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Thermal inactivation kinetics of Shiga toxin-producing *Escherichia coli* in buffalo Mozzarella curd

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ABSTRACT

The use of raw milk in the processing of buffalo Mozzarella cheese is permitted, but the heat treatment used for stretching the curd must ensure that the final product does not contain pathogens such as Shiga toxin-producing *Escherichia coli* (STEC) that may be present on buffalo dairy farms. This study carried out challenge tests at temperatures between 68°C and 80°C for 2 to 10 min to simulate curd temperatures during the stretching phase. Curd samples were inoculated with 2 STEC strains (serotypes O157 and O26), and their inactivation rates were assessed in the different challenge tests. The curd samples were digested with papain to ensure a homogeneous dispersion of bacteria. The STEC cells were counted after inoculation (range 7.1–8.7 log cfu/g) and after heat treatments using the most probable number (MPN) technique. A plot of log MPN/g versus time was created for each separate experiment. The log linear model with tail was used to provide a reasonable fit to observed data. Maximum inactivation rate (k_{max}, min^{-1}), residual population (log MPN/g), decimal reduction time (min), and time for a 4D (4-$\log_{10}$) reduction (min) were estimated at each temperature tested. A 4D reduction of the O26 STEC strain was achieved when curd was heated at 68°C for 2.6 to 6.3 min or at 80°C for 2.1 to 2.3 min. Greater resistance was observed for the O157 strain at 68°C because k_{max} was 1.48 min^{-1}. The model estimates can support cheesemakers in defining appropriate process criteria needed to control possible STEC contamination in raw milk intended for the production of Mozzarella.
Key words: Shiga toxin-producing *Escherichia coli*, Mozzarella, thermal inactivation rate, curd spinning

INTRODUCTION

The effectiveness of thermal treatments used in the production of typical Italian Mozzarella cheese (i.e., Mozzarella di bufala Campana) must be evaluated to give an adequate guarantee of the microbiological safety of the final product because it is made using raw buffalo milk. Because the organoleptic properties of Mozzarella are enhanced by the use of raw milk, the guidelines developed by the Consortium for the Protection of the Buffalo Cheese of Campania for the protection of typical Mozzarella di bufala Campana specify that milk is not to be treated at high temperatures before fermentation. In the traditional process, raw milk is inoculated and acidified with natural starters and coagulated with the addition of calf rennet broken into nut-sized pieces, and the acidified curd is separated from the serum and cut into pieces (15 to 20 cm thick). The curd mass is usually left at room temperature for 3 to 4 h, until the fermentation process reduces the pH to around 5.2, at which point the cheese can be stretched. Pasta filata is made in hot water (85–95°C), and the thermal treatment is controlled empirically by cheesemakers on the basis of the melting and stretching properties of the curd. Although producers believe this thermal treatment has a potentially antibacterial effect equivalent to pasteurization, standardized performance criteria (Gorris, 2004) must be defined to guarantee microbiological safety of the resultant cheese. Challenge tests carried out using pathogenic bacteria found in raw milk are needed to ensure that the heat treatment used for curd stretching is appropriate and that the maximum frequency and concentration of microbial hazards after this process comply with established microbiological criteria.

Microbial contamination of raw milk by Shiga toxin-producing *Escherichia coli* (**STEC**) has been reported in several areas, including buffalo herds in southern Italy (Galiero et al., 2005; Borriello et al., 2012). The carriage rates observed in the period from 2002 to 2003 were 32.3 and 14.5% of the herds and animals sampled respectively, and serotype O157:H7 isolates were de-

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tected (Galiero et al., 2005). Similarly, a study carried out from 2006 to 2009 showed that 6.8% of *E. coli* isolates detected in water buffalo calves with diarrheic syndrome were characterized as enterohemorrhagic *E. coli* (**EHEC**), possessing *stx1*, *eae*, and *HlyA* genes (Borriello et al., 2012). In 2005, 2 STEC clusters, each with 3 cases of hemolytic uremic syndrome due to serotype O26 were observed in June and September (Scavia et al., 2005). In both clusters, the common and highly probable risk factor was the consumption of fresh dairy products (e.g., Mozzarella) purchased from a local producer in the province of Salerno (southern Italy). The European Union's rapid alert system (Rapid Alert System for Food and Feed; RASFF) has never reported any alert or notification of STEC contamination of Mozzarella and the EnterNet surveillance network (the European Union's Internet system for the surveillance of *Salmonella* and STEC) has not reported any cases of hemolytic uremic syndrome or hemorrhagic colitis correlated with consumption of Mozzarella produced in Italy. Periodic monitoring of milk, in-line milk filters, and feces is useful but does not eliminate the risk. Thus, microbiological specifications for fresh milk and critical control points in the Mozzarella production process have to be defined.

In addition, thermal inactivation models could be useful to estimate process criteria during the stretching phase of Mozzarella cheese. The optimum stretching temperature to avoid breaking buffalo Mozzarella is approximately 68 to 70°C. Although the water temperature may be as high as 85 to 95°C, the interior curd mass fails to reach a higher temperature because the stretching phase must not be too long to avoid loss of fat and soluble proteins. Subsequent rapid chilling and packaging help to preserve the final product.

Four types of survival curves are commonly observed in thermal inactivation of vegetative microorganisms: linear curves, curves with a shoulder and curves with a tail (both biphasic curves), and sigmoidal curves. The first-order kinetic model for linear survival curves can be modeled by the equation $\text{Log } N(t)/N(0) = 1 - t/\text{D}$ $(t \geq 0)$, where $N(t)$ and $N(0)$ are the concentrations present at time t and zero, respectively, and D is the decimal reduction time. This method entails estimating the decimal reduction times (D-values) using linear regression to construct the straight line of best fit on a semilog plot of survivors versus time (survivor curve). This D-value is correlated with temperature and is considered characteristic for each microorganism and food matrix. Survival patterns may vary with the physiological state of the cells, growth phase (exponential or stationary phase), and preincubation conditions before applying the stress (Berney et al., 2006; van Asselt and Zwietering, 2006).

A heterogeneous stress response in clonal populations is widely observed for a range of environmental stresses, such as heat (Van Derlinden et al., 2009), acid (Steels et al., 2000), and osmotic stress or salt (den Besten et al., 2007). This nonlinear behavior is based on different resistant subpopulation patterns found in *E. coli* species in various food matrices (Álvarez et al., 2003; Aragao et al., 2007; Patil et al., 2009). Stringer et al. (2000) and van Boekel (2002) summarized additional explanations for this deviation from linearity, such as variability in heating procedure, use of mixed cultures, clumping or protective effect of the food matrix, or dead cells.

The aims of this study were to evaluate the thermal inactivation of STEC serotypes in buffalo curd at different stretching temperatures and to implement first-order thermal inactivation models to describe bacterial resistance. The experimental design was specifically aimed at assessing the effect of thermal treatments on STEC strains in mature curd. The STEC were counted by the most probable number (**MPN**)-PCR method, which allows the use of nonselective media that could hamper the detection of stressed microbial cells. Inoculum concentration was high (much higher than the natural contamination in milk) to evaluate the kinetic behavior of pathogens during inactivation, and to develop predictive models.

MATERIALS AND METHODS

Strains and Culture Conditions

Two STEC strains isolated in 2011 were used in this study. One *E. coli* O157 strain (*stx1*+, *stx2*+, *eae*+, *Hly*+, not fermenting sorbitol) was isolated from an in-line milk filter on a buffalo farm and one *E. coli* O26 strain (*stx1*+, *eae*+, not fermenting rhamnose) was isolated from cow milk. Cultures were prepared for each individual batch experiment from the same cryovials (O157/O26) stored at −80°C by streaking on trypticase soy agar plates. After 15 to 18 h of incubation at 37°C, one colony was picked and inoculated in buffered peptone water (**BPW**), and cultures were incubated at 37°C for 18 to 24 h. The transmittance (560 nm) of cultures was standardized at 40% to have a concentration of approximately 10^8 cfu/mL and this standard culture was used to spike the curd used for the challenge tests. The number of bacteria in the standardized BPW cultures was counted on trypticase soy agar plates (after incubation at 37°C for 24 h).

Samples

Curd loaves (slices cut about 15 cm thick) for individual batch experiments were taken from a local

artisanal cheese plant producing Mozzarella. The curd loaves were brought to the laboratory in an insulated box within 5 min and stored at room temperature until the pH was between 5.1 and 5.3. One-gram samples were weighed in Eppendorf tubes and used for the challenge tests.

Isothermal Inactivation Experiments

Challenge tests at different temperatures with the 2 strains were carried out on different days and repeated twice using different curd batches. Experiments were carried out at constant temperature (68, 70, 73, 77, and 80°C) to evaluate the temperature dependence of the thermal inactivation rate. For each test, thirteen 1-g curd samples (in Eppendorf tubes) were put in a thermoblock (Thermostat Plus, Eppendorf, Germany) and left for 20 to 30 min until the temperature set for the experiment was reached. The temperature was monitored continuously in one of these tubes with a MIG thermometer (Amarell GmbH & Co. KG, Kreuzwertheim, Germany). Two samples were then chilled in an ice bath and used as controls; they were inoculated with 100 μL of the STEC cultures after cooling. The remaining 10 tubes were held in the thermoblock and spiked with the same STEC cultures. After 2, 3, 5, 7, and 10 min, 2 samples were removed from the thermoblock and immediately chilled in an ice bath. The experiments were carried out in subsequent steps to ensure appropriate time control. It was not possible to ensure a homogeneous distribution of contamination in the melted curd matrix, but inoculation was done with a micropipette at the core of each tube and dispersed, as far as possible, by swirling the tip in the melted curd for 2 to 3 s.

Microbiological Analyses of Curd Samples

After chilling, the thermally treated samples were transferred to Stomacher bags (Seward Ltd., Worthing, UK). For each challenge test, the 2 replicates (i.e., the samples that received the same heat treatment or the controls) were put in the same bag. The curd samples were digested using papain as indicated for the pretreatments of insoluble proteinaceous food samples in the protocol for the enumeration methods by using the membrane filtration MPN method (Entis and Lerner, 1998; AOAC International, 2000). To this end, a 1-mL solution (10% wt/vol) of papain (Isogrid, Acumedia, Lansing, MI) was placed into the Stomacher bags, leaving the 2-g curd samples broken down into small fragments, and incubating at 37°C for 30 min. The digested curd samples were then diluted (1:10) with 47 mL of BPW and homogenized with a Stomacher for 2 min. Viable STEC in the heat-treated and control samples were counted with the MPN-PCR method (Figure 1). Ten-milliliter samples of homogenates and subsequent serial dilutions (1:10, 1:100, 1:1,000 in BPW) in triplicate were incubated at 37°C for 24 h. Bacterial growth in the BPW tubes was evaluated, and PCR was used to confirm the presence of STEC targets (O157 or O26) using the methods described by Perelle et al. (2004). Cultures from the PCR-positive tubes were then streaked on sorbitol MacConkey and rhamnose MacConkey agar plates to confirm their viability. Isolated colonies showing typical morphology (sorbitol-negative O157 or rhamnose-negative O26) were also confirmed using specific latex agglutination tests.

Data Analysis and Modeling Approach

A plot of log MPN/g versus time (min) was created for each separate experiment in Excel 2010 (Microsoft Corp., Redmond, WA). Different inactivation models were tested; for example, Weibull, biphasic, log linear, and we concluded that, given the characterized behavior of the serotypes at different temperatures, the log linear model with tail produced a reasonable fit to the observed data (Geeraerd et al., 2000). This model estimates the microbial concentration at time t, log $N(t)$, as follows:

$$\log N(t) = \log\left[\left(10^{\log N0} - 10^{\log Nres}\right)\right] \cdot e^{-k\max \cdot t} + 10^{\log Nres}, \quad [1]$$

where $k_{\max}$ = maximum inactivation rate ($\min^{-1}$); N_0 = initial contamination level (log MPN/g); N_{res} = residual subpopulation (log MPN/g; and t = treatment time (min). The kinetic parameters were calculated using the freeware tool GINaFit (Geeraerd et al., 2005).

The D-values (i.e., time needed for a 1-$\log_{10}$ reduction in viability) were calculated from $k_{\max}$ according to the equation

$$D = \frac{1}{k_{\max}} \cdot \ln(10). \quad [2]$$

For each temperature tested, the time needed to produce a 4D reduction (**TT-4D**, min) was further calculated.

The statistical goodness-of-fit indices root mean square error and coefficient of determination (R^2) were determined to assess the goodness-of-fit of the model. An ANOVA was performed to evaluate significant differences between the temperatures tested ($P < 0.05$). Statistica for Windows v10.0 (StatSoft Iberica, Lisbon, Portugal) and Microsoft Excel 2010 (Microsoft Corp.) were used for all calculations, regression procedures, and statistical analysis.

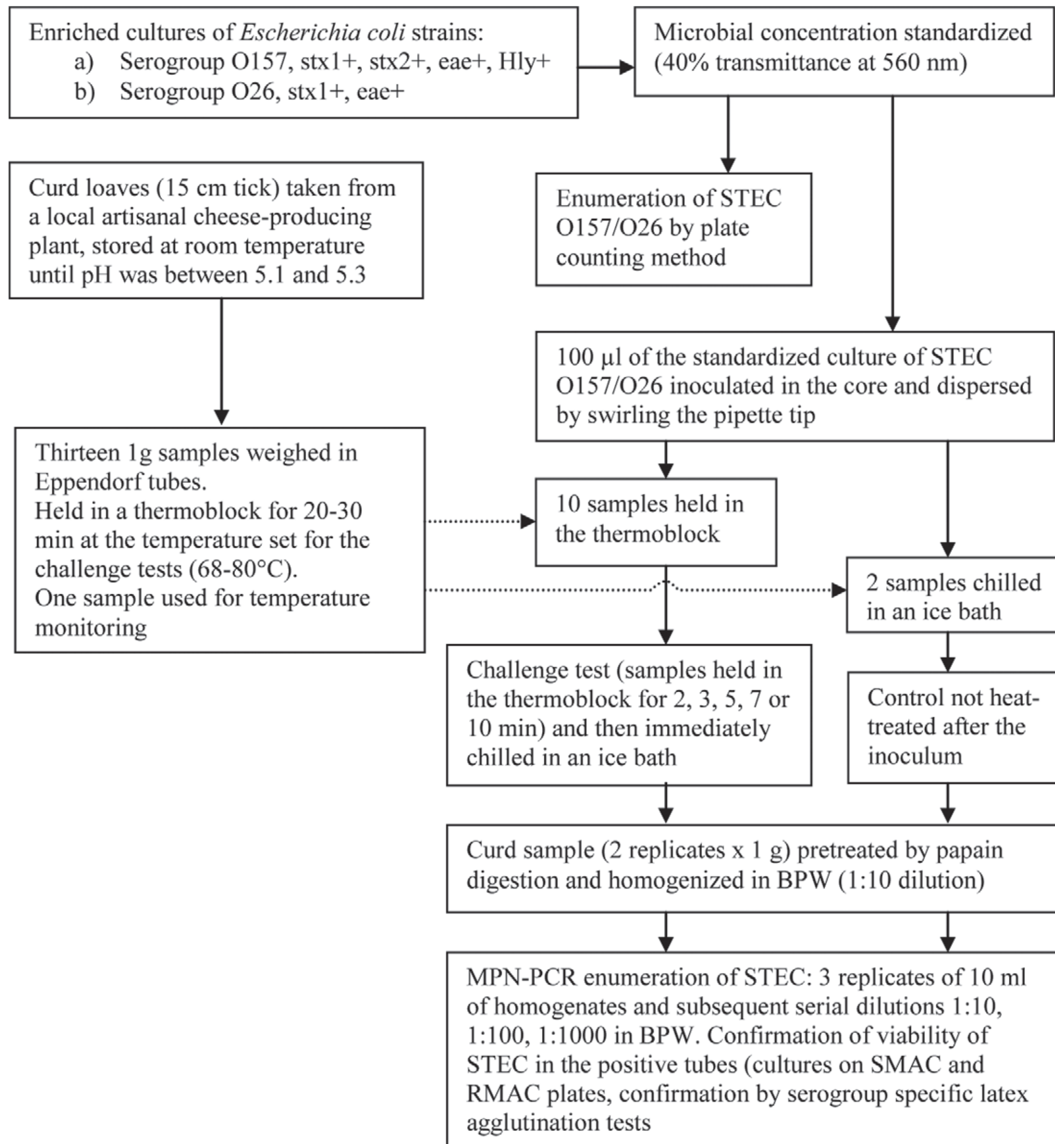

Figure 1. Applied methodology for the performance of viable Shiga toxin-producing *Escherichia coli* (STEC) serotypes O26 and O157 with most probable number (MPN)-PCR method. BPW = buffered peptone water; SMAC = sorbitol MacConkey agar; RMAC = rhamnose MacConkey agar.

RESULTS

The initial STEC counts ranged between 7.2 and 7.7 mean log MPN/g for the curd samples inoculated with the strain of serogroup O157 and in the range from 7.1 to 8.7 log MPN/g for those inoculated with the strain of serogroup O26. The pH value of mature curd ranged from 4.93 to 5.05. Survivor curves from thermal experiments at 68 to 80°C are shown in Figure 2 for *E. coli* O26:H11 and in Figure 3 for *E. coli* O157:H7. These figures show the data relative to microbial counts (log MPN/g) and the curves that best fitted the data. Average TT-4D values for *E. coli* O26 and O157 in buffalo curd at a temperature range of 68 to 80°C were 2.1 to 2.6 min and 2.1 to 6.3 min, respectively. Log-linear curves with tails were fitted to the observed data for both strains, with the exception of O157 at 68°C, in which a linear curve was fitted. The coefficients of determination (R^2) of the regression curves were always >0.89 at temperatures of 68 to 80°C (Table 1). A good adjustment was obtained with the model used because R^2 values were >0.87. For *E. coli* O26, k_{max} increased from 3.94 to 4.50 min^{-1} at 68 and 80°C, respectively. Higher resistance was observed for *E. coli* O157 at 68°C, because k_{max} was 1.48 min^{-1} (Table 1). Application of high temperatures (80°C) during the stretching

phase produced a residual population (N_{res}) lower than 10 MPN/g for both strains at the end of the treatment. Significant differences were noted ($P < 0.05$) for N_{res} between the temperatures tested for both strains, which implied the presence of resistant subpopulations of *E. coli* at temperatures <73°C. Results indicated that at 80°C, STEC O26 and O157 did not fall below the quantification limit (<0.08 MPN/g) 10 min after inoculation (Figures 2 and 3). The viable count reduction for O26 and O157 STEC ranged from 3 to 7.74 $\log_{10}$ MPN/g and from 2.72 to 7.11 log MPN/g, respectively, starting from an initial concentration of approximately 10^7 to 10^8 MPN/g. The experiments showed a reduction of 7.08 to 7.11 and 6.30 to 7.11 log MPN/g after 10 min at 77 and 80°C. The difference between initial MPN counts (lower 95% CI value) and the upper 95% CI value of MPN counts at 80°C ranged between 4.98 and 5.70 for O157 and O26 STEC, respectively (Figures 2 and 3).

DISCUSSION

Pasteurization is the traditional method of inactivating pathogenic microorganisms in milk, and several studies have shown that *E. coli* O157:H7 is rapidly inactivated at subpasteurization temperature (i.e., 64.5°C) and a holding time of 16.2 s (D'Aoust et al., 1988; Stringer et al., 2000). Other studies confirmed these data, showing a 5-log reduction of *E. coli* O157:H7 in milk by heat treatment at 64.58°C for 17.5 s (Schlesser et al., 2006). However, data on thermal inactivation in milk cannot be translated directly to milk products because factors such as water activity, pH, or chemical composition (e.g., fat content) may affect the thermal inactivation kinetics of microorganisms. This finding is correlated with an increased heat resistance for *E. coli* and *Salmonella* spp. (Line et al. 1991; Ahmed et al., 1995). The protective effect of fat on bacterial cells against the lethal effect of heat was attributed to the reduction in water activity (Jay, 2000) or to an insufficient heat penetration through the heating solvent (Ahmed et al., 1995). Other components such as low-molecular-weight solutes (lactose and salts) have also been indicated as factors affecting the thermal inactivation of *Salmonella* in milk and dairy products (Kornacki and Marth 1993). Few papers concerning production of fresh pasta filata cheese (e.g., Fior di Latte) have been published and results have been conflicting (Spano et al., 2003; Fusco et al., 2012). Spano et al. (2003) observed that stretching curd made from raw cow milk in hot water at 80°C for 5 min resulted in a reduction of approximately 5 log cfu/mL of *E. coli* O157:H7, whereas the reduction was much lower (approximately 1 log) when working the curd in hot water at 70°C. Fusco et al. (2012) reported that the stretching of curd in hot water (90°C) produced a reduction of 1.96 log MPN/g and 1.93 log MPN/g of the total cell load (viable and stressed) for *E. coli* O157:H7, but the estimated temperature in the core of the curd was around 55°C remaining in hot water for 5 to 8 min. The use of selective plating media containing bile salts (i.e., sorbitol MacConkey agar) in the study of Spano et al. (2003) might have influenced the results of counts of the heat-stressed O157 STEC (Oliver, 2010). The MPN-PCR method has been considered a good means of counting both viable and stressed bacteria if the enrichment culture is done in nonselective media (Vimont et al., 2007; Jasson et al., 2009). Confirmation of PCR results by isolation and serological characterization help to avoid false-positive results due to the presence of DNA from dead *E. coli* cells. The challenge tests carried out in this study processed both heat-treated and non-heat-treated samples by papain digestion. The incubation time (30 min at 37°C) was too short to determine appreciable changes in the number of *E. coli*, and the possible reduction of the adaptation growth phase may not negatively affect the MPN enumeration of *E. coli.* Procedures for counting bacteria by the MPN membrane filtration method have been granted AOAC Official Method status (method 997.1; AOAC International, 2000) and use enzymatic treatments to make food samples filterable. The use of papain was specifically indicated for the digestion of cheese samples in a comparative study aimed at enumerating *E. coli* (Entis and Lerner, 1998). These studies proved that the digestion method did not negatively affect the enumeration of *E. coli.*

A time of 2.1 min at 80°C or 3.2 min at 70°C was required to ensure a 4-log reduction of STEC O157 and these TT-4D are also appropriate for STEC O26. However, to take into account the uncertainty of MPN counts, 5 to 7 min at 77°C is the minimum time-temperature combination consistently allowing a 4D reduction. A reduction of 6.3 log MPN/g of *E. coli* O157:H7 was observed after 5 min and similar results were recorded after 10 min. These results are indicative of a subpopulation of O157:H7 *E. coli* that had greater thermal resistance. Moreover, due to the uncertainty of MPN counts, a reduction of 5 log MPN/g can be ensured (with 95% probability) after 7 to 10 min at 80°C (core temperature).

Significant deviations were observed from the log-linear declines (logarithmic survivor curves) with tailing. Because the curd was preheated to temperatures used for the isothermal inactivation experiments, poor heat penetration through the curd should be excluded. Therefore, the tail in the survivor curves may be attributed to a subpopulation of more resistant bacteria

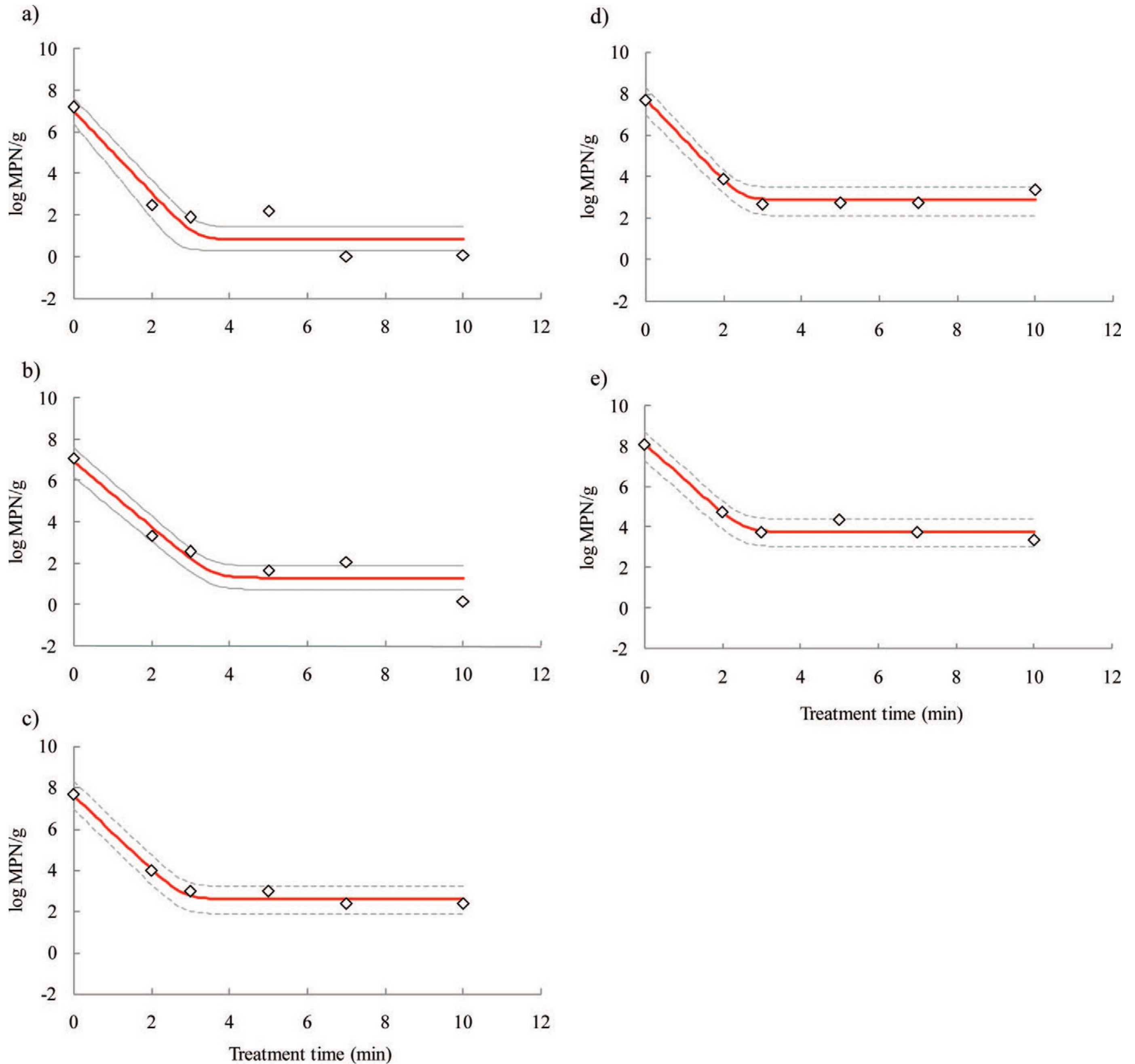

Figure 2. Fitted log linear model with tail models for the inactivation of Shiga toxin-producing *Escherichia coli* (STEC) serotype O26 at 80°C (a), 77°C (b), 73°C (c), 70°C (d), and 67°C (e). Open symbols represent the mean observed data; solid (red) lines represent the fitted mean values; dashed lines correspond to the fit of the upper and lower 95% CI. MPN = most probable number. Color version available in the online PDF.

that decline at a slower rate than the majority of cells (Juneja et al., 1997). These heat resistance patterns have already been reported for *E. coli* serotypes (Cornet et al., 2011; Velliou et al., 2011; Noriega et al., 2013). Van Derlinden et al. (2011) hypothesized that one subpopulation is able to overcome the environmental stress, whereas a second is unable to resist stress and hence is destroyed.

Non-logarithmic survivor curves (sigmoidal curves) are of public health significance. Although the exact infectious dose for STEC is unknown, it is thought to be fewer than 100 organisms, but the estimated attack rate increases the higher the number (Strachan et al., 2005; Duffy et al., 2006).

As demonstrated in the present study, a residual population of the STEC evaluated implies that high

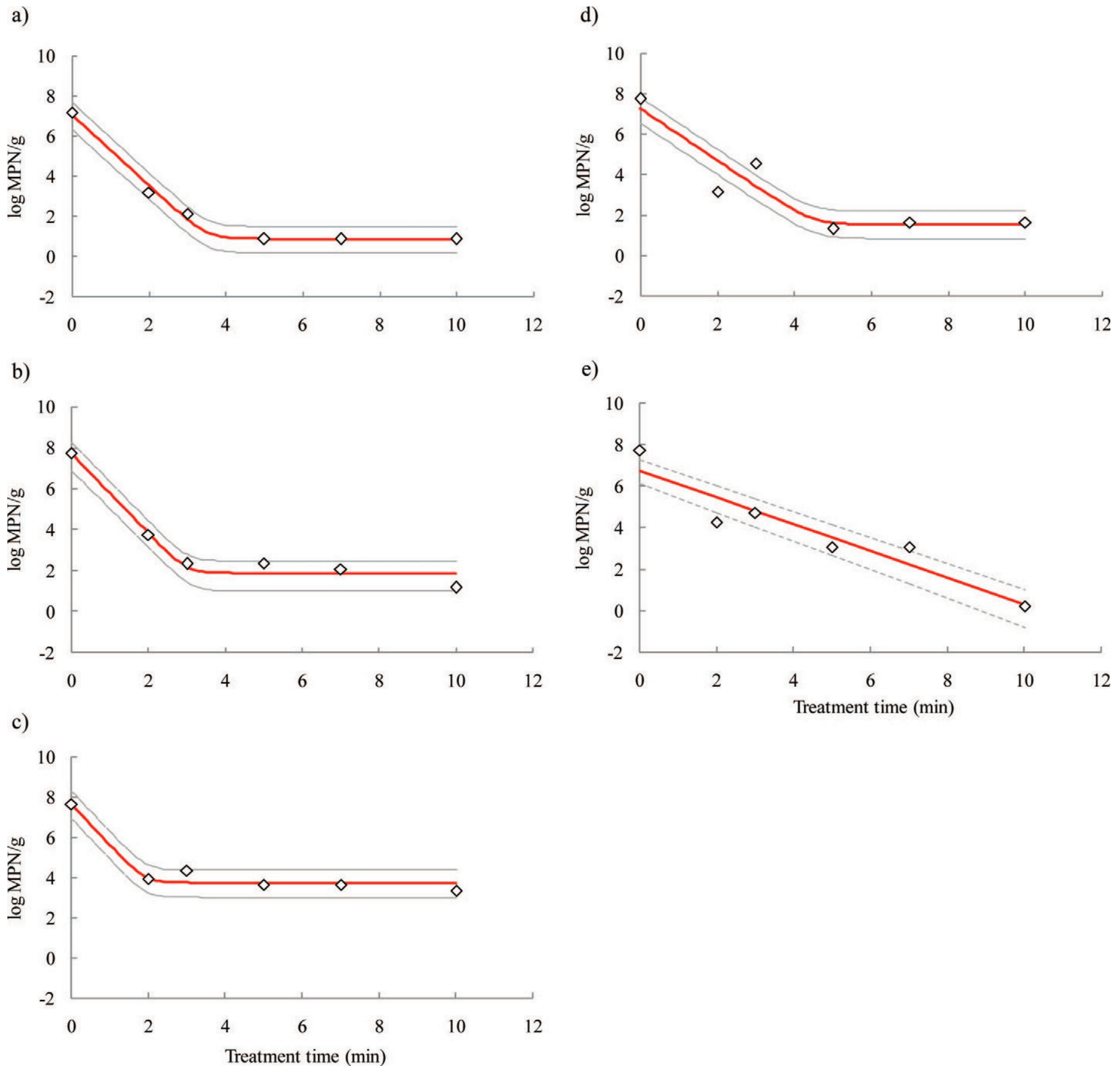

Figure 3. Fitted log linear model with tail models for the inactivation of Shiga toxin-producing *Escherichia coli* (STEC) serotype O157 at 80°C (a), 77°C (b), 73°C (c), 70°C (d), and 67°C (e). Open symbols represent the mean observed data; solid (red) lines represent the fitted mean values; dashed lines correspond to the fit of the upper and lower 95% CI. MPN = most probable number. Color version available in the online PDF.

contamination of the raw material could lead to the survival of *E. coli* cells after standardized thermal treatment. Raw milk is needed to preserve the organoleptic quality of buffalo Mozzarella cheese, but adequate control at primary production and by suppliers is essential. The maximum number and prevalence of STEC in bulk raw milk used to produce Mozzarella (a farmer's performance objective) and the thermal inactivation time and temperature ensured by the curd spinning step (a producer's process criterion) have to be monitored to ensure that the potential hazards due to the presence of STEC in buffalo or cow farms are under control.

Table 1. Kinetic parameters (SE in parentheses) estimated with the log linear model with tail model for the Shiga toxin-producing *Escherichia coli* serotypes O26 and O157

Serotype	Temperature (°C)	Inactivation parameter[1]			RMSE[2]	R^2	TT-4D[2] (min)
		k_{max} (min^{-1})	N_0 (log MPN/g)	N_{res} (log MPN/g)			
E. coli O26	67	3.94 (0.72)[b]	8.09 (0.42)[a]	3.79 (0.22)[a]	0.42	0.97	2.6[a]
	70	4.56 (0.57)[a]	7.75 (0.33)[a]	2.87 (0.17)[b]	0.33	0.98	2.1[a]
	73	4.13 (0.49)[ab]	7.63 (0.31)[a]	2.61 (0.17)[b]	0.32	0.99	2.3[a]
	77	3.64 (0.98)[b]	6.93 (0.85)[b]	1.31 (0.51)[c]	0.88	0.92	2.6[a]
	80	4.50 (1.41)[a]	6.99 (1.10)[ab]	0.82 (0.65)[d]	1.13	0.89	2.1[a]
E. coli O157	67	1.48 (0.26)[c]	6.75 (0.63)[a]	NA[3]	0.91	0.89	6.3[a]
	70	2.94 (1.21)[bc]	7.26 (1.11)[a]	1.55 (0.71)[b]	1.16	0.87	3.2[b]
	73	4.71 (1.41)[a]	7.66 (0.43)[a]	3.77 (0.21)[a]	0.43	0.96	NA
	77	4.45 (0.71)[a]	7.71 (0.51)[a]	1.89 (0.29)[b]	0.52	0.97	2.1[c]
	80	4.02 (0.32)[ab]	7.06 (0.28)[a]	0.89 (0.16)[c]	0.29	0.99	2.3[c]

[a–d]Values with different letters in the same column indicate significant differences ($P < 0.05$).

[1]k_{max} = maximum inactivation rate; N_0 = initial contamination level, log MPN/g, where MPN = most probable number; N_{res} = residual subpopulation, log MPN/g.

[2]RMSE = root mean square error; TT-4D (time to 4D, or 4 $\log_{10}$, reduction, min).

[3]Not applicable.

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