A Predictive Model for Heat Inactivation of
Listeria monocytogenes Biofilm on Stainless Steel

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ABSTRACT

Heat treatment of potential biofilm-forming sites is sometimes used for control of Listeria monocytogenes in food processing plants. However, little information is available on the heat treatment required to kill L. monocytogenes present in biofilms. The purpose of this study was to develop a predictive model for the heat inactivation of L. monocytogenes in monoculture biofilms (strains Scott A and 3990) and in biofilms with competing bacteria (Pseudomonas sp. and Pantoea agglomerans) formed on stainless steel in the presence of food-derived soil. Biofilms were produced on stainless steel coupons with diluted tryptic soy broth incubated for 48 h at 25°C. Duplicate biofilm samples were heat treated for 1, 3, 5, and 15 min at 70, 72, 75, 77, and 80°C and tested for survivors using enrichment culture. The experiment was repeated six times. A predictive model was developed using logistic regression analysis of the fraction negative data. Plots showing the probability of L. monocytogenes inactivation in biofilms after heat treatment were generated from the predictive equation. The predictive model revealed that hot water sanitation of stainless steel can be effective for inactivating L. monocytogenes in a biofilm on stainless steel if time and temperature are controlled. For example, to obtain a 75% probability of total inactivation of L. monocytogenes 3990 biofilm, a heat treatment of 80°C for 11.7 min is required. The model provides processors with a risk management tool that provides predicted probabilities of L. monocytogenes inactivation and allows a choice of three heat resistance assumptions. The predictive model was validated using a five-strain cocktail of L. monocytogenes in the presence of food soil.

Sporadic outbreaks of listeriosis due to the consumption of contaminated ready-to-eat meats have raised concern that recontamination of products may occur after processing. One possible cause of recontamination is ineffective cleaning of the food processing plant (7). Food contact surfaces and areas in processing lines such as joints, butterfly valves, pitted surfaces, equipment gaskets, and dead ends, and nonfood contact surfaces such as drains, walls, ceiling condensate, equipment surfaces, and hand trucks are all potential sources of Listeria contamination (4, 6, 13, 20). Listeria growing on equipment surfaces is difficult to remove and exhibits increased resistance to biocides (9, 18, 19). Microorganisms are more difficult to remove from abraded than from smooth surfaces (14) and are more difficult to remove from rubber than from stainless steel (11, 25). Biofilm cells are more resistant to biocide after 7 days of growth than after 2 days (15, 18).

Biofilms in open environments usually consist of heterogeneous microbial populations coexisting in specific microscopic niches. Previous biofilm formation by one species may provide a niche for another species (1); this problem can be especially significant in areas such as drains, where a nonpathogenic species can take residence and develop a biofilm and a pathogen such as Listeria can then establish residence in the preexisting biofilm. Biofilms are probably more difficult to remove when formed in the presence of food residues. Such biofilms can occur on equipment that contains niches where nutrients can accumulate and chemical cleaning agents cannot adequately access or on equipment that is not designed for easy disassembly to allow daily cleaning. This equipment may be heat treated after cleaning to kill residual Listeria.

Heat treatments for effective killing of Listeria monocytogenes present in biofilms have not been defined. In this study, we used fraction negative data analyzed using nonlinear logistic analysis to predict the inactivation of L. monocytogenes in biofilms. The objective of this research was to develop predictive models for the heat inactivation of L. monocytogenes in monoculture and in multispecies biofilms on stainless steel. The effect of adding soil to the biofilm after heat inactivation was also determined.

MATERIALS AND METHODS

Surface preparation. Stainless steel (type 304, no. 4 finish) was cut into 10-cm² (5 by 2 cm) coupons. Coupons were degreased in acetone, cleaned by sonication (Aquasonic model 550HT, VWR Scientific, Atlanta, Ga.) for 60 min at 55°C in 2 g/liter alkali detergent (Micro International Products Corp., Burlington, N.J.), rinsed with deionized water, sonicated again for 20 min in 30 g/liter phosphoric acid–based cleaner (Formula 3586, Zep Manufacturing, Atlanta, Ga.), and then rinsed three times in deionized water. Coupons were autoclaved in deionized water before use.

Culture source. Microorganisms used in this study are listed in Table 1. Thirty strains of L. monocytogenes (serotypes 4b and

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TABLE 1. Microbes used in biofilm formation on stainless steel

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Serovar</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Listeria monocytogenes</td>
<td>4b</td>
<td>Human clinical</td>
</tr>
<tr>
<td>L. monocytogenes 3990</td>
<td>4b</td>
<td>Vacherin Mont-d’Or cheese</td>
</tr>
<tr>
<td>L. monocytogenes YM 96</td>
<td>1/2a</td>
<td>Monkey environment</td>
</tr>
<tr>
<td>L. monocytogenes 303</td>
<td>1/2a</td>
<td>Monkey clinical</td>
</tr>
<tr>
<td>L. monocytogenes 17</td>
<td>4b</td>
<td>Food processing plant environmen</td>
</tr>
<tr>
<td>Pseudomonas spp. M21</td>
<td></td>
<td>Food processing plant environmen</td>
</tr>
<tr>
<td>Pantoea agglomerans</td>
<td></td>
<td>Food processing plant environmen</td>
</tr>
</tbody>
</table>

*a* All obtained from the Center for Food Safety, Griffin, Ga.

I/2a) were evaluated for biofilm formation, and five were selected for this study based on their ability to form biofilms and their heat tolerance as evaluated in preliminary experiments (data not shown). *Pseudomonas* sp. and *Pantoae agglomerans* used in this study were environmental isolates from a food processing plant. *L. monocytogenes* strains Scott A and 3990 readily produced biofilms and were heat tolerant and therefore selected to be the basis of the predictive model. *L. monocytogenes* strains YM96 and 303, which were moderate biofilm producers, and *L. monocytogenes* 17, which produced minimal biofilms, were used in the validation study along with the *L. monocytogenes* Scott A and 3990 strains.

**Biofilm preparation.** In the predictive study, cultures were activated from frozen beads (Microbank, Prolab Diagnostics, Austin, Tex.) in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) for 18 h at 32°C. Biofilms were produced by immersing stainless steel coupons in a 1:10 dilution of TSB (dTSB) for 4 h at 25°C containing inoculum of 10^8 cells/ml. Monoculture inocula of *L. monocytogenes* consisted of strains Scott A and 3990 (cultures LM Scott A and LM 3990, respectively). *Pseudomonas* sp. and *P. agglomerans* were also used to produce monoculture biofilms. Two multispecies inocula were used; the first consisted of a mixture of four parts of *L. monocytogenes* Scott A to one part of *Pseudomonas* sp. broth cultures (culture LMPs), and the second consisted of eight parts of *L. monocytogenes* 3990 to one part *Pseudomonas* sp. and one part *P. agglomerans* broth cultures (culture LMPSP). These combinations were selected based on preliminary data indicating the ability of the microorganisms to grow and maintain a viable population in a biofilm. Following a 4-h attachment period, coupons were rinsed with phosphate buffer (0.01 M, pH 7.0), transferred to dTSB (1:10), and incubated for 48 h at 25°C. Biofilms formed from this procedure were used for the low-soil condition.

**Preparation of biofilm with soil.** The soil was formulated by emulsifying rendered chicken fat (137.7 mg/ml), sterile chicken serum (211 to 338 mg protein per ml; C-5405, Sigma, St. Louis, Mo.), and lecithin (0.05 mg/ml; P-5638, Sigma). The soil mixture was emulsified for 1 min with an electric hand blender (Braun, Lynmouth, Mass.) that was disinfected (70% ethanol vol/vol) prior to use. Stainless steel coupons containing biofilm were dipped in the soil then placed in disposable centrifuge tubes and incubated at 10°C overnight (18 h). This preparation contained approximately 15 mg of soil per cm² and was used in experiments calling for soiled biofilm. This soil mixture was not sterile but contributed less than 10 CFU/cm² to the initial microbial load.

**Biofilm preparation for model validation.** Sterile stainless steel coupons were submerged into broth containing a five-strain cocktail of *L. monocytogenes* strains Scott A, 3990, 17, YM 96, and 303 present in equal portions and prepared as previously described. Following a 4-h attachment period, coupons were rinsed with phosphate buffer (0.01 M, pH 7.0), transferred to dTSB (1:10), and incubated for 48 h at 25°C. Biofilms were soiled using the previously described procedure.

**Analysis of unheated and uninoculated controls.** To analyze inoculated (positive) and uninoculated (negative) controls, the appropriate coupons were rinsed with phosphate buffer (0.01 M, pH 7.0), placed in 0.1% (wt/vol) peptone with glass beads (3 g per ml; 450-600, Sigma), and shaken on a wrist action shaker (model 75 wrist action shaker, Burrell Co., Pittsburgh, Pa.) for 3 min. The resulting suspension was serially diluted and plated using the appropriate agar media with incubation at 32°C for 24 to 48 h. *L. monocytogenes* cells were grown on *Listeria* selective agar (LSA; Oxoid, Ogsdenburg, N.Y.), *Pseudomonas* was grown on plate count agar (Difco, Becton Dickinson) or *Pseudomonas* isolation agar (Difco, Becton Dickinson) with confirmation by observing the cell morphology, and *P. agglomerans* was grown on violet red bile agar (Difco, Becton Dickinson). Mixed-culture biofilms were analyzed for surviving *Listeria* only. Counts were recorded as CFU per square centimeter.

**Heat treatment.** Duplicate samples containing either low- or soil-soaked biofilms of *L. monocytogenes* in monoculture or multispecies culture were submerged in preheated test tubes with 25 ml of phosphate buffer and heated in a circulating water bath (Precision Scientific, Winchester, Va.). Samples were heated for 1, 3, 5, and 15 min at 70, 72, 75, 77, and 80 ± 1°C. Temperatures were confirmed using a two-channel thermocouple (Traceable, Houston, Tex.). One thermocouple was suspended in the test tube with buffer and the other was attached to the coupon surface. Treated coupons were submerged in 25 ml of TSB (30 g/liter) with yeast extract (6 g/liter) and incubated at 35°C for 24 h; then a 1-ml aliquot was transferred to Fraser broth and incubated at 35°C for 48 h. Positive samples were streaked on LSA and incubated at 35°C for 24 h for presumptive identification. Only 10% of positive samples were confirmed using API-Listeria. The analysis of the heating data used the fraction negative method (21), which is based on a dichotomous response in which data were recorded as the fraction of the samples that showed either growth or no growth of *L. monocytogenes* after the heat treatment. The purpose of using fraction negative data is to overcome the tailing effect caused by clumped cells during heat inactivation. The fraction negative method allows for the inclusion of low numbers of survivors in the model that would not be included if conventional enumeration methods were used. This method also indicates whether the target population was completely inactivated (21).

**Heat treatment for model validation.** Validation data were collected using the five-strain cocktail of *L. monocytogenes* (Scott A, 3990, YM 96, 303, and 17) that was subjected to the same growth and soiling conditions previously described. Samples were
heated for 1, 8, and 15 min at 70, 76, and 80°C. The experiment was replicated six times with duplicate samples for each experiment. Data collection and analysis followed the previously described procedure.

**Experimental design for model development.** Four monoculture and two multispecies biofilms were grown on stainless steel and exposed to soil or no-soil conditions. The surfaces were subjected to five heating temperatures and four heating times. Data for the experiments were recorded as either positive or negative for the presence of *L. monocytogenes* and as positive or negative for the presence of *Pseudomonas* and *P. agglomerans*. The mixed-culture biofilms were analyzed for *Listeria* only. The experiment was replicated six times with duplicate samples for each experiment. The results from each observation were recorded as one of three possibilities: no survivors, 50% survival, or 100% survival with values of 0 of 2, 1 of 2, or 2 of 2 positive results. After removal of insignificant data and pooling of similar data, a total of 480 observations were used to develop the final model.

**Statistical analysis.** Logistic regression analysis (PROC logistic) using SAS/STAT software (SAS, Duncan, S.C.) was used to estimate the probability of complete inactivation of *L. monocytogenes* in a biofilm after heat treatment. This probability equation is in the form of

$$\ln[P/(1 - P)] = \beta_0 + \beta_1(S) + \beta_2(C) + \beta_3(TMP) + \beta_4(TIME)$$

illustrating that the probability of the binomial response (survival or inactivation) is dependent upon the coefficient estimates ($\beta_i$) and the culture (C), soil (S), temperature (TMP), and time (TIME) parameters. Culture and soil are classified variables, and time and temperature are continuous variables. Stepwise selection and the Wald statistics in the PROC logistic were used to evaluate the statistical contribution of the individual terms of the model, and the classified parameters were tested for independence using the Pearson correlation coefficient test. The final model was selected based on the Schwartz-Bayes criterion (SBC) goodness-of-fit test of the model. The SBC test is the most conservative and reliable test for evaluating the fit of a model (16).

Statistical evaluation of whether to include the initial number of *Listeria* cells in the biofilm as a variable in the model indicated that the initial cell counts (Table 2) had no significant effect in the model. The predictive power of the model was evaluated using plots of the predicted points. The predictive power of the selected models was limited by the range of the time (1 to 15 min) and temperature (70 to 80°C) parameters employed in this experiment. Predictive power was considered acceptable if the predicted points fell within the range of the heating parameters tested at or above 75% probability of total inactivation.

**Predictive model development.** Individual models were derived to predict the heat inactivation of *L. monocytogenes*, *Pseudomonas*, and *P. agglomerans* in monoculture and *L. monocytogenes* in multispecies biofilms. The monoculture biofilms of *Pseudomonas* sp. and *P. agglomerans* served as controls. Results of that initial study revealed that *Pseudomonas* cells in monoculture biofilm had an 84% probability and *P. agglomerans* had less than 0.1% probability of surviving a heat treatment of 77°C for 15 min. These control data were not used for model development because the inactivation of the non-*Listeria* cultures was assumed to be independent of *Listeria* inactivation. Stepwise selection to evaluate the statistical contribution of the model parameters indicated that culture, soil, time, and temperature contributed to the significance of the model ($P < 0.0001$). The Pearson correlation coefficient test to evaluate correlation between cultures indicated that the responses of *Listeria* in monoculture and *Listeria* within a multispecies biofilm were significantly independent ($P < 0.0001$), so culture types were analyzed as a classified variable. The resulting predictive model included time and temperature as continuous variables and soil (low soil, soiled) and culture type (Scott A, LMPs, LM 3990, LMPsP) as classified variables.

**Final predictive model.** The data for the two multispecies biofilms (LMPs and LMPsP) were merged to produce one multispecies culture type; as a result, the culture variable contains three culture types instead of four. The three culture types include monocultures of *L. monocytogenes* strains Scott A and 3990 and *L. monocytogenes* within a multispecies biofilm. Separately, the *L. monocytogenes* strains Scott A and 3990, and *L. monocytogenes* within a multispecies biofilm. Separately, the two multispecies biofilms had similar survival rates, so merging the data improved the robustness of the model. When evaluating the soil parameter, the low-soil data did not significantly contribute to the final model; therefore, this treatment was removed, resulting in the analysis of soil data only. A summary of the heat inactivation data and initial *Listeria* counts for six replicates are included in Table 2. The final predictive model was in the form

$$\ln[P/(1 - P)] = \beta_0 + \beta_1(C) + \beta_2(TMP) + \beta_3(TIME)$$

(1)

**Model validation.** The validation model was developed to assess the quality of the final prediction model using the same statistical approach as used for the prediction model. The validated model is described by equation 3, and the parameters are pre-

## Table 2. Heat inactivation of *L. monocytogenes* 3390 and Scott A and *L. monocytogenes* in mixed-culture biofilms

<table>
<thead>
<tr>
<th>Culture/time (min)</th>
<th>Initial numbers (CFU/cm²)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>LM 3990</td>
<td>5.92 ± 0.66</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>LMPs P</td>
<td>5.52 ± 0.92</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>LM Scott A</td>
<td>5.5 ± 0.25</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>

*a* Heat treatments ranged from 70 to 80°C for 1 to 15 min in the presence of soil on stainless steel surfaces. The results are a summation of six replications. Data are presented as the number of positive samples out of 12 total.

*b* Values are log mean ± standard deviation.
TABLE 3. Parameter estimates and chi-square values for the validation model

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>df</th>
<th>Estimate</th>
<th>SE</th>
<th>Chi-square P</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1</td>
<td>35.94</td>
<td>6.6</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>TEMP</td>
<td>1</td>
<td>-0.46</td>
<td>0.02</td>
<td>&lt;0.0001</td>
<td>0.63</td>
</tr>
<tr>
<td>TIME</td>
<td>1</td>
<td>-0.19</td>
<td>0.01</td>
<td>&lt;0.0007</td>
<td>0.83</td>
</tr>
</tbody>
</table>

*SBC values were 104.5 for the full model and 100 for the final (reduced) model.

sent in Table 3. The validation model was graphically compared with the predicted model (equation 2) to evaluate the bias, i.e., the over- or underprediction of the predictive model. The accuracy factor (AF) of the predictive versus the validated data was calculated and is expressed as the antilog of the average absolute logarithm ratio value for the predicted versus the validated data:

\[ AF = \exp\left(\frac{1}{n}\sum_{i=1}^{n}\log\left(\frac{\text{predicted}_i}{\text{validated}_i}\right)\right) \]

This value must be greater than or equal to 1. The larger the accuracy factor, the less accurate the prediction.

**RESULTS**

Initial numbers of *L. monocytogenes* (Table 2) in the biofilm did not have a significant effect on heat inactivation. Microorganisms within biofilms exist as clumps of various sizes held together with extracellular polymeric material (exopolysaccharide), making them difficult to break up for CFU enumeration. Therefore, the initial CFU of the biofilm may not be correlated with actual numbers of cells and or with heat treatment required for inactivation. Conventional predictive models for the inactivation of bacteria in suspension and in food matrices are based on log-linear inactivation calculations. Clumps of cells produce thermal death curves with tails because numerous cells are present in each CFU. In addition, exopolysaccharide binds the biofilm cells to the surface, making complete removal and enumeration of low numbers of surviving cells difficult.

The use of fraction negative data (Table 2) obtained through enrichment culture avoids errors associated with cell clumping and the need to detach surviving cells. The predictive model developed in this study does not assume log-linear inactivation rates. The inactivation data (Table 2) reveal a nonlinear heat inactivation trend. The model assumes a binomial distribution of the data.

The final predictive heat inactivation model for *L. monocytogenes* in a biofilm is presented in equation 2, and the statistical parameters are presented in Table 4. This model indicates that culture, temperature, and time were significant in the prediction of inactivation. The goodness-of-fit test (SBC values) comparing the full ranked factorial model with the final reduced model (Table 4) indicate that the final reduced model had a good fit to the experimental data within the range of the parameters tested.

**Final heat inactivation model for *L. monocytogenes* in a biofilm.** The final heat inactivation model for *L. monocytogenes* in a biofilm is given by

\[
\ln\left[P/(1-P)\right] = 18.0527 + [-0.4706(LM Scott A) + 0.5316(LM 3990) - 0.0616(multispecies)] - 0.2299(TEMP) - 0.1108(TIME) 
\]

**Validation model.** The validated model is described by the following equation:

\[
\ln\left[P/(1-P)\right] = 35.9399 - 0.4655(TEMP) - 0.1892(TIME) 
\]

The prediction equation (equation 2) estimates the probability of inactivation (1 - P) of *L. monocytogenes* in monoculture (Scott A and 3990) and in multispecies biofilms after heat treatment under different time and temperature conditions in the presence of soil. Plots (Figs. 1 through 3) were developed from the predictive equation to illustrate the probability of complete heat inactivation at 90, 75, and 50% in the biofilms in the presence of soil. The data indicate that to obtain 90% (P = 0.90) and 75% (P = 0.75) probabilities of total inactivation of *L. monocytogenes* Scott A, heating at 80°C for 12.5 and 2.5 min, respectively, is required. For a 50% probability of inactivation of this strain, a heating regimen of 76°C for <1 min is needed (Fig. 1). The heat inactivation requirement for *L. monocytogenes* 3990 with 90, 75, and

![FIGURE 1. Probability of heat inactivation of *Listeria monocytogenes* Scott A in biofilm on stainless steel with soil. Treatment temperatures ranged from 70 to 80°C, with treatment times of 1, 3, 5, and 15 min. Heat inactivation probabilities were plotted at the 90% (P = 0.90) and 75% (P = 0.75) levels and validated probability level at 50% (VP = 0.50).](http://meridian.allenpress.com/jfp/article-pdf/67/12/2712/1676130/0362-028x-67_12_2712.pdf)
FIGURE 2. Probability of heat inactivation of Listeria monocytogenes 3990 in biofilm on stainless steel with soil. Treatment temperatures ranged from 70 to 80°C, with treatment times of 1, 3, 5, and 15 min. Heat inactivation probabilities were plotted at the 90% (P = 0.90) and 75% (P = 0.75) levels and validated probability level at 50% (VP = 0.50).

The five-strain cocktail produced higher predicted survival the 90% (P = 0.90) and 75% (P = 0.75) levels and validated probability level at 50% (VP = 0.50).

50% probability of total inactivation at 80°C is >15 min, 11.7, and 1.7 min, respectively (Fig. 2). The prediction estimates at 90, 75, and 50% probability of total inactivation of Listeria in a multispecies biofilm indicate that heat treatment at 80°C for >15, 6.3, and <1 min, respectively, is required (Fig. 3). Additional studies are needed if predictions for heat treatments of >80°C and >15 min are desired. The predictive model demonstrates that there are differences in heat resistance between monoculture L. monocytogenes biofilms and Listeria in the multispecies biofilm in the presence of soil. L. monocytogenes Scott A in monoculture biofilm exhibited the lowest heat resistance followed by Listeria in the multispecies biofilm, and the L. monocytogenes 3990 monoculture biofilm exhibited the most heat resistance. This predictive model can therefore employ three underlying heat resistance assumptions, the highest inactivation prediction with the Scott A strain, a moderate prediction of inactivation with the mixed culture, and a conservative inactivation prediction using the 3990 strain.

The prediction model (equation 2) was verified by heating biofilms containing a five-strain cocktail of L. monocytogenes in the presence of soil. The verification plot generated from equation 3 can be graphically compared with the prediction plots of each biofilm type. The 50% probability plots indicated that the Scott A strain (Fig. 1) had lower inactivation probabilities than did the verification cocktail; for example, heat treatment at 75°C required 3 min for total inactivation of the Scott A biofilm versus 5 min for the cocktail strains. This finding suggests that the Scott A strain in the biofilm on soiled stainless steel may not be representative of other L. monocytogenes strains for heat inactivation studies of biofilm. L. monocytogenes within multispecies biofilms (Fig. 2) exhibited inactivation levels similar to those of the validation cocktail, and the L. monocytogenes 3990 biofilm had the highest heat resistance. The final model, which included strain 3990 (Fig. 3) as the representative strain in culture, is a conservative predictor of L. monocytogenes inactivation and therefore provides a safe guide for development of hot-water sanitation processes. The accuracy factors of the predicted data versus the validated data for Scott A and 3990 biofilms and L. monocytogenes within multispecies biofilms were 1.045, 1.067, and 1.055, respectively, suggesting that validation data closely follow the prediction model.

DISCUSSION

Chemical application is generally the preferred means of sanitizing food processing plant equipment and environmental surfaces. However, hot-water sanitation may be necessary to achieve a Listeria-free system. Surfaces such as pitted metal, elbows, worn gaskets, joints, and other soft parts can accumulate biofilms and organic matter, making them difficult to clean (2, 10, 14). Generally, hot-water sanitation is a good alternative to chemical application only for systems where cooking-on of organic matter is not of concern. There is little information on which to base hot-water sanitation requirements in food processing facilities. Therefore, a predictive model to estimate inactivation of L. monocytogenes in a biofilm is useful. The static heating employed in this study simulates conditions in protected or dead-end areas in a clean-in-place system such as joints with gaskets.

Although numerous strains of L. monocytogenes have been isolated from food processing plant environments, no typical or representative strain for this environment has been characterized. The predictive models developed in this study include three heat resistance assumptions based upon two L. monocytogenes strains in monoculture and one in multispecies biofilms. The probability plots generated from the predictive model also give three probability levels (90, 75, and 50%) for inactivation based on each of the three heat resistance assumptions.

The heat inactivation data indicate that L. monocytogenes 3990 biofilm is more heat resistant on stainless steel in the presence of soil than is L. monocytogenes Scott A and L. monocytogenes in the multispecies biofilm. The validation study revealed that at the 50% probability level of L. monocytogenes inactivation, the predictive model based on strain 3990 is conservative in its estimate of L. monocytogenes biofilm inactivation. The model based on this strain should be used in situations where there is high risk of L. monocytogenes contaminating a ready-to-eat product. The five-strain cocktail produced higher predicted survival
percentages than that found for the Scott A strain and survival percentages similar to those for L. monocytogenes within a multispecies biofilm. Therefore the Scott A model is not a conservative predictor of L. monocytogenes inactivation, and this model would have limited use. Inactivation of L. monocytogenes within the multispecies biofilm was similar to that of the cocktail cultures, with a slightly more conservative prediction at the higher levels of heat treatment. L. monocytogenes within a multispecies biofilm is therefore an adequate representation of L. monocytogenes biofilm inactivation and can be used in situations where there is low risk of product exposure to L. monocytogenes.

The five-strain cocktail contained L. monocytogenes strain 3990, which has above normal heat resistance and is a good biofilm producer. The low heat resistance of the biofilm produced by the strain cocktail suggests that there was an imbalance of growth among the cocktail strains during biofilm formation. We were unable to determine the predominant culture in this biofilm because of the genetic similarity (ribotyping and Rep-PCR data) of the L. monocytogenes strains. Previous studies (17) have provided evidence that strains of L. monocytogenes do not maintain balanced growth within competitive culture; e.g., L. monocytogenes grew well in the presence of Pseudomonas, but when other microorganisms were present L. monocytogenes grew only after Pseudomonas reached stationary phase (17).

Soil can have a protective effect on the heat inactivation of suspended or attached microorganisms. The heat resistance of L. monocytogenes 3990 biofilm was significantly increased in the presence of food soil (equation 2); however, this was not true of strain Scott A (data not shown). Casadei et al. (3) and Chhabra et al. (5) demonstrated that high-fat substrates increased heat resistance of suspensions of L. monocytogenes strains 1151 and Scott A, with strain 1151 exhibiting higher heat resistance than Scott A. Flint et al. (8) noted that Streptococcus thermophilus that attached to stainless steel in the presence of skim milk and was then heated in water was more heat resistant than similarly treated suspended cells. The presence of food residues could also promote bacterial growth, thereby resulting in a pH change within the biofilm. Such a change would subsequently influence heat inactivation (12).

Cells may be protected from stress if they are located in the biofilm strata where nutrients are depleted, cell growth is slow, and stress response is induced. Dense clustering of cells and production of extracellular polymers effectively change the heating menstrum. Frank and Koff (9) and Lee and Frank (19) demonstrated that bacteria in biofilm that remained attached to a surface had significantly higher survival after exposure to heat than did planktonic cells or cells in detached biofilm. For example, adherent cells survived 70°C heat treatment for 15 min, whereas planktonic cells were killed in 30 s. Lee and Frank (19) demonstrated that the greater the cell density within a biofilm the higher the survival of L. monocytogenes after heating.

When developing a predictive model, consideration should be given to selecting a representative bacterial strain or serovar because not all strains or serovars have the same level of heat resistance or biofilm production. In our model, the calculated odds ratio (Table 4) indicated that time of heating and culture type (in particular L. monocytogenes 3990) were the predominant factors contributing to the prediction of L. monocytogenes survival in biofilm after heat treatment. Our study showed that strain Scott A responded differently than did strain 3990; strain 3990 was more heat resistant in the presence of soil. Stress response resulting from biofilm growth can be different for different strains. Sorqvist (23) studied the heat resistance of more than 30 strains and serovars of L. monocytogenes and noted greater heat resistance among the 1/2 and 3b serovars tested but substantial variation within the 3b group. Vasseur et al. (24) studied the effect of osmotic, alkali, acid, or thermal stresses on L. monocytogenes and observed a variety of stress responses among strains.

The results of this study indicate the need to verify the efficacy of hot-water sanitation processes. Clean-in-place systems may not be able to exceed 77°C, and temperatures may drop lower at some points in the system. Therefore, the risk of L. monocytogenes surviving a hot-water sanitation process is significant if adequate time and temperature controls are not maintained. The predictive model, based on total inactivation of L. monocytogenes, provides three heat resistance assumptions to predict L. monocytogenes inactivation on stainless steel, with the Scott A strain having the highest probability of inactivation followed by L. monocytogenes within a multispecies biofilm and then by strain 3990, which has a conservative heat inactivation prediction. The validation data confirm the accuracy of the model within the limitations of the tested parameters. The accuracy factor indicates that the validation data closely follow the predictive data. L. monocytogenes within a biofilm on stainless steel may be effectively inactivated if heat treatment is controlled. However, it may be a challenge to control sanitation temperatures in the processing environment, especially in systems where cold spots have not been identified or temperature cannot be monitored. The predictive model developed in this study can be used for risk assessment planning, allowing processors to select a heat treatment based on the likelihood of product contamination and on the level of acceptable risk.

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