

Validation of a Tertiary Model for Predicting Variation of *Salmonella* Typhimurium DT104 (ATCC 700408) Growth from a Low Initial Density on Ground Chicken Breast Meat with a Competitive Microflora[†]

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ABSTRACT

Growth of a multiple antibiotic-resistant strain (ATCC 700408) of *Salmonella* Typhimurium definitive phage type 104 (DT104) from a low initial density ($10^{0.6}$ most probable number [MPN] or CFU/g) on ground chicken breast meat with a competitive microflora was investigated and modeled as a function of time and temperature (10 to 40°C). MPN and viable counts (CFU) on a selective medium with four antibiotics enumerated the pathogen. Data from five replicate challenge studies per temperature were combined and fit to a primary model to determine maximum specific growth rate (μ), maximum population density (N_{\max}), and the 95% prediction interval (PI). Nonlinear regression was used to obtain secondary models as a function of temperature for μ , N_{\max} , and PI, which ranged from 0.04 to 0.4 h⁻¹, 1.6 to 9.4 log MPN or CFU/g, and 1.4 to 2.4 log MPN or CFU/g, respectively. Secondary models were combined with the primary model to create a tertiary model for predicting variation (95% PI) of pathogen growth among batches of ground chicken breast meat with a competitive microflora. The criterion for acceptable model performance was that 90% of observed MPN or CFU data had to be in the 95% PI predicted by the tertiary model. For data ($n = 344$) used in model development, 93% of observed MPN and CFU data were in the 95% PI predicted by the tertiary model, whereas for data ($n = 236$) not used in model development but collected using the same methods, 94% of observed MPN and CFU data were in the 95% PI predicted by the tertiary model. Thus, the tertiary model was successfully verified against dependent data and validated against independent data for predicting variation of *Salmonella* Typhimurium DT104 growth among batches of ground chicken breast meat with a competitive microflora and from a low initial density.

In a survey conducted by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture, 45% of retail ground chicken samples (25 g) were contaminated with *Salmonella* with an average density of $10^{0.1}$ most probable number (MPN) per g (1). Rose et al. (42) estimate that the probability of infection from one cell of *Salmonella* is 7.5×10^{-3} and that the probability of death from one cell is 7.5×10^{-6} . Considering that ground chicken is usually cooked thoroughly before serving, risk of salmonellosis is considered to be low (1). However, exposure of ground chicken to temperatures (e.g., 10 to 40°C) that support growth of *Salmonella* could result in rapid growth of a single pathogen cell to levels that pose a significant risk of illness.

Although models exist for predicting growth of *Salmonella* on ground chicken incubated at temperatures that support growth of the pathogen, these models were developed with a high initial density ($N_0 > 10^3$ CFU/g) of the pathogen (34, 35, 37, 38). Recent findings (37) indicate that

a model developed with data from challenge studies conducted with a high initial density ($10^{3.8}$ CFU/g) is not a good predictor of *Salmonella* growth from a lower ($10^{0.8}$ CFU/g) initial density, as only 2.5% of model predictions were acceptable. Of note, the model predicted much less growth of *Salmonella* from the lower initial density than was observed (37). Thus, there is a need to develop a predictive model for growth of *Salmonella* from a lower initial density (i.e., $<10^1$ CFU/g) so that chicken processors can more accurately assess the safety of ground chicken that has been exposed to temperatures that support growth of the pathogen.

Another limitation of most current models is that they were developed with sterile broth (17, 32, 44) or sterile ground chicken (30, 31, 34, 35, 37, 38) and thus, effects of competitive microflora on growth of *Salmonella* are not accounted for in predictions made by the models. This is important because the competitive microflora has been shown to suppress growth of *Salmonella* during incubation of food samples in isolation broths (5, 41, 43). Thus, a model developed without competitive microflora would predict much more growth of *Salmonella* on ground chicken with a competitive microflora than would actually occur. This would result in an overly fail-safe prediction of *Salmonella* growth and the risk of salmonellosis.

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In a previous study (39), a multiple antibiotic-resistant (MAR) strain of *Salmonella* Typhimurium definitive phage type 104 (DT104) was used to successfully investigate and model (i.e., primary and secondary modeling) growth of the pathogen from a high initial density ($10^{3.8}$ CFU/g) on ground chicken breast meat with a competitive microflora. However, the tertiary model developed did not provide acceptable predictions of *Salmonella* growth because it could not predict the observed variation of pathogen growth among batches of ground chicken breast meat with a competitive microflora (39). Therefore, the current study with the same strain (ATCC 700408) of *Salmonella* Typhimurium DT104 was undertaken to develop and validate a tertiary model for predicting variation of pathogen growth among batches of ground chicken breast meat with a competitive microflora and from a lower initial density (i.e., $10^{0.6}$ MPN or CFU/g).

MATERIALS AND METHODS

Salmonella. A MAR strain (ATCC 700408, American Type Culture Collection, Manassas, Va.) of *Salmonella* Typhimurium DT104 was used for model development and validation. Stock cultures were maintained at -70°C in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, Md.) that contained 15% glycerol (Sigma, St. Louis, Mo.).

Ground chicken breast meat. Boneless chicken breast meat was purchased weekly from local retail outlets. The meat was ground ($\frac{1}{8}$ -in. plate) using an electric meat grinder (The Sausage Maker, Buffalo, N.Y.) and divided into portions (1 g) for challenge studies.

Inoculum culture. On a weekly basis, stationary-phase cells for inoculation of chicken portions were prepared by adding stock culture (5 μl) to BHI broth (5 ml) in a 25-ml Erlenmeyer flask sealed with a foam plug followed by incubation at 30°C and 150 orbits per min for 23 h. Immediately before inoculation, inoculum cultures were serially diluted in buffered peptone water (BPW; Becton Dickinson) to a final concentration of $10^{3.3}$ CFU/ml.

Challenge study. Diluted culture (10^{-7}) was inoculated (2 μl) onto chicken portions for an initial density of $10^{0.6}$ MPN or CFU/g. Inoculated portions were incubated at 10, 12, 14, 22, 30 and 40°C for model development and at 11, 18, 26, and 34°C for model validation. At selected times of incubation, a portion (1 g) was homogenized (model 80 stomacher blender, Seward, London, UK) in BPW (9 ml) for determination of pathogen density. Five replicate challenge studies, each with a different batch of ground chicken portions (12 portions per replicate) and different inoculum cultures were conducted per temperature.

Pathogen enumeration. At time zero, pathogen density (log CFU per gram) was calculated based on the viable count (CFU), dilution (10^{-7}), and volume (2 μl) of culture used for inoculation. During incubation and when a chicken portion was expected to have a pathogen density between 0 and $10^{3.28}$ /g, a 3×4 MPN assay was used for enumeration. The MPN assay was prepared in BPW followed by incubation for 24 h at 38°C before 2 μl from each of the 12 dilution tubes and 2 μl from the remaining stomachate in the stomacher bag were spot inoculated onto xylose lysine (XL) agar medium (Becton Dickinson) that contained 25 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] or HEPES (H; Sigma) and 25 $\mu\text{g}/\text{ml}$ of the following antibiotics from Sigma: chloramphenicol (C), ampicillin (A), tetracycline (T)

and streptomycin (S); hereafter, referred to as XLH-CATS. After incubation of the XLH-CATS plates at 38°C for 24 h, a black colony formed within the inoculation areas for each positive dilution tube, whereas inoculation areas for negative dilution tubes were clear with no signs of microbial growth.

The method reported by Thomas (45) was used to calculate the MPN. This method produces results similar to MPN tables but has the advantage of allowing MPN determinations from assay designs, such as the one used in the present study, with an arrangement of tubes that differs from those found in MPN tables. The MPN was calculated using the following equation:

$$\text{MPN/g} = P/\sqrt{N \times T}$$

where P was the number of positive tubes, N was the total amount of sample (g) in all negative tubes, and T was the total amount of sample (g) in all tubes (45). For example, for an MPN assay result of 3-1-0-0:

$$P = 3 + 1 + 0 + 0 = 4$$

$$N = [(0.1 \times 0) + (0.01 \times 2) + (0.001 \times 3) + (0.0001 \times 3)] = 0.0233 \text{ g}$$

$$T = 3 \times (0.1 + 0.01 + 0.001 + 0.0001) = 0.3333 \text{ g}$$

$$\text{MPN/g} = 4/\sqrt{0.0233 \times 0.3333} = 45 \text{ or } 10^{1.65} \text{ MPN/g}$$

When the MPN assay result was 0-0-0-0 and the bag was positive for *Salmonella*, this indicated that pathogen density was greater than 0 but less than $10^{0.55}$ MPN/g, which corresponded to an assay result of 1-0-0-0. In this situation, it was assumed that pathogen density was $10^{0.275}$ MPN/g. When the MPN assay result was 0-0-0-0 and the bag was negative for *Salmonella*, this indicated that pathogen density was 0 MPN/g.

During incubation and when the inoculated chicken portion was expected to have a pathogen density greater than $10^3/\text{g}$, direct plating on XLH-CATS was used for enumeration. Stomachate was serially diluted in BPW and then 50 μl was spiral plated (Whitley Automatic Spiral Plater, Microbiology International, Frederick, Md.) onto XLH-CATS in duplicate. Spiral plates were incubated at 38°C for 24 h before automated counting of colonies (ProtoCol, Microbiology International).

Primary modeling. Calculated log CFU at time zero, and log MPN and log CFU data for pathogen density at times greater than zero, and from all five replicate challenge studies per temperature were combined, graphed as a function of time and fit (Prism version 4.0, GraphPad Software, San Diego, Calif.) to the following primary model:

$$N(t) = N_{\max}/\{1 + [(N_{\max}/N_0) - 1]\exp(-\mu \times t)\}$$

which was obtained from the logistic with delay model (2) and where $N(t)$ was pathogen density (log MPN or CFU per gram) at time t (h), N_{\max} was maximum population density (log MPN or CFU per gram), N_0 was initial density (log MPN or CFU per gram), and μ was maximum specific growth rate (h^{-1}). In addition, a 95% prediction interval (PI) for pathogen density (log MPN or CFU per gram) was obtained for each growth curve (25). The 95% PI quantified variation of pathogen growth among batches of ground chicken breast meat with a competitive microflora.

Secondary modeling. Secondary models were fit to growth parameter data from primary modeling using the Prism software program. The best-fit values of μ from primary modeling were graphed as a function of temperature (T , $^{\circ}\text{C}$) and were fit to the following secondary model:

$$\mu = \begin{cases} \mu_i & \text{if } T \leq T_0 \\ \frac{\mu_{\text{opt}}}{1 + [(\mu_{\text{opt}}/\mu_i) - 1]\exp[-\mu_{\text{rate}}(T - T_0)]} & \text{if } T > T_0 \end{cases}$$

which was identical to the logistic with delay model (2) but with different parameter names: μ_i (h^{-1}) was initial predicted μ for the growth conditions studied, T_0 was the temperature at which μ was predicted to increase, μ_{opt} (h^{-1}) was optimal predicted μ , and μ_{rate} ($\text{h}^{-1}/^\circ\text{C}$) was the maximum predicted rate of increase of μ as a function of temperature.

Best-fit values for N_{max} from primary modeling were graphed as a function of temperature and were fit to the following secondary model:

$$N_{\text{max}} = \exp\{[a((T - T_{\text{min}})/(T - T_{\text{submin}}))]\}$$

which was obtained from the asymptote model (50) and where a was a regression coefficient, T_{min} was the minimum predicted growth temperature, and T_{submin} was a predicted temperature below T_{min} .

Ninety-five-percent prediction intervals (PI) from primary modeling were graphed as a function of temperature and were fit to the following four-phase linear secondary model:

$$\text{PI} = \begin{cases} \text{PI}_1 & \text{if } T \leq T_1 \\ \text{PI}_1 + [(\text{PI}_2 - \text{PI}_1)/(T_2 - T_1)](T - T_1) & \text{if } T_1 < T \leq T_2 \\ \text{PI}_3 + [(\text{PI}_2 - \text{PI}_3)/(T_2 - T_3)](T - T_3) & \text{if } T_2 < T \leq T_3 \\ \text{PI}_3 & \text{if } T_3 < T \end{cases}$$

which was obtained from the three-phase linear model (8) and where PI_1 , PI_2 , and PI_3 were predicted PI at predicted temperatures T_1 , T_2 , and T_3 , respectively. To obtain model convergence, T_1 was constrained to 10°C , which was the lowest temperature investigated. To reduce the size of 95% confidence intervals for PI_1 , PI_2 , and PI_3 , T_2 and T_3 were constrained to fit values after the first round of nonlinear regression (26). The purpose of the secondary model for PI was to allow the tertiary model to predict the 95% PI around the predicted growth curve for temperatures within the range (10 to 40°C) used in model development.

The maximum time of sampling (Ω) at each temperature was graphed as a function of temperature and fit to the following reverse two-phase linear secondary model:

$$\Omega = \begin{cases} \Omega_{\text{min}} & \text{if } T \geq T_d \\ \Omega_{\text{min}} + [\Omega_{\text{rate}}(T - T_d)] & \text{if } T < T_d \end{cases}$$

which was obtained from the three-phase linear model (8) and where Ω_{min} was the minimum predicted Ω , T_d was the predicted temperature at which Ω stopped decreasing, and Ω_{rate} was the predicted linear rate of decrease of Ω from 10°C , which was the lowest temperature investigated, to T_d . The purpose of the secondary model for Ω was to prevent predictions of pathogen growth in the tertiary model that were beyond sampling times used in model development or beyond sampling times where growth was observed.

Performance of secondary models was evaluated using the acceptable prediction zone method (37, 38). Relative errors (RE) for individual prediction cases were calculated:

$$\text{RE for } \mu \text{ and } N_{\text{max}} = (\text{observed} - \text{predicted})/\text{predicted}$$

$$\text{RE for PI} = (\text{predicted} - \text{observed})/\text{predicted}$$

such that RE of less than 0 represented fail-safe predictions and RE greater than 0 represented fail-dangerous predictions. Model performance (prediction bias and accuracy) was quantified using the percentage of RE (% RE) in an acceptable prediction zone

that was twice as wide in the fail-safe direction as in the fail-dangerous direction (37, 38). The criterion for acceptable model performance was that 70% of RE had to be in the acceptable prediction zone (37, 38). Widths of the acceptable prediction zones are based on an assessment of experimental error associated with determining individual growth parameters (37). Width of the acceptable prediction zone for evaluation of model performance is -0.3 to 0.15 for μ and -0.8 to 0.4 for N_{max} and PI (37).

Tertiary modeling. Secondary models for μ , N_{max} , PI, and Ω were combined with the primary model in a computer spreadsheet (Excel 2000, Microsoft Corporation, Redman, Wash.) to create a tertiary model (37). The tertiary model predicted variation (95% PI) of *Salmonella* Typhimurium DT104 growth from a low initial density ($10^{0.6}$ MPN or CFU/g) on ground chicken breast meat with a competitive microflora as a function of time and temperature (10 to 40°C). Outputs of the tertiary model were a predicted growth curve, its 95% PI, and minimum, most likely, and maximum predicted pathogen density for a specified time. The 95% PI was expected to contain 95% of all future MPN and CFU data collected using the same methods (25).

Predictions of the tertiary model were verified by comparison with MPN and CFU data used in model development (dependent data), whereas predictions of the tertiary model were validated by comparison with MPN and CFU data not used in model development (independent data) (38). The criterion used for model verification and validation was that 90% of observed MPN or CFU data had to be in the 95% PI predicted by the tertiary model. As stated above, one would expect 95% of future MPN and CFU data to be in the predicted 95% PI (25). Applying the accepted criterion in statistics that a 5% probability of incorrectly rejecting the null hypothesis is acceptable; then the 90% acceptable criterion was calculated as $95\% \text{ PI} \times (100\% - 5\%)$ or $95\% \text{ PI} \times 95\% = 90.25\%$, which was rounded to 90%. Thus, if the tertiary model predictions had 90% concordance with the observed MPN and CFU data, the tertiary model was classified as acceptable.

Independent data for validation of the tertiary model were collected in five replicate challenge studies conducted at temperatures (11, 18, 26, and 34°C) that were intermediate to those (10, 12, 14, 22, 30, and 40°C) used in model development. The experimental and modeling methods used to collect MPN and CFU data for model development were also used to collect independent MPN and CFU data for model validation so as not to confound comparison of observed and predicted values (38). Moreover, the independent data provided good coverage of the response surface in dimensions of both time and temperature and thus, provided a proper test of the ability of the tertiary model to predict variation of *Salmonella* Typhimurium DT104 growth within its entire response surface (38).

RESULTS AND DISCUSSION

To model variation of *Salmonella* Typhimurium DT104 growth on ground chicken breast meat with a competitive microflora and from a low initial density ($10^{0.6}$ MPN or CFU/g), both MPN and CFU methods were used. Agreement between these methods was compared for eight samples that were in the overlap region of the two assays. The MPN assay had a range from 0 to $10^{3.28}$ MPN/g, whereas the CFU assay had a lower range of 10^3 CFU/g. On average, for the comparative samples, the MPN assay indicated a lower ($P = 0.03$; paired Student's t test in Excel) pathogen density than the CFU assay with a mean difference of 0.27 log MPN or CFU/g (Table 1). Thus, using both

TABLE 1. Comparison of most probable number (MPN) and viable counts (CFU) among samples of ground chicken breast meat inoculated with *Salmonella Typhimurium* DT104 ($10^{0.6}$ MPN or CFU/g) and incubated for selected times at 11 to 40°C

Sample	Temp (°C)	Time (h)	Log MPN/g	Log CFU/g
1	11	175.9	3.28	3.08
2	14	38.7	3.09	3.30
3	14	68.0	3.28	3.38
4	26	8.7	2.95	3.65
5	26	9.7	3.28	3.66
6	30	6.0	2.95	3.00
7	30	6.8	3.00	3.51
8	40	4.4	3.09	3.56
		Mean	3.12	3.39

methods to quantify *Salmonella Typhimurium* DT104 introduced some experimental error into the modeling process. However, it could not be avoided because the objective was to investigate and model growth of the pathogen from an initial density ($10^{0.6}$ MPN or CFU/g) that was below the detection limit (10^3 CFU/g) of the CFU assay to a pathogen density that was above the upper limit ($10^{3.28}$ MPN/g) of the MPN assay.

The maximum time of sampling (Ω) decreased as a function of temperature as planned (Fig. 1). However, there was considerable scatter in the data because Ω was not precisely controlled. To produce a conservative model for prediction of Ω in the tertiary model, only data points at 11, 12, 22, and 34°C were included in the final fit. A secondary model for Ω was needed for predicting growth of *Salmonella Typhimurium* DT104 because when ground chicken breast meat with a competitive microflora was incubated for extended time and spoiled, reductions in pathogen density were observed (data not shown). Thus, it was important to develop a tertiary model with a secondary model for Ω that did not allow predictions for times of storage beyond those used in model development or beyond those where growth was observed. Parameters of secondary models for Ω , PI, μ , and N_{max} and their 95% confidence intervals are presented in Table 2.

As expected (39), there was variation of *Salmonella Typhimurium* DT104 growth among batches of ground chicken breast meat with a competitive microflora. Rather than fit the primary model to MPN and CFU data from individual replicates as was done previously (39), the primary model was fit to combined data from all five replicates per temperature. This simplified modeling in two ways. First, one rather than five sets of growth parameters were obtained per temperature, which simplified secondary modeling. Second, it provided a simple method (95% PI) to quantify variation of pathogen growth among batches of ground chicken breast meat with a competitive microflora (Fig. 2). Lag phase was not always apparent in the growth curves (Fig. 2), which made it difficult to develop a secondary model, and consequently, λ was not modeled. Parameters of primary model fits and 95% PI for data used in model development (i.e., dependent data) and data not

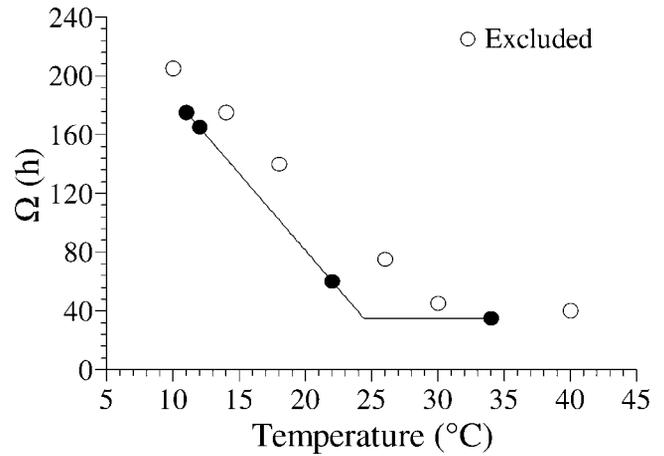


FIGURE 1. Secondary model (—) for maximum time of sampling (Ω) as a function of temperature for growth of *Salmonella Typhimurium* DT104 from a low initial density ($10^{0.6}$ MPN or CFU/g) on ground chicken breast meat with a competitive microflora. The indicated data points were excluded from the final model fit to yield a conservative model for predicting Ω as a function of temperature in the tertiary model.

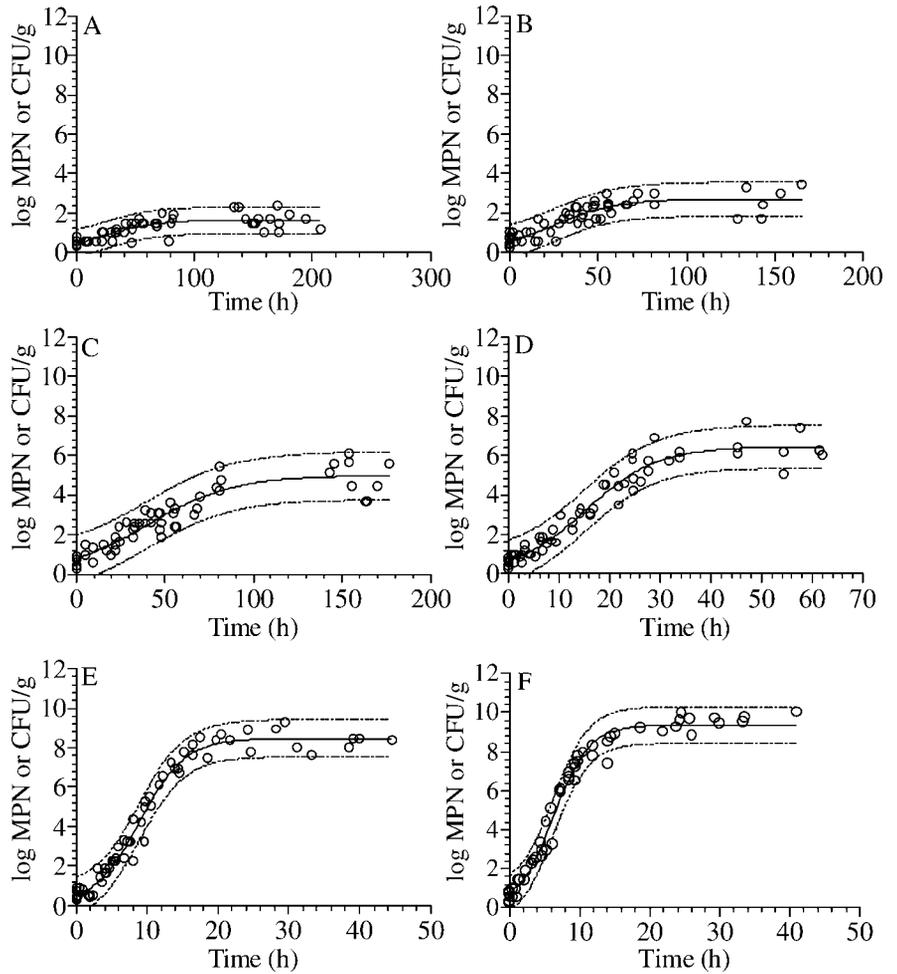
used in model development (i.e., independent data) are shown in Table 3.

Corradini and Peleg (12) found that sigmoid growth curves are adequately described by primary models with three (N_0, μ, N_{max}) rather than four ($N_0, \lambda, \mu, N_{max}$) parameters. This finding is consistent with results of the current study (Fig. 2). An advantage of a primary model without a parameter for λ over a primary model with a term for λ is that it simplifies modeling of pathogen growth under dynamic conditions from data collected under constant conditions. The most common approach for predicting pathogen growth under dynamic conditions from models

TABLE 2. Best-fit values and 95% confidence intervals for parameters of the secondary models for maximum time of sampling (Ω), 95% prediction interval (PI), maximum specific growth rate (μ), and maximum population density (N_{max})

Secondary model	Parameter	Best-fit value	95% confidence interval
Ω	T_d	24.39	23.74 to 25.04
	Ω_{min}	35.00	30.74 to 39.26
	Ω_{rate}	-10.47	-10.97 to -9.98
PI	PI ₁	1.330	1.098 to 1.562
	PI ₂	2.576	2.303 to 2.849
	PI ₃	1.944	1.774 to 2.113
	T_1	10.00	Fixed
	T_2	14.85	Fixed
	T_3	26.92	Fixed
μ	μ_i	0.04733	0.02804 to 0.06663
	T_0	15.59	11.25 to 19.93
	μ_{rate}	0.2188	0.1244 to 0.3131
	μ_{opt}	0.4084	0.3573 to 0.4595
N_{max}	α	2.473	2.051 to 2.895
	T_{min}	9.114	6.209 to 12.02
	T_{submin}	5.655	-1.131 to 12.44

FIGURE 2. Best-fit (—) and 95% prediction interval (-----) for primary model fits to observed MPN and CFU data (○) for growth of *Salmonella* Typhimurium DT104 from a low initial density ($10^{0.6}$ MPN or CFU/g) on ground chicken breast meat with a competitive microflora at A) 10, B) 12, C) 14, D) 22, E) 30, or F) 40°C. These data from five replicate challenge studies per temperature were used in model development.



developed under constant conditions is to calculate cumulative λ before each temperature shift and then to apply the prediction of growth rate once cumulative λ has been exhausted (19). By using a primary model without λ to model pathogen growth, the latter calculation and its associated assumptions can be avoided. The result is a more parsimonious approach for modeling pathogen growth under dynamic conditions from data collected under constant conditions (11).

To model variation of *Salmonella* Typhimurium DT104

growth among batches of ground chicken breast meat with a competitive microflora, a 95% PI was used. The 95% PI, which were obtained during primary modeling, increased in a non-linear manner as a function of temperature and ranged from 1.4 to 2.4 log MPN or CFU/g (Fig. 3). A secondary model with six parameters was used to model PI as a function of temperature. To evaluate performance of this model (3), independent MPN and CFU data were collected, subjected to primary modeling (Fig. 4) and the resulting PI (i.e., independent data) were included in the

TABLE 3. Observed (O), predicted (P), and relative error (RE) for data used (dependent) and not used (independent) in development of secondary models for maximum specific growth rate (μ), maximum population density (N_{max}), and the 95% prediction interval (PI) as a function of temperature (T)

T (°C)	Type of data	μ (h^{-1})			N_{max} (log MPN or CFU/g)			PI (log MPN or CFU/g)		
		O	P	RE	O	P	RE	O	P	RE
10	Dependent	0.045	0.047	-0.058	1.63	1.66	-0.055	1.38	1.33	-0.122
11	Independent	0.050	0.047	0.063	2.28	2.39	-0.226	2.02	1.59	-1.711
12	Dependent	0.056	0.047	0.183	2.70	3.08	-0.588	1.74	1.84	0.213
14	Dependent	0.041	0.047	-0.127	4.98	4.25	4.292	2.42	2.36	-0.154
18	Independent	0.080	0.074	0.084	5.34	5.93	-0.746	2.00	2.41	0.612
22	Dependent	0.142	0.142	-0.002	6.43	7.03	-0.744	2.18	2.20	0.049
26	Independent	0.228	0.229	-0.003	7.63	7.79	-0.300	1.96	1.99	0.071
30	Dependent	0.308	0.308	-0.001	8.49	8.34	0.404	1.92	1.94	0.054
34	Independent	0.337	0.360	-0.064	9.29	8.77	2.350	1.86	1.94	0.176
40	Dependent	0.394	0.394	-0.001	9.36	9.24	0.307	1.88	1.94	0.137

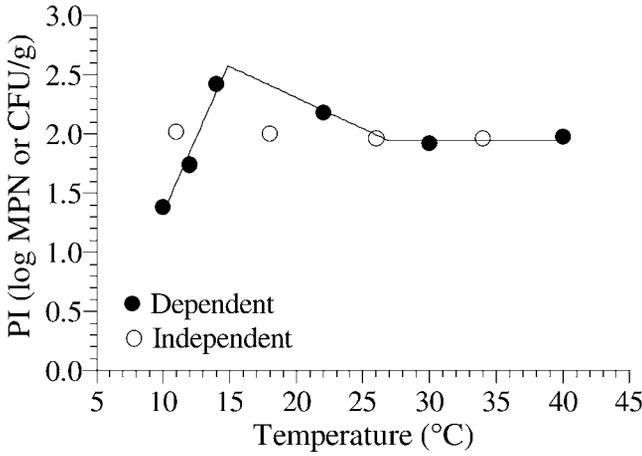


FIGURE 3. Secondary model (—) for the 95% prediction interval as a function of temperature for growth of *Salmonella* Typhimurium DT104 from a low initial density ($10^{0.6}$ MPN or CFU/g) on ground chicken breast meat with a competitive microflora. Data used in model development (dependent data) and data not used in model development (independent data) are shown.

graph for the secondary model (Fig. 3). In addition, performance of the secondary model for PI was evaluated using the acceptable prediction zone method (37, 38). For data ($n = 6$) used in model development, 100% of RE for PI were acceptable (i.e., in the acceptable prediction zone from an RE of -0.8 to 0.4), whereas for data ($n = 4$) not used in model development 50% of RE for PI were acceptable. The criterion for acceptable model performance in the acceptable prediction zone method is 70% or more acceptable predictions (37, 38). Thus, the secondary model for PI had acceptable goodness-of-fit (i.e., acceptable prediction of dependent data) but did not provide acceptable predictions of data not used in model development.

As expected, μ increased in a nonlinear manner as a function of temperature and ranged from 0.04 to 0.4 h^{-1} (Fig. 5). Between 10 and 15.6°C , there was not an apparent

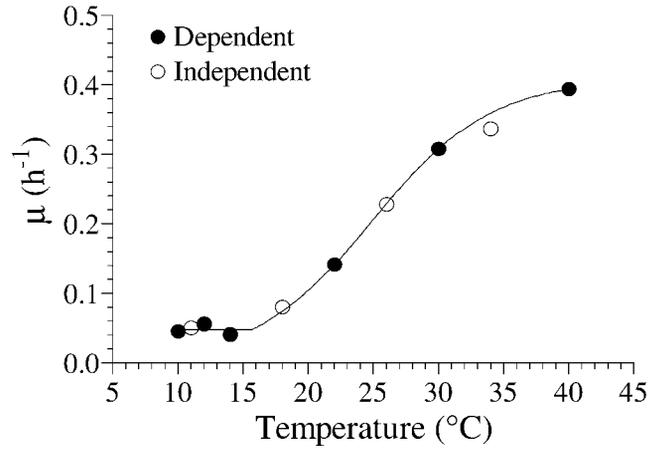


FIGURE 5. Secondary model (—) for maximum specific growth rate (μ) as a function of temperature for growth of *Salmonella* Typhimurium DT104 from a low initial density ($10^{0.6}$ MPN or CFU/g) on ground chicken breast meat with a competitive microflora. Data used in model development (dependent data) and data not used in model development (independent data) are shown.

change in μ . This result is similar to a previous study (37) in which μ of *Salmonella* Typhimurium on sterile chicken did not change between 10 and 11.4°C . However, it differs from results of other studies for *Salmonella* and sterile chicken (30, 31, 34, 35, 38) or sterile broth (17, 32, 44).

To validate the secondary model for μ , independent MPN and CFU data were collected, subjected to primary modeling (Fig. 4) and the resulting independent data for μ were included in the graph for the secondary model (Fig. 5). In addition, performance of the secondary model for μ was evaluated using the acceptable prediction zone method (Table 3). For data ($n = 6$) used in model development, 83% of predictions were acceptable (i.e., in an acceptable prediction zone from -0.3 to 0.15), whereas for data ($n = 4$) not used in model development 100% of RE for μ were

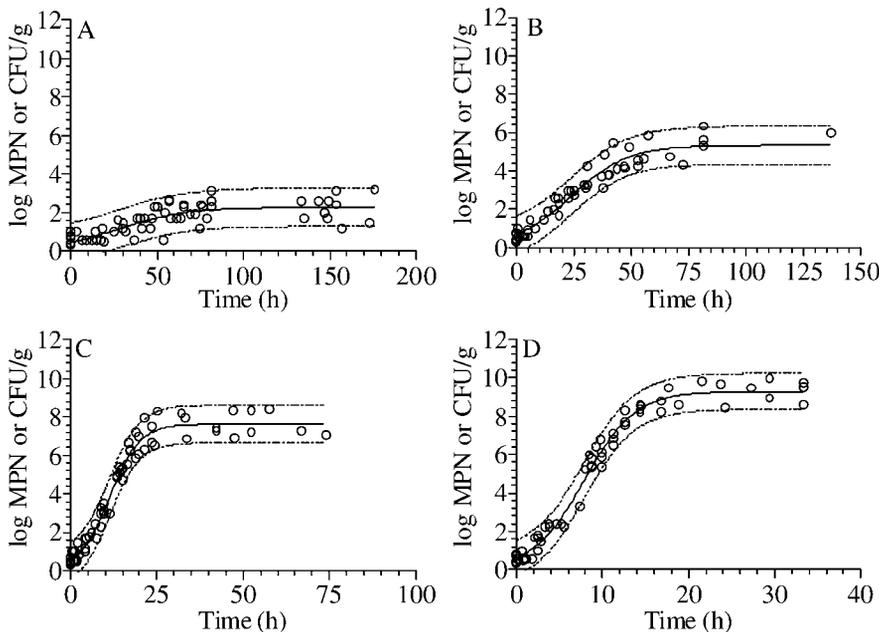


FIGURE 4. Best-fit (—) and 95% prediction interval (---) for primary model fits to observed MPN and CFU data (○) for growth of *Salmonella* Typhimurium DT104 from a low initial density ($10^{0.6}$ MPN or CFU/g) on ground chicken breast meat with a competitive microflora at A) 11°C , B) 18°C , C) 26°C , or D) 34°C . These data, which were obtained in five replicate challenge studies per temperature, were not used in model development but rather were used to validate secondary models and the tertiary model.

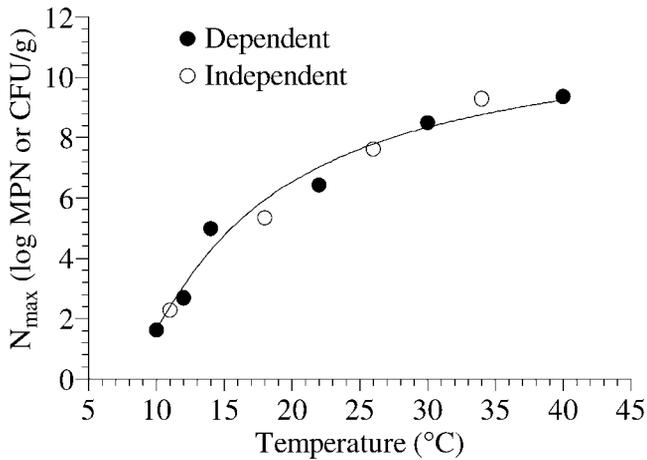


FIGURE 6. Secondary model (—) for maximum population density (N_{max}) as a function of temperature for growth of *Salmonella* Typhimurium DT104 from a low initial density ($10^{0.6}$ MPN or CFU/g) on ground chicken breast meat with a competitive microflora. Data used in model development (dependent data) and data not used in model development (independent data) are shown.

acceptable. Both of these values (i.e., % RE) exceeded the criterion of 70% for acceptable model performance in the acceptable prediction zone method. Thus, the secondary model for μ had acceptable goodness-of-fit for data used in model development and was validated against independent data.

In a previous study (37), when growth of *Salmonella* Typhimurium from a high initial density ($10^{4.8}$ CFU/g) on sterile chicken was investigated, N_{max} increased from 8.5 log CFU/g at 10°C to 10.2 log CFU/g at 12 to 40°C. In contrast, when growth of *Salmonella* Typhimurium DT104 from a high initial density ($10^{3.8}$ CFU/g) on ground chicken breast meat with a competitive microflora was investigated, N_{max} increased from 4.9 log CFU/g at 10°C to 9.8 log CFU/g at 26 to 40°C (39). In the present study, when growth of *Salmonella* Typhimurium DT104 from a low initial density ($10^{0.6}$ MPN or CFU/g) on ground chicken breast meat with a competitive microflora was investigated, N_{max} increased from 1.6 log MPN/g at 10°C to 9.4 log CFU/g at 34 to 40°C (Fig. 6). Although comparison of these studies is confounded by differences in initial density, the strain of *Salmonella* and other experimental variables, these results suggest that the competitive microflora of ground chicken breast meat reduces N_{max} and that the extent of the reduction of N_{max} depends on the temperature and initial density. In fact, greater reductions of N_{max} by the competitive microflora were observed at low temperatures and low initial density. However, at higher growth temperatures, N_{max} was much less affected by the competitive microflora.

The reduction in N_{max} by the competitive microflora can be explained by the effect described by Jameson (20) in which final density of *Salmonella* is determined by its growth rate relative to the competitive microflora. At low temperatures, growth rate of *Salmonella* is less than growth rate of cold-tolerant organisms in the competitive microflora (46, 47). Consequently, nutrient exhaustion by the faster

growing competitors at low temperatures prevents *Salmonella* from achieving the high final density observed in sterile food systems. At higher temperatures, growth rate of *Salmonella* is similar (47) or higher (46) than that of the competitive microflora resulting in N_{max} that are closer to those observed in sterile food systems. In addition, to the Jameson effect, reductions in N_{max} occur through other mechanisms such as those involving production of anti-*Salmonella* products (e.g., inhibitory peptides and organic acids) by competitors (4, 7, 10, 27). Thus, numbers and types or strains of competitors, which vary among chickens (13, 48), interact with storage conditions (e.g., temperature) to determine N_{max} of *Salmonella* Typhimurium DT104, which was observed to vary among batches of ground chicken breast meat in this and another study (39).

To evaluate performance of the secondary model for N_{max} , independent MPN and CFU data were collected, subjected to primary modeling (Fig. 4), and the resulting independent data for N_{max} were included in the graph for the secondary model (Fig. 6). In addition, performance of the secondary model for N_{max} was evaluated using the acceptable prediction zone method (Table 3). For data ($n = 6$) used in model development, 83% of predictions were in the acceptable prediction zone from an RE of -0.8 to 0.4 , whereas for data ($n = 4$) not used in model development 75% of predictions for N_{max} were acceptable. Both of these values (i.e., % RE) exceeded the criterion of 70% for acceptable model performance in the acceptable prediction zone method. Thus, the secondary model for N_{max} had acceptable goodness-of-fit for data used in model development and was validated against independent data.

The secondary models for Ω , PI, μ , and N_{max} were combined with the primary model in a computer spreadsheet to create a tertiary model for predicting the variation of *Salmonella* Typhimurium DT104 growth on ground chicken breast meat with a competitive microflora and from a low initial density as a function of time and temperature (10 to 40°C). To evaluate performance of the tertiary model (Fig. 7), the times and temperatures for individual MPN and CFU data ($n = 344$) used in model development (dependent data) were entered into the tertiary model and then if the observed MPN or CFU data were in the 95% PI predicted by the tertiary model, the tertiary model prediction was classified as acceptable. For dependent data, 322 of 344 MPN or CFU data were in the 95% PI predicted by the tertiary model for 93.3% acceptable predictions. This percentage of acceptable predictions exceeded the stated criterion (see "Materials and Methods" for an explanation) of 90% for acceptable performance and thus, the tertiary model was successfully verified.

To further evaluate performance of the tertiary model (Fig. 7), individual times and temperatures for MPN and CFU data ($n = 236$) not used in model development (independent data) were entered into the tertiary model and then if observed MPN or CFU data were in the 95% PI predicted by the tertiary model, the prediction was classified as acceptable. For independent data, 223 of 236 observed MPN and CFU data were in 95% PI predicted by the tertiary model for 94.5% acceptable predictions. This

Parameter	Units	Range	Input
Initial Density	log MPN or CFU/g		0.60
Time	h	156	20
Temperature	°C	10 to 40	12.8
			Output
Predicted Density	log MPN or CFU/g	Maximum	2.25
		Most likely	1.23
		Minimum	0.20

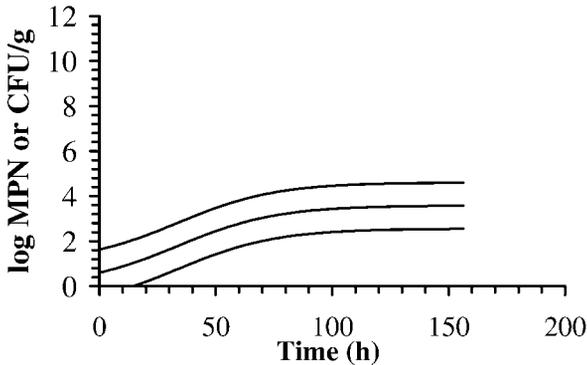


FIGURE 7. Tertiary model for predicting, as a function of time and temperature (10 to 40°C), variation of *Salmonella Typhimurium DT104* growth from a low initial density ($10^{0.6}$ MPN or CFU/g) on ground chicken breast meat with a competitive microflora. Most likely growth curve (middle line) and its 95% prediction interval (defined by lower and upper lines) for 12.8°C (55°F) and predicted minimum, most likely and maximum density of *Salmonella Typhimurium DT104* on ground chicken breast meat with a competitive microflora after 20 h at 12.8°C.

percentage of acceptable predictions exceeded the 90% criterion for acceptable performance and thus, the tertiary model was successfully validated.

The tertiary model developed and validated in this study predicts growth of *Salmonella Typhimurium DT104* from an initial average density of 0.6 log MPN or CFU/g or about four cells per gram of ground chicken breast meat. The reported size of *Salmonella Typhimurium* grown in the laboratory and inoculated onto chicken skin is $1.45 \pm 0.29 \mu\text{m}^2$ (22). If one examines electron micrographs at the micrometer level, it is apparent that these four inoculated cells of the pathogen would have experienced a heterogeneous microenvironment with respect to nutrients and competitive microflora (21). Consequently, ground chicken was not analyzed for nutrient or competitive microflora content because it was felt that average values for 1-, 10-, or 25-g samples would not accurately reflect the microenvironment experienced by the inoculated cells. In addition and as previously discussed (39), a pathogen growth model with an input for the competitive microflora would not find practical application in the food industry because it would take about 7 days to quantify the initial numbers of competitors and longer to determine the types of competitors present. Thus, by the time these test results would become available, the meat would either have been consumed or spoiled.

A similar conclusion was reached by Lebert et al. (23) who stated that “even if the average water content of a food sample is easily measured, it does not reflect the water content at the surface of the products.” Moreover, it is not possible to measure water activity at the surface of a food

(i.e., a microenvironment) because water activity is dynamic and methods do not exist for measuring water activity on such a small sample (23). Consequently, to predict effects of dynamic changes in water activity at the surface of food on microbial growth, Lebert et al. (23) developed a global model, which includes a microbial growth model, a water transfer model, and a thermodynamic model.

In addition to water activity, other factors in food are dynamic in both time and space (51); for example, pH, temperature, and the competitive microflora. Using the global modeling approach of Lebert et al. (23) to predict pathogen growth on food would require additional models to predict dynamic changes in time and space of these additional factors. The result would be a very complex global model that would be difficult to validate. Likewise, using the Bayesian approach of Delignette-Muller et al. (14) to model variability and uncertainty of the competitive microflora on pathogen growth would result in a very complex model that would be difficult to validate.

An alternative and more parsimonious approach to modeling pathogen growth in response to dynamic changes in time and space of both abiotic and biotic factors in food microenvironments was demonstrated in this research and in a previous study by Geysen et al. (16), namely, use of a 95% prediction interval to capture and predict variation of pathogen growth among batches of food with competitive microflora. For example, for the temperature abuse scenario (20 h at 12.8°C) illustrated in Figure 7, the tertiary model developed in this study predicted a 95% PI of 2.05 log MPN or CFU/g and a minimum, most likely and maximum pathogen density of $10^{0.20}$, $10^{1.23}$, and $10^{2.25}$ MPN or CFU/g, respectively. When interpreting this prediction, no assumptions are made or necessary as to why pathogen density after 20 h at 12.8°C is predicted to vary from $10^{0.20}$ to $10^{2.25}$ MPN or CFU/g. Rather, it is recognized that variation of abiotic and biotic factors in the food among batches and in time and space can explain the predicted range of pathogen density for a specified time and temperature scenario. Such stochastic predictions of pathogen growth will be of particular value for improving predictions of microbial risk assessments for *Salmonella* and poultry meat (6, 29, 36, 49).

The tertiary model developed and validated in this study was not tested for the ability to predict growth of other strains of *Salmonella* because there were no other strains available that had a phenotype that would allow collection of growth data in ground chicken breast meat with a competitive microflora. The strain of *Salmonella Typhimurium DT104* used in this study has several characteristics that make it a good strain for modeling *Salmonella* behavior in chicken with a competitive microflora. First, the strain occurs in beef, but is rarely isolated from chicken (18). Thus, interference from indigenous populations is not a concern in modeling growth from low initial densities. Second, the strain is resistant to multiple antibiotics, with typical resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines (ACSSuT) (9). Thus, a selective media with multiple antibiotics can be used to inhibit competitors and follow behavior of the strain even

in the presence of high numbers of competitive microflora. Third, the antibiotic resistance genes are carried in the chromosome, which means the MAR phenotype is stable (9). Fourth, growth of the DT104 strain used in this study is similar to other strains of *Salmonella* in sterile food systems (data not shown). Although this does not validate that it behaves similar to other strains of *Salmonella* in chicken with a competitive microflora it does offer a degree of assurance that it does provide reasonable predictions of *Salmonella* growth in chicken with a competitive microflora.

There are clearly important strain variations in the growth of *Salmonella* (15, 28, 33, 40). To better characterize this variation and develop better models, methods are needed where the strains common to a particular food can be isolated and transformed to a phenotype that can be followed in the presence of competitive microflora. We have attempted to do this by genetically engineering strains of *Salmonella* to express the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (35, 40). However, thus far, our attempts to produce GFP strains that behave the same as the parents have been unsuccessful. Under most growth conditions examined, the GFP strain grows slower than the parent strain (35).

Similar to the current study, Mackey and Kerridge (24) modeled growth of *Salmonella* in a ground meat (i.e., minced beef) with a competitive microflora. However, they used a cocktail of strains (Thompson, Stanley, and Infantis) selected for resistance to multiple antibiotics (nalidixic acid and rifampin) rather than a single MAR strain that occurs in nature. Nonetheless, similar to our research, a selective medium (brilliant green agar) with multiple antibiotics was used to quantify pathogen growth from a low (40 CFU/g) or high (10,000 CFU/g) initial density. In contrast to our research, initial density did not affect λ or μ as a function of temperature (10 to 35°C) in minced beef. In ground chicken breast meat with a competitive microflora, λ was not always apparent when *Salmonella* Typhimurium DT104 was inoculated at a low ($10^{0.6}$ MPN or CFU/g) initial density, but λ was consistently observed when the pathogen was inoculated at a high ($10^{3.8}$ CFU/g) initial density (39). Another difference was that N_{\max} of *Salmonella* in minced beef was $10^{8.5}$ CFU/g at 15°C (24) as compared to a predicted N_{\max} of $10^{4.7}$ CFU/g (95% PI = $10^{3.4}$ to 10^6 CFU/g) at 15°C in this research. Differences in strains of *Salmonella*, type of meat, types and numbers of competing microflora, and other experimental conditions and modeling methods can explain differences in results between our research and that of Mackey and Kerridge (24). Regardless, these studies demonstrate that it is possible to investigate and model growth of *Salmonella* in poultry and red meat with competitive microflora using strains resistant to multiple antibiotics.

In summary, objectives of this research to develop and validate a tertiary model that predicts variation of *Salmonella* growth among batches of ground chicken breast meat with a competitive microflora and from a lower initial density were accomplished. Of note, MPN and CFU data were used in tandem to model growth of *Salmonella* from a low initial density (i.e., $10^{0.6}$ MPN or CFU/g) that is very close

to the mean initial density (i.e., $10^{0.1}$ MPN/g) of *Salmonella* levels reported in a recent survey of retail ground chicken samples by FSIS (1). In addition, a simple stochastic method (i.e., 95% PI) and a simple validation method (i.e., 90% concordance) were developed for modeling variation of *Salmonella* growth among batches of ground chicken breast meat with a competitive microflora. These methods offer a simple alternative to more complex modeling approaches being developed for predicting growth of pathogens in food with competitive microflora. The validated model will help chicken processors and risk assessors to better predict safety of ground chicken breast meat exposed to times and temperatures that support growth of *Salmonella*.

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