Predicting the concentration of verotoxin-producing *Escherichia coli* during processing and storage of fermented raw meat sausages

Quinto E.J.\(^1^a\), Arinder P.\(^2\), Axelsson L.\(^3\), Heir E.\(^3\), Holck A.\(^3\), Lindqvist R.\(^4^5\), Lindblad M.\(^4\), Andreou P.\(^6\), Lauzon H.L.\(^7^b\), Marteinsson VÆ\(^7\) and Pin C.\(^1^*\)

\(^1\) Institute of Food Research, Norwich, NR4 7UA, United Kingdom.
\(^2\) SIK-The Swedish Institute for Food and Biotechnology, Ideon Science Park, SE-223 70 Lund, Sweden.
\(^3\) Nofima - Norwegian Institute of Food, Fisheries, and Aquaculture Research, P.O. Box 210, NO-1431 Ås, Norway.
\(^4\) National Food Agency, P.O. Box 622, SE-751 26 Uppsala, Sweden.
\(^5\) Department of Microbiology, Swedish University of Agricultural Sciences, SE-75007, Uppsala, Sweden.
\(^6\) h.s.i. Foodtech Laboratories Ltd, P.O. Box 27066, 1641, Nicosia, Cyprus.
\(^7\) Matís ohf., Food Safety, Environment & Genetics, Vínlandsleið 12, 113-Reykjavík, Iceland.

Current addressees:

\(^a\) Food Science and Nutrition Department, Faculty of Medicine, University of Valladolid, Valladolid E-47005, Spain

\(^b\) Primex ehf., Oskarsgata 7, 580-Siglufjörður, Iceland.

*Corresponding author:
carmen.pin@ifr.ac.uk
Tel: +441603255000
Fax: +441603507723

**Running Title:** Predicting VTEC concentration in fermented meats

**Key words:** VTEC, STEC, fermented meats, predictive models
Abstract

A model to predict the population density of verotoxigenic *Escherichia coli*, VTEC, throughout the elaboration and storage of fermented raw meat sausages, FRMS, was developed. Probabilistic and kinetic measurement datasets collected from publicly available resources were completed with new measurements when required and used to quantify the dependence of VTEC growth and inactivation on the temperature, pH, \(a_w\), and concentration of lactic acid.

Predictions were compared with observations in VTEC contaminated FRMS manufactured in a pilot plant. Slight differences in the reduction of VTEC were predicted according to the fermentation temperature, 24 or 34°C, with greater inactivation at the highest temperature. The greatest reduction was observed during storage at high temperatures. A population decrease greater than 6 decimal logarithmic units was observed after 66 days of storage at 25°C while only ca. 1 logarithmic unit reduction was detected at 12°C.

The performance of our model and other modelling approaches were evaluated throughout the processing of dry and semi-dry FRMS. The greatest inactivation of VTEC was predicted in dry FRMS with long drying periods while the smallest reduction was predicted in semi-dry FMRS with short drying periods.

The model is implemented in a computing tool, *E. coli* SafeFerment, EcSF, freely available from http://www.ifr.ac.uk/safety/EcoliSafeFerment. EcSF integrates growth, probability of growth, and thermal and non-thermal inactivation models to predict VTEC concentration throughout FRMS manufacturing and storage under constant or fluctuating environmental conditions.
INTRODUCTION

Verotoxin-producing *Escherichia coli* (VTEC), including the serotype O157:H7, are foodborne pathogens causing severe illness in humans (1). A subgroup of VTEC, capable of causing haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS), forms the enterohaemorrhagic *E. coli* (EHEC). From those, a range of serogroups (i.e. O157, O26, O103, O91, O145 and O111) are very frequently associated with public health issues (2).

Cattle are the principal reservoir of VTEC (3, 4). Transmission mainly occurs by eating undercooked infected beef, unpasteurized dairy products, vegetables, or contaminated water (3). Person-to-person transmission has also been reported (5). The dose associated with food-borne *E. coli* O157:H7 outbreaks has been estimated to be between 2 and 2,000 bacteria. Such a low dose means that this microorganism does not need to grow in a product to be able to cause illness (6).

In the USA, VTEC is responsible for an estimated 260,000 illness annually, with 3,700 annual hospitalizations and 20 annual deaths (7). In Europe, the estimated number of cases per year is approximately 3,000-4,000 (5).

Fermented raw meat sausages, FRMS, are products in which, a combination of fermentation, drying, spices, salt, sodium nitrite, and the starter culture activity, generating lactic acid and reducing the medium pH, inhibits the growth and/or reduces the survival of pathogenic bacteria. Several food-borne outbreaks involving FRMS contaminated with VTEC have been reported (8, 9). In EU, minced and/or fermented meat and products thereof, are considered as a hazard to public health because of the possibility of VTEC contamination (10). In USA, a reduction of 5 logarithmic units in the population of *E. coli* during the manufacturing of FRMS is required (11). In Canada, a 5-logarithmic unit reduction is recommended while in Australia the required reduction is 3 logarithmic units (12).

The increasing number of food-borne outbreaks caused by VTEC and the severity of VTEC
infections are the reasons behind the intense efforts to understand its population dynamics regarding growth and decay rates and also growth limits under several environmental conditions (13, 14). Models to predict the population kinetics of *E. coli* and other bacteria in foods are available at several public resources such as the ComBase portal (15), the PMP (16) or FoodRisk.org (17). The dependence of the growth of *E. coli* on the environment has been modelled in a wide range of food conditions (18-24). Growth/no-growth boundaries (13, 22) and inactivation of *E. coli* (25-30) have also been estimated in broth and foods including FMRS. Specifically the inactivation of *E. coli* O157:H7 has been modelled as a function of changes in the medium due to the metabolic activity of the starter culture and yeast during fermentation in olives (31) and as a function of pH and a$_w$ in soujouk-style fermented sausages during the process of fermentation and drying (32). The publically available THERM software predicts the growth or no growth of *E. coli* O157:H7 and other pathogens as a function of the time-temperature history of raw meat products (33); more specifically, tools implementing models to predict the inactivation of *E. coli* O157:H7 in raw meat (19) and in fermented meat products (34, 35) are available from the Australia Food Safety Center of Excellence. However, predicting the response of VTEC throughout the entire range of conditions that comprise growth and no growth environments characteristic to FRMS manufacturing and storage has not been approached yet.

The aim of this work was to quantify the response of VTEC under FRMS manufacturing and storage conditions, and to develop a user-friendly dynamic modelling framework to predict the concentration of VTEC throughout those processes. We carried out an extensive search in the literature and publicly available data sources regarding growth and inactivation rates, and growth/no-growth limits under different conditions of temperature, pH, a$_w$, and lactic acid in laboratory media and food products. In addition, new data were experimentally generated to address data gaps and FRMSs were manufactured in a pilot plant to evaluate the predictions of
the model of the population density of VTEC during manufacturing and storage.

With the aim of facilitating the application of the model, it has been implemented in an Excel add-in called “E. coli SafeFerment” (EcSF) with is freely available at http://www.ifr.ac.uk/safety/EcoliSafeFerment. The EcSF tool combines growth, probability of growth, and inactivation models to give predictions of VTEC concentration at any stage of the production and storage of FRMS under constant or fluctuating environmental conditions.

MATERIAL AND METHODS

Data collection. The datasets used for developing the models for VTEC were obtained from the ComBase database (15), from the literature (13, 20, 22, 35-38) and kindly provided by the Food Safety Centre, University of Tasmania (39, 40). Additional data were generated in this work to address data gaps (Supplementary Table 1). Supplementary Table 2 shows the serotypes and strains used in publicly available data and for newly data generation.

Data generation. i) Strains, inoculum and medium preparation. Two verotoxigenic E. coli strains were used in this work to measure non-thermal inactivation rates as well as to generate growth/no growth observations at various combinations of temperature, pH and a_w and in the presence of several concentrations of lactic acid. These were the O103:H25 strain implicated in an outbreak caused by fermented sausages containing mutton beef (“Morr”) in 2006 in Norway (41), and the O157:H7 218 strain implicated in an outbreak caused by cold-smoked fermented sausages in Sweden in 2002 (42).

Rifampicin-resistant strains, RifR, E. coli O157:H7 218Rif and O103:H25 RifR, previously constructed and evaluated for growth and inactivation in laboratory media under various environmental conditions, were used to inoculate FRMS (28, 30). Growth of wild type and RifR mutant strains was evaluated in Tryptone Soya Broth (TSB, Oxoid, UK) and in TSB added with
10000 ppm lactic acid at pH 5.5; inactivation was measured in TSB added with 45000 ppm lactic acid at pH 4.0 and $a_w$ value equal to 0.90. The growth and inactivation responses of Rif$^R$ strains were identical to those of the wild type strains in all tested conditions (data not shown).

Strain cultures were stored at -70°C with 20% glycerol. Inocula of wild strains were sub-cultured successively three times in TSB at 37°C for 24 h, immediately prior to the experiments. Rif$^R$ strains inocula were prepared as previously described (28). When required, media pH was adjusted aseptically with 6 N NaOH and 1N HCl and lactic acid (90.08 g/L; 90% acid) and NaCl were added to the media prior to autoclaving. Water activity was based on NaCl supplementation to TSB, already containing 0.5%, and it was estimated according to the equation $a_w = -0.0071 \times [% \text{NaCl}] + 1.0054$ as previously reported (43).

(ii) Non-thermal inactivation experiments. A new dataset of 96 non-thermal inactivation curves, in which $a_w$ was the inhibitory factor, was generated at all possible combinations of several temperatures (12, 16, 21, and 25°C), pH values (4, 5, and 6), $a_w$ values (0.90 and 0.94) and lactic acid concentrations (5000, 10000, 20000, and 45000 ppm). TSB was conditioned according to the experimental design and maintained at the target temperature prior to the inoculation of approximately $10^7$ colony forming units (cfu)/ml of E. coli O103:H25 or E. coli O157:H7. At adequate time intervals, samples were plated out on Tryptone Soy Agar (TSA, Oxoid); plates were incubated at 37°C for 48 h. The reason for this extended period of 48 h of incubation against the traditional 24 h is that 37°C may not be an optimum temperature for the recovery of stressed bacteria and for the subsequent colony formation. Despite this precaution, the absolute number of colony counts is likely to be affected. However, the recovery environment is assumed to have the same impact on the absolute counts at all sampling times during the inactivation process so that the estimation of the population inactivation rate should not be significantly affected by the recovery conditions. However, this is not experimentally proven and
could be one of the reasons behind the complexity and lack of linearity reported for bacterial
inactivation.

iii) Growth/no-growth experiments. Two new datasets were generated to evaluate: a) the
ability to grow in 288 conditions resulting from combining several temperatures (10, 16, and
20°C), pH values (4.8, 5.1, 5.4, 5.7, 6.0, and 6.3), aw (0.94, 0.95, 0.96, and 0.97), and lactic acid
concentrations (5000, 10000, 20000, and 45000 ppm). TSB was conditioned and set to the target
temperature. Volumes of 225 µl were dispensed into Bioscreen microplates and inoculated with
25 µl of 100-fold diluted of *E. coli* O103:H25. b). In addition, the ability of *E. coli* to grow was
evaluated in 144 conditions resulting from combining a wider range of temperatures (10, 16, 20
and 25°C), and higher aw (0.96, and 0.99) with the same pH values and lactic acid concentrations
as in dataset i). TSB was prepared as described in i) and inoculated with 25 µl of a mixture of
equal parts of a 100-fold diluted culture of *E. coli* O103:H25 with a 100-fold diluted culture of *E.
coli* O157:H7.

The borders of the plates were filled with 250 µl of sterile TSB or with 225 µl of TSB
inoculated with 25 µl of 100-fold diluted *E. coli* to follow normal growth in TSB. Optical density
measurements were performed at 600 nm (Bioscreen C analyser) upon inoculation and then daily
for up to 33 days. Microplates were stored in plastic boxes to avoid liquid evaporation and
incubated at the target temperatures. At least two replicates were recorded for each strain and
combination of environmental conditions.

iv) Manufacturing of FRMS in a pilot plant. Sausage production was carried out in a
Biosafety level 3 laboratory as previously described(28). The “Norwegian sausage” recipe used
was as follows (g per kg): beef, 378.5; pork, 378.5; pork back fat, 200; glucose, 40; sodium
nitrite, 0.08; ascorbic acid, 0.5; sausage spices (commercial premix), 3. LS-25 starter culture
(Gewürzmüller, Germany) was added according to the manufacturer’s instructions. After
chopping and mixing, approximately $10^7$ cfu/g of *E. coli* O157:H7 218Rif was added together with the starter culture with gentle mixing before stuffing in fibrous casings (diameter: 70 mm) to sausages of approximately 500-700 g. To cover all samplings (one sausage per sampling), at least 10 sausages were made for each batch. The temperature of the batter at stuffing was -2 - 0°C. The sausages were conditioned at ambient temperature and humidity for approximately 2 h and then transferred to the climate chamber. The temperature and relative humidity (RH) regime in the climate chamber was as follows: 3 days at 24°C and 94% RH, 3 days at 18°C and 85% RH, 4 days at 17°C and 81% RH and 17 days at 15°C and 75% RH (Table 2). The internal sausage temperature was monitored by an automatic temperature logging device (Termometerfabriken, Viking AB, Eskilstuna, Sweden). For the four periods of the regime described above, the actual internal temperatures were on average 24.2°C, 18.6°C, 17.0°C and 14.2°C, respectively, with deviations of ±0.4°C.

Meat batter representing a typical “Swedish mettwurst”-type sausage was delivered frozen in portions directly from a Swedish sausage factory (exact recipe not provided).

*E. coli* O157:H7 218Rif and starter culture (FSC 111, Chr. Hansen A/S, Denmark) were mixed into the batter before stuffing and weighing, as described above. The temperature and RH regime in the chamber were: 24 h at 19-21°C and 40-50% RH, 3 days at 22°C and 70% RH, 2 days at 20°C and 60% RH and 4 days at 16°C and 60% RH (Table 2). The actual internal temperatures were on average 20°C, 22.4°C, 20.7°C and 15.7°C ± 0.4°C, respectively. The finished sausages were vacuum-packed and stored in the dark at 4°C.

To study the effect of the fermentation temperature and of storage conditions, Norwegian sausages were inoculated with ca. $10^7$ cfu/g of an equal parts mixture of *E. coli* O157:H7 218Rif and *E. coli* O103:H25 RifR. The temperature regime was 3 days at 24°C or 34°C, 3 days at 18°C,
7 days at 17°C and 7 days at 15°C (Table 2). The finished product was vacuum-packed and stored at different temperatures 12, 16, 21, or 25°C for up to 66 days (Table 2).

At appropriate sampling times, an entire sausage was removed; 10 g of the sausage middle section were mixed in a stomacher with 90 ml of 0.4% peptone water. Water activity was analysed using the Aqualab instrument (Decagon Devices, Pullman, WA, USA) according to the manufacturer’s instructions. The pH was measured on the water phase in the stomacher mixing. Concentration of lactic acid was determined by a commercial analytical laboratory (Eurofins Norge, Moss, Norway). Samples were plated out on TSA plates (Oxoid) containing 100 μg/ml rifampicin (Sigma-Aldrich, Norway).

v) Parameter estimation. When required, the maximum specific growth rate was estimated by fitting the model of Baranyi and Roberts (44) to growth curves, while thermal and non-thermal specific inactivation rates were estimated from the slope of the linear part of the inactivation curve expressed as the natural logarithm of the concentration vs time.

vi) Model development. The bacterial concentration, $x$, at any time, $t$, in fluctuating environmental conditions was modelled as follows:

$$\frac{dx(t)}{dt} = r(t)u(t)x(t)$$  \hspace{1cm} (1)

where $u(t)$ describes the transition from the exponential growth phase to the stationary phase

$$u(t) = 1 - \left(\frac{x(t)}{x_{max}}\right)$$  \hspace{1cm} (2)
The maximum bacterial concentration, $x_{\text{max}}$, is taken to be constant and equal to $10^9$ cfu per ml or gram.

$$r(t) = \begin{cases} 
\mu_{\text{max}}(t) & \text{if } P(t) > 0.5 \text{ and } \text{temp} < 47^\circ C \\
\varphi(t) & \text{if } P(t) < 0.5 \text{ and } \text{temp} < 47^\circ C \\
\tau(t) & \text{if } \text{temp} \geq 47^\circ C 
\end{cases}$$

(3)

where $\mu_{\text{max}}(t)$ describes the maximum specific growth rate, $\varphi(t)$ the non-thermal specific inactivation rate, $\tau(t)$ the thermal specific inactivation rate and $P(t)$ the probability of growth according to the environmental conditions at a given time, $t$.

The dependence of $\mu_{\text{max}}$ on the temperature, pH, $a_w$, and lactic acid concentration was modelled with a second order polynomial:

$$\ln(\mu_{\text{max}}) = a_0 + a_1 T + a_2 pH + a_3 b_w + a_4 T pH + a_5 T b_w + a_6 pH b_w$$

$$+ a_7 T^2 + a_8 pH^2 + a_9 b_w^2 + a_{10} UL$$

(4)

where $a_i$ are the model parameters, $T$ is the temperature in Celsius scale ($^\circ$C), $b_w = \sqrt{1-a_w}$ (45), and $UL$ is the concentration of undissociated lactic acid (mM) which was estimated according to the Henderson-Hasselbalch equation as $UL = L/(1+10^{(pH-pK_a)})$, where $L$ is the total amount of lactic acid (mM) produced during fermentation and the $pK_a$ is the pH value at which the undissociated and dissociated forms of the acid are balanced; this value is 3.86 for lactic acid. Parameters in equation 4 were fitted in two linear regression steps as previously described (46).

In the first step the $a_0$ coefficient, as well as all the coefficients accounting for the effect of the temperature, pH and $a_w$, were fitted to the data described in Supplementary Table 1 in order to
obtain the three factors core model. The differences between the predictions of this model and the observed rates in the presence of lactic acid (Supplementary Table 1) were used to estimate the $a_4$ coefficient and extend the model with the fourth environmental factor, $UL$. All environmental factors exerted a significant effect on the growth rate.

The relationship between $\varphi(t)$, i.e. the non-thermal specific inactivation rate, and the temperature, pH, $a_w$, and lactic acid was modelled by an Arrhenius-type function as previously reported (23):

$$\ln(\varphi) = b_0 + b_1 \left( \frac{1}{TK} \right) + b_2 \left( \frac{1}{pH} \right) + b_3 \left( \frac{1}{b_w} \right) + b_4 UL + F$$

(5)

where $b_i$ and $F$ are model parameters and $TK$ is the absolute temperature in Kelvin scale. As previously described (46), the $b_0$, $b_1$, $b_2$ and $b_3$ parameters of the core model were estimated by linear regression in a first step from data described in Supplementary Table 1. $F$ tests (47) were carried out to evaluate if the effect of each environmental variable on the inactivation rate was significant. The apparent activation energy, $E_a$, a typical parameter of the Arrhenius model, can be estimated as $E_a = b_1/K$, where $K$ is the universal gas constant equal to ca. 8.314 JK$^{-1}$ mol$^{-1}$. In a second linear regression step, the core model was extended with the effect of the lactic acid concentration by estimating the $b_4$ coefficient from the differences between model predictions and the observed rates in the presence of lactic acid (Supplementary Table 1). The parameter $F$ was a factor estimated from the bias between model predictions and rates measured with the most resistant strain studied in this work.

The dependence of $\tau$, i.e. the thermal specific inactivation rate at temperatures greater than 47 °C, on the temperature was modelled as follows:
$\ln(\tau) = c_0 + c_1T$  \hspace{1cm} (6)

c_0$ and $c_1$ were estimated by linear regression.

$P(t)$ expresses the relationship between the probability of growth and the environmental conditions. This was estimated according to a logistic regression model as previously described (13):

$$
\ln \left( \frac{P}{1-P} \right) = d_0 + d_1 \ln(T - T_{\min}) + d_2 \ln(pH - pH_{\min}) + d_3 \ln(a_w - a_{w_{\min}}) \\
+ d_{12} \ln(T - T_{\min}) \ln(pH - pH_{\min}) + d_{13} \ln(T - T_{\min}) \ln(a_w - a_{w_{\min}}) \\
+ d_{23} \ln(pH - pH_{\min}) \ln(a_w - a_{w_{\min}}) + d_4 \ln \left( 1 - \left( \frac{UL}{UL_{MIC}} \right) \right)
$$  \hspace{1cm} (7)

where $d_i$ are the model coefficients estimated by logistic regression, and $T_{\min}$, $pH_{\min}$, $a_{w_{\min}}$ and $UL_{MIC}$ are the limiting values of the temperature (°C), pH, $a_w$ and undissociated lactic acid (mM), respectively, that prevent growth of *E. coli*. $T_{\min}$, $pH_{\min}$, and $a_{w_{\min}}$ were fixed to 7, 3.5 and 0.94, respectively, as estimated from Combase, while $UL_{MIC}$ was fixed to 25 as previously reported (48). The core model coefficients for the temperature, pH and $a_w$ were estimated by linear logistic regression using SAS 9.3 (49) with backwards parameter selection in order to omit unnecessary coefficients from the model. The core model was extended with the effect of lactic acid by estimating the value of $d_4$ that maximizes the likelihood between model predictions and growth/no growth observations with lactic acid (Supplementary Table 1).
Computing tool development. A forward Euler method (50) has been implemented in Visual basic code to simulate equation 1 under fluctuating conditions of temperature, pH, a_w and lactic acid. The program is published as an Excel add-in named “E. coli SafeFerment” (EcSF) freely available at http://www.ifr.ac.uk/safety/EcoliSafeFerment.

Model performance indices. Bias and discrepancy percentages between model predictions, pred, and observations, obs, were estimated as previously reported (51):

\[
Discrepancy(\%) = \left( e^A - 1 \right) \times 100 \tag{5}
\]

where

\[
A = \left( \sum_{i=1}^{n} (\text{Ln pred} - \text{Ln obs})^2 \right)^{1/2} \tag{6}
\]

where \( n \) is the number of observations, and

\[
Bias(\%) = \text{sign}(B) \left( e^{|B|} - 1 \right) \times 100 \tag{7}
\]

with

\[
B = \frac{\sum_{i=1}^{n} (\text{Ln pred} - \text{Ln obs})}{n} \tag{8}
\]
and

\[ \text{sign}(B) = \begin{cases} 
1 \text{ if } B \geq 0 \\
-1 \text{ if } B < 0 
\end{cases} \]  

(9)

**RESULTS**

The description of the datasets used in this work on the responses of VTEC to environmental conditions relevant to the production and storage of FRMS are shown in Supplementary Table 1. Supplementary Table 2 shows the serotypes and strains used for data generation. Despite the high variety in data generation conditions and the large number of serotypes and strains involved, our joint dataset showed consistent trends and the models were successfully validated with new observations and independent data from literature and public resources.

**Model development.** i) Modelling the growth rate. Supplementary Fig 1A shows the comparison between the predicted and the observed growth rates used to fit the model under different conditions of temperature, pH, and aw. The core model developed with these three environmental factors was extended with a term for the effect of the concentration of undissociated lactic acid which was fitted to the data generated in this study. Supplementary Fig 1B shows the comparison between the predictions and the observations generated with several concentrations of lactic acid used to extend the model. Table 1 shows the values of the estimated model parameters. As previously reported for *Listeria monocytogenes* (48), we also observed for *E. coli* that the inhibition of the natural logarithm of the growth rate is proportional to the
concentration of undissociated acid lactic regardless of pH or total lactic acid concentration (data not shown).

ii) Modelling the probability of growth. The model describing the dependence of the probability of growth on temperature, pH and $a_w$ was fitted by logistic regression. A backward coefficient selection procedure indicated that five out of the ten initial model terms were not significant and could be omitted from the model. The model coefficients are shown in Table 1. This model was extended with a term for the effect of the concentration of undissociated lactic acid as fourth environmental factor. Supplementary Fig 2 shows the predictions of the model for the probability of growth of VTEC according to three environmental factors: temperature, pH and $a_w$, and the predictions of this model extended with the term for undissociated lactic acid. The predicted boundary conditions at which the probability of growth is equal to 0.5 changed significantly when lactic acid was added (Supplementary Fig 2). The growth-no growth boundary pH was close to 4 in the absence of lactic acid, while it reached values of up to 6 when 45000 ppm of total lactic acid was added into the medium.

iii) Modelling survival/non-thermal inactivation rates. Survival or non-thermal inactivation refers in this context to the decay of the population under environments characteristic to FRMS in which other factors distinctive from the temperature prevent the growth of *E. coli*. Fig 1A shows model predictions according to the temperature together with the inactivation rates used to fit the model. Temperatures varied from 2 to 37°C and therefore included no growth temperatures and a wide temperature range at which the growth of *E. coli* is possible. The core model developed to study the dependence of the population decay rate on temperature, pH and $a_w$ showed that only the temperature exerted a significant effect on the inactivation rate of *E. coli* (Figs 3A-C). Thus, when other factors, such as low pH and/or low $a_w$, inhibited the growth of *E. coli*, the rate of inactivation was dependent exclusively on the temperature, which was not lethal in itself, while it
did not depend on the environmental factors causing the growth inhibition and/or inactivation (Figs 3BC).

In order to study the effect of the concentration of undissociated lactic acid on the non-thermal inactivation of *E. coli*, a new dataset comprising 96 survival curves with several concentrations of lactic acid under various combinations of temperature, pH, and *a*<sub>w</sub> was generated with two *E. coli* serotypes, O157:H7 and O103:H25. The comparison of the inactivation rates between these two serotypes is shown in Fig 1D. The decay rates of *E. coli* O103:H25 were significantly faster than those of *E. coli* O157:H7 (*p* value < 0.0001). *E. coli* O157:H7 was more resistant to inactivation, mainly at low pH values. The non-thermal inactivation model predicted faster inactivation rates than those observed with this strain, mainly at the most stringent conditions. The model was modified in order to predict VTEC survival according to the most resistant strain-. To do this, a factor, *F*, calculated from the bias between model predictions and the observed inactivation rates in *E. coli* O157:H7, was added to the model. The data generated with this strain was also used to extend the model for non-thermal inactivation of VTEC with a term for the lactic acid effect. Fig 1E shows the comparison between the predictions and the observed inactivation rates in *E. coli* O157:H7. The model coefficients for the dependence of the non-thermal decay rate on temperature and lactic acid are shown in Table1.

iv) Modelling thermal inactivation rate. At temperatures greater than approximately 47°C, the temperature in itself exhibits a lethal effect on *E. coli* and there is a sharp change of the effect of the temperature on the inactivation of *E.coli*, which requires the development of a thermal inactivation model (35). Therefore, a thermal inactivation model was developed to describe the lethal effect of temperatures on *E.coli*. To do this, thirty eight thermal inactivation rates measured at temperatures between 49°C and 55°C and at pH 7 and reported in ComBase were compared with those reported by Duffy *et al.* at the same temperature and pH (36, 52). In the latter studies,
the response of *E. coli* O157:H7 to several heat treatments, between 49 and 62°C, was quantified in pepperoni at pH 4.4 and 4.8. The greatest heat resistance was observed in the dataset of Duffy *et al.* (36, 52) generated at pH 4.8 (Supplementary Fig 3). This dataset was used to fit the thermal inactivation model parameters (Table 1).

**Model validation.** i) Validation of the model for the growth rate. The predictions of our model were compared with the predictions of the model for the growth rate of *E. coli* developed by Ross *et al.* (20) (Supplementary Fig 4A). The model of Ross *et al.* (20) predicted on average 50% faster growth rates. Ross *et al.* (20) already warned on possible systematic differences when comparing predictions and observations by other workers attributable to data generation methodology and to the way of estimation of the growth rates. Systematic differences can be quantified by analysing the two components of the error as previously described when measuring the differences between predictions and observations in foods (53). These two components of the error are the bias and the variability or error of the unbiased parameter or model. These concepts are applicable to the analysis of the overall discrepancy between two models. The estimations of bias and discrepancy percentages between two models can be carried out as previously described (51). A constant bias throughout all the region of comparison means that one of the models gives on average greater or smaller predictions by a factor or systematic difference. Disregarding this systematic difference by centring predictions from both models around the same expected value, the discrepancy between our model and the model of Ross *et al.* (20) is of ca. 25% which is similar to the original error percentage observed between predictions and observations used to fit the models.

Predicted growth rates were also compared with observations in several food types collected from ComBase (Supplementary Table 3, Supplementary Fig 4B). On average, the model was unbiased and the discrepancy percentage between observations and predictions was
ca. 120%. Values of 50% for the discrepancy between model predictions and food observations have been reported when analysing the sources of error in laboratory control experiments (54). However, larger bias factors and discrepancies are commonly found when comparing predictions and observations in complex foods, which comprise numerous factors that affect bacterial growth but are not included in the model such as the natural food microbiota, food ingredients and preservatives, heterogeneous structure, etc. (55). For instance, errors of 300-400% have been estimated between the growth rates of _L. monocytogenes_ predicted by four models and those observed in seafood inoculated and naturally contaminated with _L. monocytogenes_ (56). In a different study, a discrepancy greater than 200% has been reported between predicted rates by broth-based aerobic growth models and observations in ground beef inoculated with _E. coli_ (25).

ii) Validation of the model for probability of growth. Growth and non-thermal inactivation rates obtained from ComBase were coded as 1 and 0, respectively, and used to validate the predicted growth-no growth boundaries. Supplementary Fig S5 shows a good agreement between the predicted boundary conditions, at which the probability of growth is equal to 0.5, and this dataset. However, the ComBase dataset did not include measurements obtained in the presence of lactic acid.

New datasets were generated in this study in order to explore the performance of the model for the probability of growth under several lactic acid concentrations at various combinations of temperature, pH and _a_w_. Fig 2 shows the predicted boundary conditions with and without lactic acid, and independent previously published (37, 38) as well as newly generated growth-no growth data. In general, the predictions of the model extended with a term for the effect of lactic acid were in good agreement with the independently generated growth-no growth data. The percentage of concordance, estimated as the sum of the number of conditions at which growth was observed and the predicted probability of growth was greater than 0.5 and the number of
conditions at which growth was not detected and the predictive probability of growth was smaller than 0.5, was equal to 86% for the extended model including lactic acid while it was equal to 77% for the original model without the term for the lactic acid (Fig 2).

iii) Validation of the survival/non-thermal inactivation rate model. The non-thermal inactivation rate model developed in the present work shows a deviation from the model of McQuestin et al. (35). This is due to the correction factor applied to the model developed here in order to predict non-thermal inactivation according to the most resistant *E. coli* strain studied in our work (Fig 1D). Supplementary Fig 6 shows the comparison between predicted inactivation rates given by both models from 2 to 37°C. Mainly at high temperatures, the model developed here predicts inactivation rates up to 40% slower than the rates predicted by the model of McQuestin et al. (35). A similar discrepancy between the predictions of McQuestin et al. (35) and the observed non-thermal inactivation rates is reported in a previously published study using the same VTEC strains as in our work (38).

iv) Validation of the model for the concentration of *E. coli* in VTEC contaminated FRMS manufactured in a pilot plant. The concentration of VTEC predicted as a function of the four environmental factors: temperature, pH, *a*<sub>w</sub> and lactic acid, was compared with the observed concentration during the manufacturing and storage of VTEC contaminated Norwegian sausage (Fig 3A) and Swedish mettwurst (Fig 3B) in a pilot plant. The fermentation took place at ca. 24 and 22°C, respectively (Table 2). No significant differences in the inactivation of *E. coli* were observed between these two products (Table 2).

Two batches of Norwegian sausage, initially contaminated with a cocktail of the strains O157:H7 218Rif and O103:H25 Rif<sup>R</sup>, were fermented at 24 and 34°C and stored at 12, 16, 21, and 25°C, for ca. 66 days (Table 2, Fig 3C-E). The models implemented in EcSF predicted slight differences in the reduction of VTEC during fermentation, with greater reductions at higher
fermentation temperatures (Table 3). However, these differences were small and could not be observed in the data measured in the manufactured sausages because of the measurement error inherent to food plate counts (Table 2). In general, during the 27 days of fermentation plus maturation, VTEC concentration decreased by ca. 1 decimal log, regardless the temperature of fermentation. The greatest reduction of VTEC was observed during storage at high temperatures (Table 2). At 25°C, VTEC was not detected after 66 days of storage (Fig 3DF, Table 2). At 21°C, reductions of 3-4 decimal logarithmic units were observed during the storage period, while decreases of less than 2 decimal logarithmic units were observed after 66 days of storage either at 16°C or at 12°C (Table 2, Fig 3CE). The predicted concentrations were in good agreement with the observations for all VTEC curves throughout FRMS elaboration and storage, showing discrepancy percentages between 3 and 16% of (Table 2).

v) Comparison of the model performance throughout FRMS production and storage with other modelling approaches. For further validation, we compared the predicted concentrations by our modelling tool, *E. coli* SafeFerment- EcSF, with the approaches of Ross *et al.* (20) and Mellefont *et al.* (19) (Meat & Livestock Australia Refrigeration Index Calculator; MLA-RIC), and Ross and Shadbolt (34) (Meat & Livestock Australia *E. coli* Inactivation in Fermented Meat model; MLA-EcIFM). The EcFS simulates the solution for equation 1 to predict the concentration of VTEC under environments fluctuating between growth, survival and thermal inactivation conditions characteristic to FRMS manufacturing and storage. The MLA-RIC predicts the Refrigeration Index which is the decimal logarithmic increase in the concentration of *E. coli* in meat at a given time temperature profile. The MLA-EcIFM model predicts the inactivation of *E. coli* in uncooked, comminute, fermented meat products or analogous environments in which inactivation conditions are determined by low $a_w$ and/or pH, at a given time temperature profile.
Three flow diagrams in Fig 4 describe meat processing (F1), FRMS elaboration (F2), and the concatenation of both operations in a continuous process (F1+F2). Fig 4 shows times and conditions for each phase in each diagram.

The processing of meat (F1) starts after the slaughter process of the animal in the slaughterhouse and the post-mortem inspection by the competent authority. Meat must be stored and manipulated in accordance with the requirements laid down in Anonymous (57). Two scenarios were taken into account (Fig 4): the standard process with immediate refrigeration of the carcass (scenario A), and the hot boning and cutting alternative (scenario B). These processing scenarios were combined with two production options: minced meat and meat preparations produced from animals other than poultry 6 days after processing, and meat preparations produced with boned, vacuum-packed beef and veal meats 15 days after processing.

The response of VTEC was simulated with the MLA-RIC and the EcSF in all scenarios and production options. For scenario A, with immediate refrigeration of the carcass previous to the boning and cutting operation, the EcSF predicted an initial increase of 0.5 log_{10} cfu/g VTEC in the first 2.5 hours followed by a decrease of 0.10 log_{10} cfu/g VTEC in meat preparations produced 6 days after processing, and the same initial increase followed by a reduction of 0.22 log_{10} cfu/g in meat prepared 15 days after processing. For the hot boning and cutting alternative or scenario B, the EcSF predicted an increase of 0.91 log_{10} cfu/g VTEC in the first 3.5 hours followed by the same predicted reductions as in Scenario A. The MLA-RI considers only growth and predicted similar increases of 0.73 and 1.03 log_{10} cfu/g VTEC in scenarios A and B, respectively.

The VTEC concentration throughout FRMS manufacturing according to flow F2 (Fig 4), was predicted with the MLA-EcIFM and the EcSF. Two general types of FRMS were considered for the comparison: dry and semi-dry (34, 58). Two possible initial meat temperatures were taken
into account for the production of FRMS (Fig 4, Table 3). These temperatures were the standard refrigeration temperature for meat preparations, 4°C, and the maximum permitted room temperature in the slaughterhouse for boning and cutting, 12°C. Due to the wide variability of temperatures, pH and $a_w$, values, lactic acid concentrations and time intervals used in FRMS production, average values were used as input environmental conditions (Fig 4, Table 3).

Both approaches, MLA-EcIFM and EcSF, predicted the smallest VTEC reduction, i.e. smaller than 1 decimal logarithmic unit, in the Northern Europe semi-dry FRMS because of the short, drying step of 17.5 h. Greater reductions were predicted in dry FRMS and the Northern America semi-dry variety (Table 3). According to the predictions of both approaches, the safest product, i.e. with the greatest reduction of VTEC concentration, would be the traditional dry Southern Europe type due to a long, drying period of 11 weeks and thus a longer exposure of VTEC to inactivation conditions. The EcSF tool predicted in general lower reductions of VTEC concentration than the MLA-EcIFM approach. This difference was due to the correction of the non-thermal inactivation model implemented in the EcSF with a factor calculated from the inactivation rates generated in this study with the most resistant strain which was *E. coli* O157:H7 (Fig 1B).

Predictions on VTEC concentration were obtained at the unified flows, F1+F2, representing the entire process from animal slaughtering to the final product using the EcSF tool. The predicted reduction of VTEC concentration throughout these processes was lower than those observed alone in flow F2 because of the increase in the concentration predicted during meat processing in flow F1 (Table 3). As predicted in the flow for FRMS manufacturing only, inactivation of VTEC was greater in general in dry than in semi-dry fermented sausages, and the greatest inactivation was predicted in the dry Southern Europe FRMS type traditionally elaborated (Table 3). An increase of VTEC concentration was predicted in all cases for the
Northern Europe semi-dry variety as well as in other FMRS types manufactured after hot boning and cutting (Table 3).

These scenarios for FRMS production were simulated to illustrate the potential use of the prediction tools available for VTEC, although some of the specific conditions used in the simulations may not reflect real manufacturing environments.

DISCUSSION

Kinetic and probability models have been integrated to give dynamic predictions at environments that fluctuate in a range of growth, survival and inactivation conditions. Predictions for the concentration of VTEC throughout FRMS manufacturing and storage have been compared with independent observations. The model is implemented in the EcSF computing tool, which predicts the concentration of VTEC during FRMS elaboration and allows the estimation of storage conditions and manufacturing steps required to reach a given reduction in the concentration of VTEC.

Predictions when models are empirical as in this work are reliable only if inside the interpolation region of the model. The interpolation region of the model was defined in a landmark paper as the minimum convex region containing all the environmental conditions at which measurements used to fit the model were obtained (59). The interpolation region of the model is different from the nominal region or product of the ranges of the environmental variables where observations were obtained (59). These ranges are described in Supplementary Table 1 for our models. The interpolation region of the EcSF tool was estimated using the DMFit tool (60) from the non-thermal inactivation, thermal inactivation and growth datasets used to fit the population kinetic models and its vertices are reported in Supplementary Table S4. The interpolation region is inside the model nominal region but generally smaller; the percentage of
overlap between both regions can be estimated by Monte Carlo methods as previously described (61). We have estimated that the interpolation region of the EcSF tool is 30% of its nominal region. Therefore there is a high risk of extrapolation if conditions are chosen randomly inside the nominal region. This is of high importance because the error of model predictions obtained in environmental conditions lying outside the interpolation region of the model is increasingly greater as the distance to the edge of the interpolation region increases (53). While large variations in FRMS manufacturing conditions are not expected and therefore are likely to lie within the interpolation region of the EcSF tool, it is recommended to check that the vertices of the set of conditions forming the dynamic environmental profile are inside the interpolation region of the model.

We found that when factors distinctive from the temperature, such as low pH and/or low \( a_w \), inhibited the growth of \textit{E. coli}, the rate of inactivation was dependent on the temperature and to a smaller degree also on the undissociated acid lactic, which were not lethal in themselves, while it did not depend on the environmental factors causing the growth inhibition and/or inactivation, i.e. pH and/or \( a_w \). Similar results have been previously reported for \textit{E.coli} (35, 62).

We observed that the rates of inactivation of \textit{E. coli} increased as the temperature increased from 2 to 37°C when the values of pH and/or \( a_w \) were growth inhibitory. The increase of the rates could be due to the acceleration of the inactivation cellular processes as the temperature increases in this range. For most of bacteria, enzymatic rates increase proportionally to the temperature in the range of 20-40°C. Another explanation for the greater sensitivity of \textit{E. coli} to inactivating pH and/or \( a_w \) values at high temperatures could be the change in the properties of the membrane. Bacterial acid tolerance mechanisms are associated with membrane composition, such as lipid content that changes proton permeability (63). Acid tolerance is also based in the effectiveness of ion transport through the cell membrane to maintaining a constant internal pH (64), and in the
induction of proteins responsible for repairing the membrane (65). On the other hand, the
mechanism responsible of the death of *E. coli* during exposure to increasing osmotic pressure has
been reported to be a combination of membrane deformation and structural changes of the
membrane lipids affecting permeability (66). Therefore, perturbations of the membrane
properties associated with relatively high temperatures could be one of the factors causing the
decrease of *E. coli* survival at inactivating pH and/or *a*<sub>w</sub> values. It has been observed that as
temperature increases there is an increase in saturated fatty acids together with a decrease in
unsaturated fatty acids of *E. coli* cell membranes affecting their consistency (67-69). Heat shocks
at 42°C have been reported to destabilize the membrane changing the phospholipids and fatty
acid profiles and increasing its permeability (32, 69, 70). Other researchers (71, 72) indicated that
exposure to 42°C decreased the membrane anisotropy and increased its rigidity.

In our study we have observed that the concentration of undissociated lactic acid affected
growth and inactivation rates and had a considerable impact on the growth/no-growth boundary
conditions (Supplementary Fig 2). Organic acids are widespread used preservatives in the food
industry (73). Despite this use, their antimicrobial mode of action is still not fully understood
(74). It is generally agreed that the ability of weak acids to inhibit microbial growth is related to
their membrane permeability. At pH values lower than the pKa of their acidic group, the acid is
majorly uncharged and in this form of the acid can pass freely through the cell membrane. At
higher pH values the acid is mainly in dissociated or charged form; the transport of the
dissociated acid through the membrane cannot take place by free diffusion but it requires a less
efficient secondary transport mechanism (75). Early studies suggested that effects on intracellular
pH were key to understanding the toxicity of weak acids. Inside the cell, lactic acid dissociates
releasing protons that decrease the internal pH, and anions that in addition specifically inhibit
different aspects of metabolism and can have osmotic effects on the cell resulting in impaired
growth (74, 76-78). Available evidence indicates that it can also affect membrane function (74). Undissociated lactic acid has been shown to disrupt the lipopolysaccharide layer of the outer membrane in *E. coli*, increasing its permeability (79-81). These actions on the cell are likely to contribute to the growth inhibitory effect of other environmental factors and to narrow the growth environmental region.

The EcSF predictive tool can be applied for the evaluation of the impact of modifications, interventions or unexpected events during the manufacturing process and/or storage period on VTEC survival. As an example, EcSF predicts that the raise of the fermentation temperature from 20 to 28°C results in ca. 100% increase of VTEC inactivation. Similarly the impact of changes during maturation or storage can be evaluated at the exact conditions for each particular FRMS manufacturing process. Thus, the EcSF program can be used to optimize FRMS production in order to achieve the required reduction in the concentration of *E. coli* set by the relevant Food Safety Authorities (11, 12). The models implemented in EcFS are based in the most resistant strains of *E. coli* found in our study and therefore, EcSF is likely to over predict VTEC concentration in FRMS. The design of an optimum FRMS manufacturing and storage process to achieve a required reduction of VTEC together with the current efforts directed towards minimizing the prevalence of VTEC in raw meat, are approaches able to enhance the safety of these meat products regarding VTEC.

**Acknowledgments**

We would like to acknowledge the staff of the Food Safety Centre, University of Tasmania, specially Lyndal Mellefont and Tom Ross for useful discussions and their kind assistance in providing the data.
We thank Ahmed Abdelgani, Birgitta Baardsen, Janina Berg, Tom Chr. Johannessen, Karin Solgaard, Anette Wold Áslí and Erla Heiðrún Benediktsdóttir for excellent technical assistance and Dr. Camilla Sekse, Norwegian Veterinary Institute and Dr. Sven Löfdahl, Swedish Institute of Infectious Disease Control, for providing the O103:H25 and O157:H7 218 strains, respectively.

EJQ thanks the support of the International Exchange Program from the Spanish Ministry of Education. CP acknowledges an IFR career progression fellowship.

This work was funded by the EC, SAFEFOODERA call, project 06458 “SafeFerment: Increased safety of fermented sausage by the application of production exposure assessment for VTEC”.

References


5. ECDC (European Centre for Disease Prevention and Control), EFSA (European Food Safety Authority). 2011. Shiga toxin/verotoxin-producing *Escherichia coli* in humans, food and animals in the EU/EEA, with special reference to the German outbreak strain STEC O104. Stockholm.


13. **Presser KA, Ross T, Ratkowsky DA.** 1998. Modelling the growth limits (growth no
growth interface) of Escherichia coli as a function of temperature, pH, lactic acid
concentration, and water activity. Appl Environ Microb **64:**1773-1779.

Australian red meat industry: Risk ratings of hazard-product pairings. Int J Food Microbiol
**105:**221-232.


16. **PMP.** 2003. US Department of Agriculture, Agricultural Research Service Pathogen
Modeling Program, Eastern Regional Research Center, Wyndmoor, Pennsylvania, USA.
Version 7.0.

17. **FoodRisk.org.** 2011. Simulation and modeling software. JIFSAN, University of Maryland.

as a function of pH and lactic acid concentration. Appl Environ Microb **63:**2355-2360.

describing the effects of temperature, water activity, pH and lactic acid concentration on the

20. **Ross T, Ratkowsky DA, Mellefont LA, McMeekin TA.** 2003. Modelling the effects of
temperature, water activity, pH and lactic acid concentration on the growth rate of
Escherichia coli. Int J Food Microbiol **82:**33-43.


biomedical aspects of verocytotoxigenic E. coli including serotype O157:H7, an emerging pathogen (CT98-3935).


**Figure Captions**

Fig. 1. Analysis of the non-thermal inactivation rate of *E. coli* as a function of temperature, pH, aw, and lactic acid. A) Observed inactivation rates and predictions by a model that considers only the effect of the temperature – one factor model. B and C) The effect of the pH and aw on the inactivation rates was not significant as shown in the respective residuals plots of the model. D) Comparison of *E. coli* O157:H7 and *E. coli* O103:H25 non-thermal inactivation rates. E) Comparison between the observed rates in *E. coli* O157:H7 and the predictions by the one factor model extended with the effect of the undissociated lactic acid and corrected according to the greater survival of this strain.

Fig. 2. Comparison of the predicted growth/no-growth boundary conditions for VTEC in the presence of lactic acid and independent growth-no growth data newly generated in this work, upper 8 plots, and previously published(37, 38), lower plot with data obtained at 27°C with 4500 ppm of total lactic acid. The continuous line shows conditions at which the probability of growth is 0.5 as predicted by the three factors model without considering the effect of the lactic acid. The discontinuous line shows the boundary prediction by the model extended with lactic acid.
Symbols denote the observed growth (+) and no growth (○) conditions independently generated in our study.

Fig. 3. Predictions and observations of VTEC during the elaboration process of Norwegian sausages (A) and Swedish mettwurst (B) fermented at 24 and 22°C respectively, and during elaboration and storage of Norwegian sausages fermented at 24°C and storage for 66 days at 12°C (C) or at 25°C (D) and fermented at 34°C and storage for 66 days at 12°C (E) or at 25°C (F).

Fig. 4. Flow diagram of meat processing (F1) and of manufacturing several types of FRMS (F2). In F1, a_w in meat was assumed to be equal to 0.995 and the value of the meat pH equal to 5.8. Temperature refers to surface temperature in carcasses and meat pieces.

**Supplementary Figure Captions**

Supplementary Fig. 1. Comparison between predictions and observations used to fit the model for the dependence of the growth rate A) on the temperature, pH and a_w – three factors model, and B) on those three factors in the presence of several concentrations of lactic acid–extended model.

Supplementary Fig. 2. Growth/no-growth boundary temperature and pH values for VTEC at several concentrations of lactic acid and a_w fixed to 0.99. The continuous line shows the conditions at which the probability of growth is equal to 0.5 predicted by the model developed for the dependence of the probability of growth on the temperature, pH and a_w – three factors model. The discontinuous line shows the boundary predicted by the three factors model extended with the effect of undissociated lactic acid. Symbols denote the observed growth (+) and no-growth (○) conditions used to fit the model.
Supplementary Fig. 3. D value (minutes), which is the time required for one decimal reduction of the population at constant temperature, observed in *E. coli* at several temperatures. ComBase data generated at pH 7 (●). Observations reported by Duffy *et al.* (36, 52) at pH 4.8 (■) and at pH 4.4 (□). The line shows the model fitted to the observations at pH 4.8, which showed the greatest resistance to inactivation.

Supplementary Fig. 4. A) Comparison of the predictions of our model, EcFS, for the dependence of growth rate on the temperature, pH, *a*<sub>w</sub> and lactic acid and the predictions by the model developed by Ross *et al.* (20). B) Comparison between the predicted growth rates by our model and observed rates in foods recorded in ComBase. Few food products included lactic acid (○).

Supplementary Fig. 5. Comparison of the predicted growth/no-growth boundary conditions for VTEC at several temperature intervals and independent growth-no growth data. Predictions are estimated at the centre of the temperature interval. The continuous line shows conditions at which the probability of growth is 0.5 as predicted by the logistic model. Symbols denote the observed growth (+) and no growth (○) conditions derived independently from kinetic measurements in ComBase.

Supplementary Fig. 6. Comparison of the non-thermal inactivation rates of VTEC predicted by our model, EcFS, and by the model of McQuestin *et al.* (35).
Table 1. Estimates and standard error, se, for the coefficients of the models and error of the fit.

<table>
<thead>
<tr>
<th>Model / Modelled quantity / Equation</th>
<th>Coefficient</th>
<th>Estimate</th>
<th>se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum specific growth rate, $\mu_{max}$ / (\ln(\mu_{max})) / Equation 4</td>
<td>$a_0$</td>
<td>-9.95</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>$a_1$</td>
<td>0.188</td>
<td>0.0584</td>
</tr>
<tr>
<td></td>
<td>$a_2$</td>
<td>1.89</td>
<td>0.253</td>
</tr>
<tr>
<td></td>
<td>$a_3$</td>
<td>-3.65</td>
<td>4.22</td>
</tr>
<tr>
<td></td>
<td>$a_{12}$</td>
<td>0.0290</td>
<td>0.00789</td>
</tr>
<tr>
<td></td>
<td>$a_{13}$</td>
<td>-0.279</td>
<td>0.0955</td>
</tr>
<tr>
<td></td>
<td>$a_{23}$</td>
<td>4.55</td>
<td>0.572</td>
</tr>
<tr>
<td></td>
<td>$a_{11}$</td>
<td>-0.00514</td>
<td>0.000295</td>
</tr>
<tr>
<td></td>
<td>$a_{22}$</td>
<td>-0.223</td>
<td>0.0184</td>
</tr>
<tr>
<td></td>
<td>$a_{33}$</td>
<td>-131</td>
<td>8.81</td>
</tr>
<tr>
<td></td>
<td>$a_4$</td>
<td>-0.146</td>
<td>0.0167</td>
</tr>
<tr>
<td></td>
<td>RMSE&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.25</td>
<td>0.0584</td>
</tr>
</tbody>
</table>

| Non-thermal specific inactivation rate, $\varphi$ / \(\ln(\varphi)\) / Equation 5 | $b_0$ | 23.4 | 1.96 |
|                                                                                   | $b_1$ | -8000 | 567 |
|                                                                                   | F | -1.51 | - |
|                                                                                   | $b_4$ | 0.00796 | - |
|                                                                                   | RMSE | 0.693 | 0.000737 |

| Thermal specific inactivation rate, $\tau$ / \(\ln(\tau)\) / Equation 6 | $c_0$ | -20.3 | 1.61 |
|                                                                          | $c_1$ | 0.391 | 0.0281 |
|                                                                          | RMSE | 0.267 | - |

| Probability of Growth, $P$ / \(\ln(P/1-P)\) / Equation 7 | $d_0$ | 21.3 | - |
|                                                           | $d_3$ | 7.38 | 1.26 |
|                                                           | $d_{12}$ | 0.642 | 0.468 |
|                                                           | $d_{13}$ | -0.583 | 0.157 |
|                                                           | $d_{23}$ | -0.496 | 0.0521 |
|                                                           | $d_4$ | 16.5 | 0.121 |
|                                                           | Concordance<sup>b</sup> | 97.2% | - |
|                                                           | AIC<sup>c</sup> | 660 | - |

---

<sup>a</sup> RMSE, root mean square error.

<sup>b</sup> Percent concordant between predicted probabilities and observed responses.

<sup>c</sup> AIC, Akaike information criterion
Table 2. Observed and predicted reduction in the concentration of VTEC -log\textsubscript{10} cfu/g- during FRMS elaboration and storage and discrepancy and bias percentages between observed and predicted concentrations throughout the process.

<table>
<thead>
<tr>
<th>Food</th>
<th>Fermentation: temp(°C)/time(days)</th>
<th>Maturation: temp(°C)/time(days)</th>
<th>Storage: temp(°C)/time(days)</th>
<th>Observed/Predicted log reduction fermentation</th>
<th>Observed/Predicted log reduction maturation</th>
<th>Observed/Predicted log reduction storage</th>
<th>Num. data points</th>
<th>Discrepancy (%)</th>
<th>Bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norwegian sausage</td>
<td>24°/3d + 19°/3d</td>
<td>17°/4d +14°/17d</td>
<td>-</td>
<td>0.41/0.26</td>
<td>0.79/0.14</td>
<td>-</td>
<td>8</td>
<td>5.19</td>
<td>1.35</td>
</tr>
<tr>
<td>Swedish mettwurst</td>
<td>22°/4d + 21°/2d</td>
<td>16°/4d</td>
<td>-</td>
<td>0.55/0.28</td>
<td>0.28/0.20</td>
<td>-</td>
<td>6</td>
<td>6.82</td>
<td>1.72</td>
</tr>
<tr>
<td>Norwegian sausage</td>
<td>24°/3d + 19°/3d</td>
<td>17°/7d +14°/7d</td>
<td>12°/66d</td>
<td>0.59/0.35</td>
<td>0.44/0.51</td>
<td>1.19/1.69</td>
<td>7</td>
<td>3.98</td>
<td>0.0044</td>
</tr>
<tr>
<td></td>
<td>Id.</td>
<td>Id.</td>
<td>Id.</td>
<td>Id.</td>
<td>Id.</td>
<td>Id.</td>
<td>7</td>
<td>10.53</td>
<td>-3.39</td>
</tr>
<tr>
<td></td>
<td>Id.</td>
<td>Id.</td>
<td>16°/66d</td>
<td>Id.</td>
<td>Id.</td>
<td>1.34/2.49</td>
<td>7</td>
<td>11.81</td>
<td>3.12</td>
</tr>
<tr>
<td></td>
<td>Id.</td>
<td>Id.</td>
<td>21°/66d</td>
<td>Id.</td>
<td>Id.</td>
<td>4.30/3.92</td>
<td>7</td>
<td>13.49</td>
<td>6.59</td>
</tr>
<tr>
<td></td>
<td>Id.</td>
<td>Id.</td>
<td>25°/66d</td>
<td>Id.</td>
<td>Id.</td>
<td>&gt;6.00/5.58</td>
<td>7</td>
<td>13.49</td>
<td>6.59</td>
</tr>
<tr>
<td>Norwegian sausage</td>
<td>34°/3d + 19°/3d</td>
<td>17°/7d +14°/7d</td>
<td>12°/66d</td>
<td>0.59/0.55</td>
<td>0.26/0.55</td>
<td>0.77/1.61</td>
<td>7</td>
<td>7.90</td>
<td>-2.02</td>
</tr>
<tr>
<td></td>
<td>Id.</td>
<td>Id.</td>
<td>Id.</td>
<td>Id.</td>
<td>Id.</td>
<td>Id.</td>
<td>7</td>
<td>6.64</td>
<td>-1.57</td>
</tr>
<tr>
<td></td>
<td>Id.</td>
<td>Id.</td>
<td>16°/66d</td>
<td>Id.</td>
<td>Id.</td>
<td>1.80/2.34</td>
<td>7</td>
<td>11.86</td>
<td>-5.10</td>
</tr>
<tr>
<td></td>
<td>Id.</td>
<td>Id.</td>
<td>21°/66d</td>
<td>Id.</td>
<td>Id.</td>
<td>3.34/3.74</td>
<td>7</td>
<td>16.33</td>
<td>2.95</td>
</tr>
<tr>
<td></td>
<td>Id.</td>
<td>Id.</td>
<td>25°/66d</td>
<td>Id.</td>
<td>Id.</td>
<td>&gt;5.94/5.20</td>
<td>7</td>
<td>16.33</td>
<td>2.95</td>
</tr>
</tbody>
</table>
Table 3. Predicted reduction of VTEC concentration (log$_{10}$ cfu/g) by the Meat & Livestock Australia *E. coli* Inactivation in Fermented Meat tool (MLA-EcIFM) and the *E. coli* SafeFerment tool (EcSF) during manufacturing of several types of FRMS according to the production flows described in Fig 4.

<table>
<thead>
<tr>
<th>Flows$^a$</th>
<th>Tools</th>
<th>F1</th>
<th>F1 + F2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Processing of meat$^b$</td>
<td>MLA-EcIFM</td>
<td>EcSF*</td>
<td>EcSF**</td>
</tr>
<tr>
<td>Initial temperature$^c$</td>
<td>4°C</td>
<td>12°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Dry FRMS types:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Northern Europe</td>
<td>1.46</td>
<td>1.46</td>
<td>0.46</td>
</tr>
<tr>
<td>Southern Europe</td>
<td>3.29</td>
<td>3.29</td>
<td>1.05</td>
</tr>
<tr>
<td>Southern Europe (traditional)</td>
<td>6.55</td>
<td>6.55</td>
<td>2.13</td>
</tr>
<tr>
<td>Semi-dry FRMS types:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Northern America</td>
<td>1.41</td>
<td>1.41</td>
<td>0.43</td>
</tr>
<tr>
<td>Northern Europe</td>
<td>0.45</td>
<td>0.45</td>
<td>0.11</td>
</tr>
</tbody>
</table>

$^a$ See flow diagrams in Fig 4.

$^b$ Possible scenarios for meat processing according to Fig 4, cold and hot cutting and boning and 6 and 15 days at 7°C before preparation.

$^c$ Initial temperatures of the meat used in FRMS manufacturing according to Fig 4.

$^d$ Δ denotes increase in concentration (log$_{10}$ cfu/g)

*In F2 simulation with EcSF: The final value for lactic acid is 4657 ppm as reported in MLA- EcIFM documentation; $a_w$ was assumed to decrease at a rate of 0.014 units per day during the drying step.

**In F1+F2 simulation with EcSF: the final concentration of lactic acid is the average final value observed in the sausages manufactured in the pilot plant in this work, i.e. 25850 ppm.
\[
\ln \varphi = -7999.2 \times \frac{1}{\text{Temp}} + 23.4
\]

\( R^2 = 0.6163 \)
F1. Flow diagram of meat processing

Scenario A. Boning and cutting after refrigeration

(a) Post-mortem inspection after slaughter (time 0 h; carcass at ca. 40°C)

(b) Refrigeration time for the surface of the carcass (2-3 h for achieving 7°C)

(c) Refrigeration time for the interior of the carcass (maximum 24h for achieving <7°C)

(d) Boning and cutting (ca. 1 h)

(e.1.) Elaboration of minced meat (MM) after the slaughter (maximum 6 days for animals other than poultry). 7°C

(f.1.) Immediate refrigeration (2°C), storage and transport of MM

(e.2.) Elaboration of meat preparations (MP) after the slaughter (maximum 15 days for boned, vacuum-packed beef and veal meats). 7°C

(f.2.) Immediate refrigeration (4°C), storage and transport of MP

Scenario B. Hot boning and cutting

F2. Flow diagram of FRMS elaboration

Meat at 4 and 12°C

Dry FRMS types

- Northern Europe
  - 25°C, 33 h
  - 4.6-5.1
  - 0.95
  - 4657-25850

- Southern Europe
  - 23.5°C, 2.5 days
  - 5.1-5.5
  - 0.95
  - 4657-25850

Semi-dry FRMS types

- Northern America
  - 34°C, 17.5 h
  - 4.4-5.0
  - 0.95
  - 4657-25850

- Northern Europe
  - 26°C, 33 h
  - 4.4-5.0
  - 0.95
  - 4657-25850

Fermentation conditions:
- Final pH: 4.6-5.1
- Final a_w: 0.95
- Lactic acid (ppm): 4657-25850

Drying conditions:
- Final pH: 4.6-5.1
- Final a_w: 0.80-0.91
- Lactic acid (ppm): 4657-25850