n-p}}$$

A measure of the reliability of the parameters to predict the loss of viability (log RF) caused by a high-pressure homogenisation treatment was obtained by comparing the experimentally determined log RF values to the ones fitted by the model. The fits obtained for the various models were also examined for the distribution of residuals. Residuals of an appropriate fit should be distributed randomly and not systematically related to the applied pressure or temperature.

Furthermore, a statistical comparison of the different nonlinear models was made by calculating Mallows's criterion (C_p) (Mallows, 1973), Akaike's information criterion (AIC) (Akaike, 1969), Schwarz Bayesian criterion (SBC) (Schwarz, 1978) and the predictive residual sum of squares (PRESS). These criteria, except PRESS, compare models by their sum of squares of residuals (SSE), corrected for the number of parameters and the number of observations.

$$C_p = \left(\frac{SSE_p}{s^2}\right) - (n - 2p)$$

where s^2 is the MSE for the full model and SSE_p is the sum of squares of errors for a model with p parameters including the intercept, if any.

$$AIC = n\ln\frac{SSE}{n} + 2p$$

$$SBC = n\ln\frac{SSE}{n} + p\ln n$$

$$PRESS = \sum(logRF_{experimental} - logRF_{fitted-i})^2$$

log RF$_{fitted-i}$ is the fitted value for the ith observation that results from dropping the ith observation from the parameter estimates.

This comparison is only valid when the errors are normally distributed. The model with the lowest C_p,

AIC, SBC and PRESS is the best choice from a statistical point of view.

Based on log RF values calculated with the selected models, pressure–temperature diagrams were drawn using Microsoft Excel to show the pressure–temperature combinations resulting in the same inactivation (log RF).

3. Results and discussion

3.2. Inactivation of Y. enterocolitica and S. aureus by high-pressure homogenisation

The inactivation of *Y. enterocolitica* and *S. aureus* caused by high-pressure homogenisation treatments (100–300 MPa and 5–50 °C) is represented, respectively, in Fig. 1A and B. It appears that in the entire studied pressure–temperature domain, *S. aureus* is more resistant to high-pressure homogenisation than *Y. enterocolitica*. High-pressure homogenisation caused almost no inactivation of *S. aureus* at 35 °C and below, even up to a process pressure of 300 MPa. However, above a process temperature of 35 °C, the inactivation degree abruptly increased for high-pressure homogenisation treatments with a process pressure higher than 100 MPa. A homogenisation pressure of 100 MPa caused already 1 log unit inactivation of *Y. enterocolitica* even at low temperature. An increase in the process pressure resulted in a gradual increase of inactivation, which is more pronounced when the process temperature is higher.

From Fig. 1, it can also be concluded that for *S. aureus* compared to *Y. enterocolitica*, the effect of the process pressure on the inactivation is considerably smaller. Also, temperature between 5 and 40 °C does not seem to affect inactivation of *S. aureus* by high-pressure homogenisation, while *Y. enterocolitica* inactivation is affected by temperature over a much wider range.

3.2. Mathematical modelling

In a first approach, we tried to fit the data with a complex second-order polynomial model (model 1). The model parameters and the adjusted r^2 and RMSE are given in Table 2, the analysis of the optimisation criteria in Table 3. Subsequently, one by one the
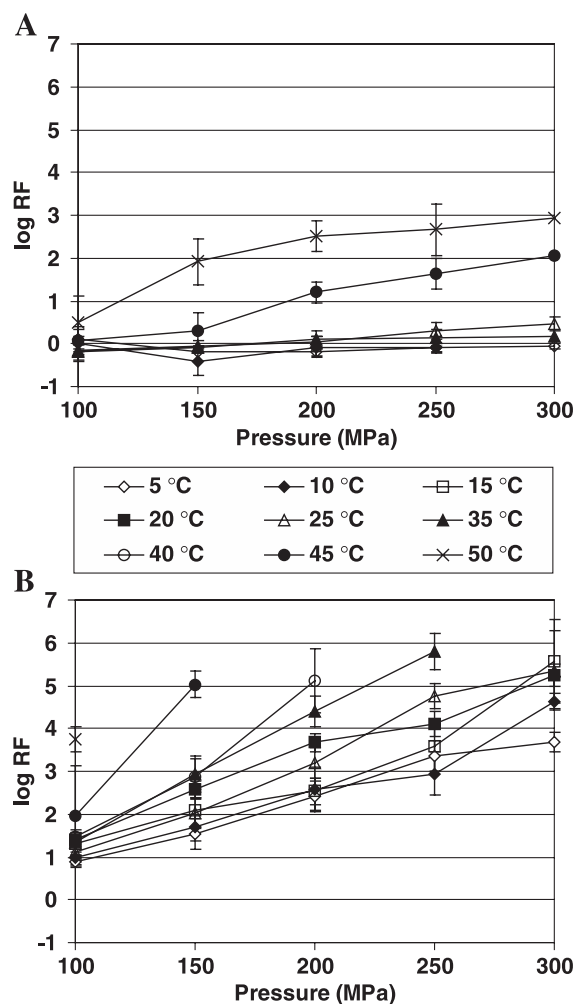

Fig. 1. Inactivation of *S. aureus* (A) and *Y. enterocolitica* (B) as a function of the homogenisation pressure at different process temperatures. log RF = log N_o/N. The presented data are the mean value of three experiments ± standard deviation.

nonsignificant variables were removed by using the backward regression method until a model with only significant variables remained. This resulted for both *S. aureus* and *Y. enterocolitica* in the elimination of the quadratic pressure term (model 2, see Table 2). Elimination of other variables resulted in an increase of the C_p criterion and RMSE and in a reduction of the adjusted r^2 (data not shown). However, for *Y. enterocolitica*, the estimation of the intercept *a* was very bad ($t = -0.150$), as can be seen from the high standard error. Therefore, we carried out a separate back-

Table 2

Estimated parameters ($\pm$ standard error) of different models describing inactivation of *S. aureus* and *Y. enterocolitica* in PBS buffer as function of the process temperature (°C) and pressure (MPa)

Parameter	Estimated value			
	Model 1	Model 2	Model 3	Model 4
S. aureus				
a	0.891 ± 0.532	1.03 ± 0.335		
b	-0.130 ± 0.016	-0.130 ± 0.016	-0.115 ± 0.015	-0.0899 ± 0.0111
c	0.00204 ± 0.00025	0.00205 ± 0.00024	0.00193 ± 0.00024	0.00174 ± 0.00024
d	-0.0018 ± 0.0050	-0.00345 ± 0.00153	0.00584 ± 0.00217	0.000647 ± 0.000791
e	$-4.21e-06 \pm 0.000012$		$-2.00e-04 \pm 7.86e-06$	
f	0.00029 ± 0.00005	0.000291 ± 0.000045	0.000248 ± 0.000039	0.000184 ± 0.000030
	adjusted $r^2 = 0.817$	adjusted $r^2 = 0.806$	adjusted $r^2 = 0.845$	adjusted $r^2 = 0.806$
	RMSE $= 0.417$	RMSE $= 0.414$	RMSE $= 0.422$	RMSE $= 0.438$
Y. enterocolitica				
a	0.0956 ± 0.6042	-0.059 ± 0.394		
b	-0.0825 ± 0.0273	-0.0819 ± 0.0271	-0.0799 ± 0.0214	-0.0855 ± 0.0133
c	0.0016 ± 0.0004	0.00160 ± 0.00039	0.00157 ± 0.000357	0.00164 ± 0.000297
d	0.0112 ± 0.0054	0.0130 ± 0.0016	0.0120 ± 0.00244	0.0127 ± 0.000806
e	$4.29e-06 \pm 0.000013$		$2.77e-06 \pm 8.26e-06$	
f	0.000039 ± 0.000072	$0.000386 \pm 7.10e-05$	0.000383 ± 0.000055	$0.000395 \pm 4.10e-05$
	adjusted $r^2 = 0.870$	adjusted $r^2 = 0.870$	adjusted $r^2 = 0.972$	adjusted $r^2 = 0.973$
	RMSE $= 0.587$	RMSE $= 0.585$	RMSE $= 0.585$	RMSE $= 0.583$

Model 1: log RF $= a + bT + cT^2 + dP + eP^2 + fTP$; model 2: log RF $= a + bT + cT^2 + dP + fTP$; model 3: log RF $= bT + cT^2 + dP + eP^2 + fTP$; model 4: log RF $= bT + cT^2 + dP + fTP$.

ward regression on the model without an intercept. Application of the backward regression method showed in this case all parameters to be significant for *S. aureus* (model 3) ($P < 0.1$), and the further

Table 3

Optimisation criteria of different models describing inactivation of *S. aureus* and *Y. enterocolitica* in PBS buffer as function of the process temperature and pressure

Optimisation criteria	Model 1	Model 2	Model 3	Model 4
S. aureus				
AIC	-129	-131	-128	-123
SBC	-115	-119	-116	-114
PRESS	14.3	14.0	14.6	15.6
C_p	6.00	4.12	5.00	9.52
Y. enterocolitica				
AIC	-137	-139	-138	-141
SBC	-120	-124	-124	-129
PRESS	50.5	49.4	49.6	48.8
C_p	6.00	4.11	5.00	3.11

Model 1: log RF $= a + bT + cT^2 + dP + eP^2 + fTP$; model 2: log RF $= a + bT + cT^2 + dP + fTP$; model 3: log RF $= bT + cT^2 + dP + eP^2 + fTP$; model 4: log RF $= bT + cT^2 + dP + fTP$.

elimination of variables resulted in an increase of the C_p criterion and RMSE and in a reduction of adjusted r^2 (data not shown). For *Y. enterocolitica*, the backward regression method applied to the model without an intercept resulted in the elimination of the quadratic pressure term (model 4). Elimination of additional variables resulted in an increase of the C_p criterion and RMSE and in a reduction of adjusted r^2 (data not shown).

The residual plots and experimental-fitted plots for model 2 (*S. aureus*) and model 4 (*Y. enterocolitica*) are shown, respectively, in Figs. 2 and 3. The plots for the other models were similar. Based on the adjusted r^2 (see Table 2), RMSE (see Table 2), the residual plots and experimental-fitted plots, we can conclude that all of the proposed models fit well the data of *Y. enterocolitica*, but somewhat less so for *S. aureus*. Depending on the model, the adjusted r^2 is between 0.87 and 0.98 for *Y. enterocolitica*, and between 0.80 and 0.85 for *S. aureus*. On the other hand, based on the RMSE as a criterion, the fit is better for *S. aureus* (0.41–0.44) than for *Y. enterocolitica* (0.58–0.59). Furthermore, the experimental-fitted plots show no
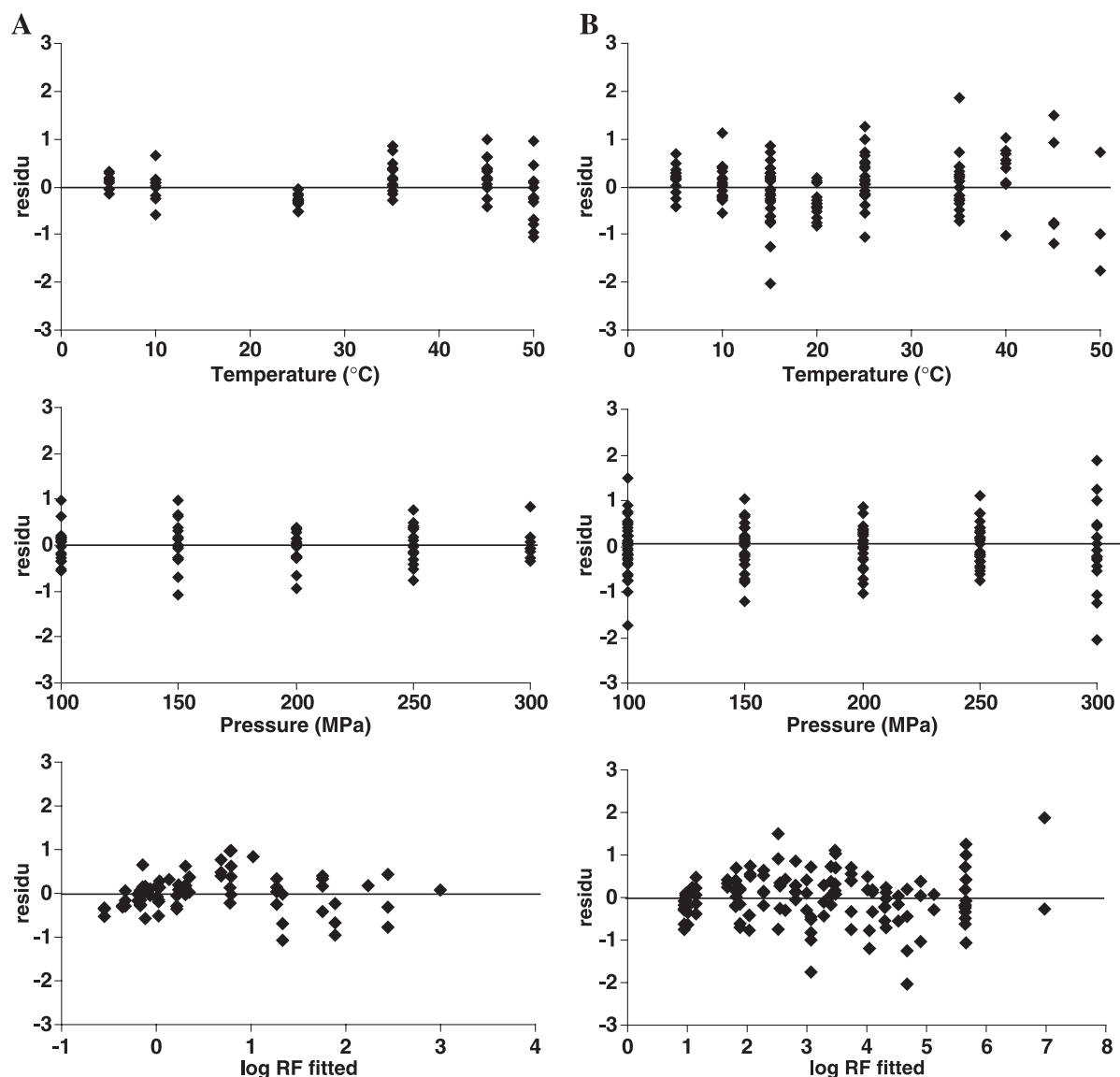
A

B

Fig. 2. Residual analysis of the inactivation of *S. aureus* (A) and *Y. enterocolitica* (B) according to, respectively, model 2 and model 4.

local over- or underestimation of *Y. enterocolitica* inactivation by the proposed models. For *S. aureus* in contrast, the analysis shows a slightly underestimated inactivation when the experimental log RF is higher than 2. However, from a safety point of view, such an underestimation of inactivation is acceptable and certainly to be preferred above an overestimation. The residual plots show a random distribution for both *Y. enterocolitica* and *S. aureus*, except the plot of the

residuals against the fitted log RF for the latter organism, which shows a rather uneven distribution of the residuals around the zero line, probably due to the underestimation of the inactivation when the experimental log RF is higher than 2 (or the fitted log RF higher than 1.2).

For *Y. enterocolitica*, it can be concluded that model 4 results in the best fit (highest adjusted r^2 (0.973) and lowest RMSE (0.583)) and good estima-
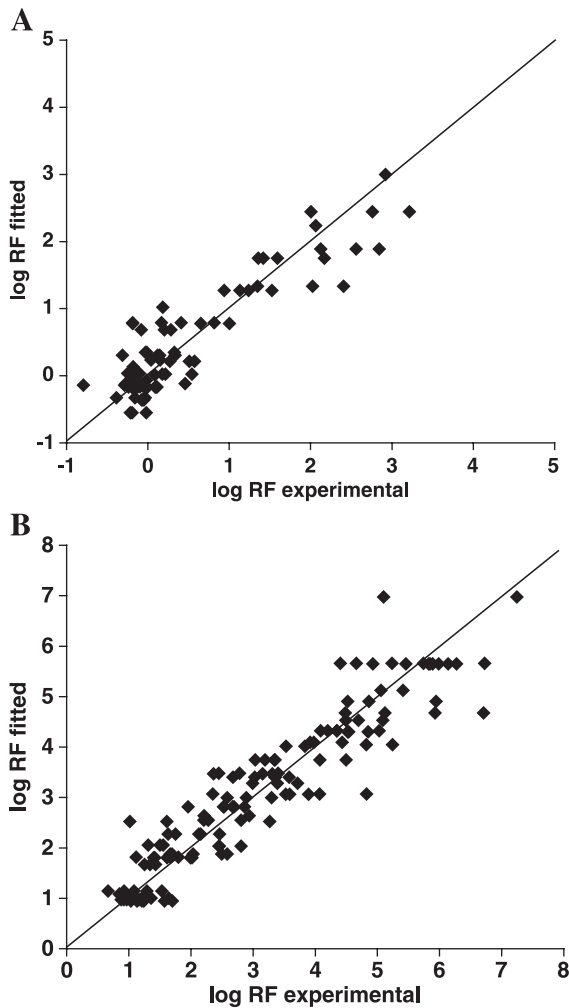
Fig. 3. Correlation between the experimentally determined log RF of *S. aureus* (A) and *Y. enterocolitica* (B) after high-pressure homogenisation and log RF calculated according to, respectively, model 2 and model 4. log RF = log N_o/N.

tica, since those models have the lowest C_p, AIC, SBC and PRESS (Table 3). Each of these individual criteria can be criticised, but since all give the same answer, the result is quite convincing.

A closer look on model 2 and 4 learns that the latter is a special case of the former in which the intercept is zero. Interestingly, when model 2 is applied to *Y. enterocolitica*, the estimated intercept is indeed very close to zero, and in fact, model 2 describes the data equally well as model 4, however the addition of an extra parameter in model 2 is punished in our selection criteria since they take in account the number of parameters. Probably, since the intercept is so close to zero, it is difficult to accurately

tion of all parameters ($|t| > 5$). For *S. aureus*, model 3 resulted in the highest adjusted r^2 (0.845), while model 2 resulted in the lowest RMSE (0.414). Both models resulted in fairly good parameter estimation ($|t| > 2$). As further criteria for model selection, we applied statistical comparison of the models by calculating AIC, SBC and PRESS of log RF. This comparison is valid because the errors of log RF can be assumed to be normally distributed. It appears from this analysis that model 2 and model 4 are most suitable, respectively, for *S. aureus* and *Y. enterocoli-*
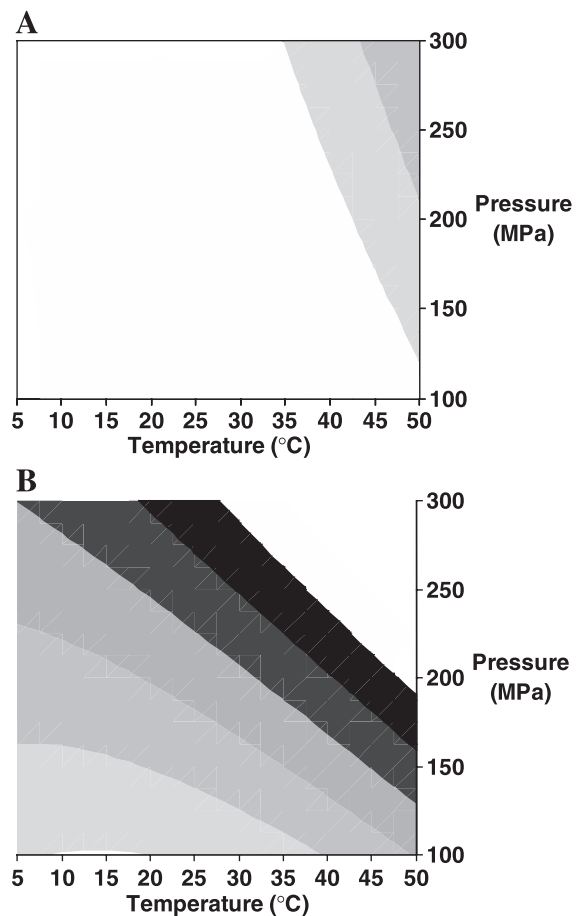

Fig. 4. Fitted iso-inactivation contour plot of *S. aureus* (A) and *Y. enterocolitica* (B) based on log RF calculated, respectively, with model 2 and model 4. log RF between 1 and 2 ▨; 2 and 3 ▨; 3 and 4 ▨; 4 and 5 ▨; 5 and 6 ■.

predict, which explains the high p-value in the t-test ($P = 0.881$). In conclusion, inactivation by high-pressure homogenisation can be described by model 2 as a common model structure for both bacteria. It will be interesting to see whether this model structure is also valid for other bacteria.

Based on log RF values calculated with the aid of model 2 for *S. aureus* and model 4 for *Y. enterocolitica* iso-log RF contours were determined. This is illustrated in Fig. 4A for *S. aureus* and in Fig. 4B for *Y. enterocolitica*.

Acknowledgements

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