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Neural Network Model for Survival and Growth of *Salmonella enterica* Serotype 8,20:–:z_6 in Ground Chicken Thigh Meat during Cold Storage: Extrapolation to Other Serotypes†

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ABSTRACT

Mathematical models that predict the behavior of human bacterial pathogens in food are valuable tools for assessing and managing this risk to public health. A study was undertaken to develop a model for predicting the behavior of *Salmonella enterica* serotype 8,20:–:z_6 in chicken meat during cold storage and to determine how well the model would predict the behavior of other serotypes of *Salmonella* stored under the same conditions. To develop the model, ground chicken thigh meat (0.75 cm^3) was inoculated with 1.7 log *Salmonella* 8,20:–:z_6 and then stored for 0 to 8 days at –8 to 16°C. An automated miniaturized most-probable-number (MPN) method was developed and used for the enumeration of *Salmonella*. Commercial software (Excel and the add-in program NeuralTools) was used to develop a multilayer feedforward neural network model with one hidden layer of two nodes. The performance of the model was evaluated using the acceptable prediction zone (APZ) method. The number of *Salmonella* in ground chicken thigh meat stayed the same ($P > 0.05$) during 8 days of storage at –8 to 8°C but increased ($P < 0.05$) during storage at 9°C (+0.6 log) to 16°C (+5.1 log). The proportion of residual values (observed minus predicted values) in an APZ (pAPZ) from –1 log (fail-safe) to 0.5 log (fail-dangerous) was 0.939 for the data ($n = 426$ log MPN values) used in the development of the model. The model had a pAPZ of 0.944 or 0.954 when it was extrapolated to test data ($n = 108$ log MPN per serotype) for other serotypes (*S. enterica* serotype Typhimurium var 5–, Kentucky, Typhimurium, and Thompson) of *Salmonella* in ground chicken thigh meat stored for 0 to 8 days at –4, 4, 12, or 16°C under the same experimental conditions. A pAPZ of $\geq$0.7 indicates that a model provides predictions with acceptable bias and accuracy. Thus, the results indicated that the model provided valid predictions of the survival and growth of *Salmonella* 8,20:–:z_6 in ground chicken thigh meat stored for 0 to 8 days at –8 to 16°C and that the model was validated for extrapolation to four other serotypes of *Salmonella*.

Salmonella bacteria are a leading cause of foodborne illness in the United States *(24),* and chicken meat is often identified as an important source of human exposure to this pathogen *(2)*. Although chicken producers, in general, do a good job of delivering product to consumers that has low prevalence and numbers of *Salmonella* bacteria at retail *(23, 26, 31),* failure of consumers to properly refrigerate chicken can result in rapid growth of low numbers of *Salmonella* cells to high and dangerous levels. The time for cold storage of chicken meat in the home has been found to range from 0 to 5 days with a most likely time of 2 days *(28)* or from 0.5 to 10 days with a most likely time of 2 days *(4),* depending on the population of consumers surveyed. The temperature of cold storage in the home refrigerator has been found to range from 0.8 to 12.6°C *(5)* or from –7.9 to 20.7°C *(10, 21),* depending on the population whose refrigerators are surveyed. There are also significant temperature gradients within refrigerators and significant fluctuations in temperature within refrigerators depending on loading conditions, frequency and duration of door openings, and type of refrigerator *(7, 8)*. Thus, under some cold-storage conditions and practices in the home, low levels of *Salmonella* in chicken meat could grow to high and dangerous levels at the time of meal preparation and result in considerable risk of foodborne illness from increased bacterial survival during undercooking or from increased cross-contamination of ready-to-eat food.

Mathematical models that predict the behavior of *Salmonella* in chicken meat during cold storage are valuable tools for helping to assess and manage this risk to public health, and several models of this type have been developed. Zhou et al. *(32)* developed a model for predicting the growth of *Salmonella enterica* serotype Typhimurium (10^3/g) in chicken meat as a function of time, temperature (4, 8, 20, or 37°C), and sodium chloride level (0 to 9%). Pradhan et al. *(22)* modeled the survival and growth of *Salmonella* Typhimurium ($10^{4.7}$/g) on chicken breast meat as a function of time (0 to 20 days) and temperature (–20, –12, 0, 4, or 8°C). Juneja et al. *(9)* developed a model for the growth of a

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Salmonella (10^3/g) cocktail ($n = 5$ serotypes) on irradiated chicken breast meat (i.e., with no microbial competition) as a function of time and temperature (10 to 45°C). Oscar *(17)* developed a model for the survival and growth of *Salmonella* Typhimurium strain DT104 ($10^{0.9}$ per portion) on chicken skin stored for 0 to 10 days at 4 to 12°C. However, these models do not cover the entire range of temperatures encountered during storage of chicken meat in a domestic refrigerator, some were developed with high inoculum sizes, they are not validated for other types of *Salmonella*, and they do not all account for microbial competition. Thus, there is a need to develop additional models that predict the behavior of *Salmonella* in chicken meat during cold-storage conditions encountered in the home.

In a recent study *(19),* the survival and growth of *Salmonella* Typhimurium DT104, a multiple-antibiotic-resistant strain, on chicken meat ($10^{2.8}$ per portion) during cold storage as a function of time (0 to 8 days), temperature (–8 to 16°C), and type of meat (white, dark, or skin) was investigated and modeled. Although this model covers the entire range of temperatures encountered during cold storage of chicken meat in the home refrigerator and accounts for microbial competition, the enumeration method used to collect data for the development and validation of the model is limited to strains of *Salmonella* with the same multiple-antibiotic-resistant profile as *Salmonella* Typhimurium DT104, and thus, the model cannot be validated for all types of *Salmonella* found in chicken meat. Consequently, a new method (i.e., an automated miniaturized most-probable-number (MPN) method for *Salmonella*) that does not require an antibiotic-resistant strain or antibiotics in the enumeration medium was developed and used for the first time in the present study to investigate and model the behavior of *Salmonella* in chicken meat during cold storage. Specifically, the current study was undertaken to develop and validate a model for predicting the behavior of *S. enterica* serotype 8,20:–:z_6 in ground chicken thigh meat during cold storage and then to evaluate the model for its ability to predict the behavior of other serotypes of *Salmonella* in ground chicken thigh meat stored under the same conditions. In the previous study *(19),* the growth of *Salmonella* Typhimurium DT104 on chicken meat during cold storage was highest on dark meat, intermediate on skin, and lowest on white meat. Consequently, thigh meat was used in the present study to develop a model that would be fail-safe when extrapolated to skin or white meat.

MATERIALS AND METHODS

Salmonella. *S. enterica* serotypes 8,20:–:z_6, Typhimurium var 5–, Kentucky, Typhimurium, and Thompson were isolated from chicken meat obtained at retail *(20)*. Stock cultures of these *Salmonella* serotypes were maintained at –80°C in brain heart infusion broth (BD, Sparks, MD) that contained 15% glycerol (Sigma, St. Louis, MO).

Chicken meat preparation. Chicken thigh meat with native microflora obtained from a local retail store was cubed and then ground through coarse and fine plates of a table-top meat grinder (model 586.8 Zelmer, The Sausage Maker, Buffalo, NY). Portions (75 g) of the ground meat were packed into plastic petri dishes (100 by 15 mm), frozen at –20°C, and then cut into cylindrical portions (0.75 cm^3) with a cork borer (#5). The small portions (0.75 cm^3) were transferred to 1.5-ml microcentrifuge tubes (Eppendorf Flex-Tubes, Thomas Scientific, Swedesboro, NJ) and stored at –20°C until used in experiments.

Inoculum culture. A 5-μl amount of the appropriate serotype of *Salmonella* from stock culture was transferred to 9 ml of buffered peptone water (BPW; BD) in a glass dilution tube (16 by 125 mm), and the dilution tube was capped. Cultures were then incubated for 72 h at 22°C without shaking. Just before the initiation of a storage trial, the culture was serially diluted (1:10) in BPW to 10^{-5}.

Storage trials. Small portions of chicken (0.75 cm^3) were thawed (30 min at 22°C) and then inoculated (5 μl of a 10^{-5} dilution of the inoculum culture) in their centers to an initial *Salmonella* level of ca. 1.7 log. Inoculated portions at 22°C were then incubated for 0 to 8 days at –8 to 16°C in a heating and cooling block (ThermoStat Plus, Eppendorf, Hamburgh, Germany, or Grant-bio PCH1, Grant Instruments [Cambridge] Ltd., Shepreth, Cambridgeshire, UK). This was done to simulate and model the dynamic temperature shifts experienced by *Salmonella* on chicken meat when it is stored by consumers in a domestic refrigerator/freezer.

Experimental designs. To develop the model, a 6×13 full factorial design that included time (0, 1, 2, 4, 6, and 8 days) and temperature (–8, –4, 0, 4, 8, 9, 10, 11, 12, 13, 14, 15, and 16°C) with *Salmonella* serotype 8,20:–:z_6 was used. However, it was not always possible to collect samples on the planned sampling days; for this reason, some samples were collected on days 3 and 5 of cold storage. To validate the model for extrapolation, a $4 \times 6 \times 4$ full factorial design including *Salmonella* serotype (Typhimurium var 5–, Kentucky, Typhimurium, and Thompson), time (0, 1, 2, 4, 6, and 8 days), and temperature (–4, 4, 12, and 16°C) was used. From two (survival conditions) to six (growth conditions) replicate trials were conducted per combination of temperature and serotype.

Sampling. One or two samples of chicken portions inoculated with *Salmonella* (ca. 1.7 log) were processed individually at each sampling time in a storage trial. The *Salmonella*-inoculated chicken portion (0.75 cm^3) was transferred to a 207-ml plastic bag with a filter screen (Whirl-Pak, Nasco, Fort Atkinson, WI) that contained 9 ml of BPW. The sample was processed with a Pulsifier (model PUL 100, Microbiology International, Frederick, MD) for 1 min to recover *Salmonella* into BPW for enumeration.

MPN assay. A 3 (replicate) × 8 (dilution) MPN assay was performed in 2.0-ml 96-well deep-well plates (Axygen Scientific, Union City, CA). A 1-ml amount of a 10^0, 10^{-1}, or 10^{-2} serial dilution of a Pulsifier-treated sample in BPW was added to an empty cell in the first row of a 96-well deep-well plate that contained 0.9 ml of BPW in all the other wells. After the first row was filled with samples ($n = 3$ replicates per chicken sample), serial dilutions (1:10) were performed by a robotic pipettor (SOLO Plus, Hudson Robotics, Springfield, NJ). After incubation (24 h at 40°C), 10 μl from each well of the 96-well deep-well plate used for preenrichment in BPW was transferred by the robotic pipettor to corresponding wells of a second 96-well deep-well plate that contained 1 ml of Rappaport-Vassiliadis broth (RVB; BD) per well. After incubation (24 h at 42°C), 2 μl from each well of the 96-well deep-well plate used for selective enrichment in RVB was

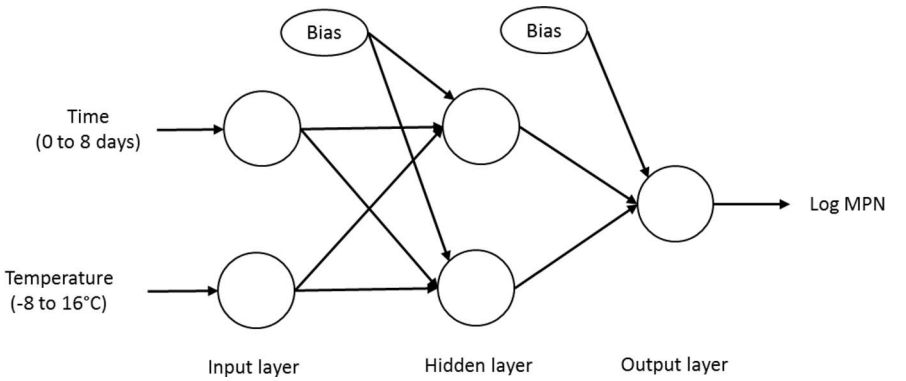

FIGURE 1. *Structure of the multilayer feedforward neural network model with a single hidden layer of two nodes for predicting log most probable number (MPN) values of* Salmonella *8,20:−:z_6 in ground chicken thigh meat as a function of time (0 to 8 days) and temperature (−8 to 16°C) of cold storage.*

drop plated by the robotic pipettor onto xylose lysine Tergitol 4 (XLT4; BD) agar plates ($n = 4$ per 96-well deep-well plate) in a 6-by-4 pattern. After incubation (24 h at 40°C), positive (black) and negative (no visible result) drops on XLT4 were recorded. The XLT4 step was discovered to be optional because there was consistent agreement between positive (black) and negative (no visible result) drops on XLT4 and positive (white) and negative (blue) wells in RVB. In addition, an AOAC International–approved lateral flow assay for *Salmonella* (Reveal 2.0, Neogen, Lansing, MI) was used to validate the results of the miniaturized MPN method where in all cases white wells in RVB tested positive for *Salmonella,* whereas blue wells in RVB tested negative for *Salmonella*. MPN values (log per portion) were calculated by the method of Thomas *(29)* as previously described *(19)*.

Model development. Data ($n = 426$ log MPN values) for *Salmonella* 8,20:−:z_6 were used for model development. A data set was created in a computer spreadsheet (Excel 2013, Microsoft Corporation, Redmond, WA) with four columns: (i) tag, (ii) temperature, (iii) time, and (iv) MPN. Within the data for a temperature and time, the highest 70 to 80% of log MPN values were tagged as training data ($n = 310$), and the lowest 20 to 30% of log MPN values were tagged as testing data ($n = 116$). This planned tagging of data was done to develop a fail-safe model and to improve the model's performance as described below. To evaluate this approach, a second data set was created with only three columns: (i) temperature, (ii) time, and (iii) MPN. Data for training ($n = 310$ log MPN values) and testing ($n = 116$ log MPN values) in this second data set were randomly tagged, using a random number generator seed of 1 to initiate the random tagging process.

A spreadsheet add-in program (NeuralTools 6.12, Palisade Corporation, Ithaca, NY) was used to randomly tag MPN data in the second data set and to develop a general regression neural network (GRNN) model, as previously described *(16),* as well as multilayer feedforward neural network models with single hidden layers of two (MLF2), three (MLF3), or four (MLF4) nodes. Figure 1 shows the structure of the MLF2 model.

To develop the MLF models, the software (NeuralTools) used the hyperbolic tangent function as the activation function for neurons in the hidden layer and the identity function as the activation function for the output neuron. Connection weight (w_{ij}) and bias (b_j) terms for each connection and neuron, respectively, were determined using a combination of the conjugate gradient descent and simulated annealing methods *(11)*; this was done to reduce the risk of finding the local minimum rather than the desired global minimum. The formula used to calculate input from the jth neuron (X_j) into the activation functions *(27)* was as follows:

$$X_j = \sum_{i=1}^{n} f(w_{ij} y_i + b_j)$$

where y_i was the ith input value and n was the number of neurons. Connection weights and bias terms were not provided by the neural network software for proprietary reasons. However, a stand-alone version of the model will be made available on the author's Web site, www.ars.usda.gov/naa/errc/PoultryFARM.

Model performance. The acceptable prediction zone (APZ) method was used to evaluate the model's performance *(14, 19)*. This method involves three sequential evaluation steps: (i) goodness of fit, (ii) validation for interpolation, and (iii) validation for extrapolation. The APZ method has criteria for test data and model performance that must be met in each sequential step for a model to be classified as validated. The criteria for test data for interpolation are that they must be independent (i.e., not used to train or develop the model), they must be collected with the same methods as used to collect dependent data for model development so as not to confound comparison of observed and predicted values, and they must provide sufficient and appropriate coverage of the model's predictions to allow an unbiased and complete assessment of the model's performance.

For models that predict log counts, such as those in the current study, a model is considered to provide predictions with acceptable bias and accuracy when the proportion of residual values (observed minus predicted values) in an APZ (pAPZ) from −1 log (fail-safe) to 0.5 log (fail-dangerous) is ≥0.7 *(14, 15)*. Moreover, a neural network model is considered validated for interpolation when the pAPZ is ≥0.7 for data used to train (i.e., dependent data) and test (i.e., independent data for interpolation) the model and the criteria for test data are satisfied.

The analytical units for evaluation of model performance in this study were complete data sets, individual survival or growth curves, and individual combinations of independent variables. A local prediction problem was defined as three consecutive times within a combination of serotype and temperature where the pAPZ was <0.70. To validate the model for extrapolation to other serotypes of *Salmonella,* the same test data and model performance criteria were used, except that test data could differ from dependent data in one respect and one respect only—the test variable, or in this case, the serotype of *Salmonella* used to collect the data.

Statistical analysis. To provide an objective assessment of *Salmonella* behavior in ground chicken thigh meat, one-way analysis of variance (ANOVA) was performed within temperature and serotype to determine whether the *Salmonella* numbers stayed the same (survival), decreased (death), or increased (growth) as a function of the time of cold storage. When time had a significant effect ($P < 0.05$), the mean *Salmonella* numbers for days 1 to 8 of

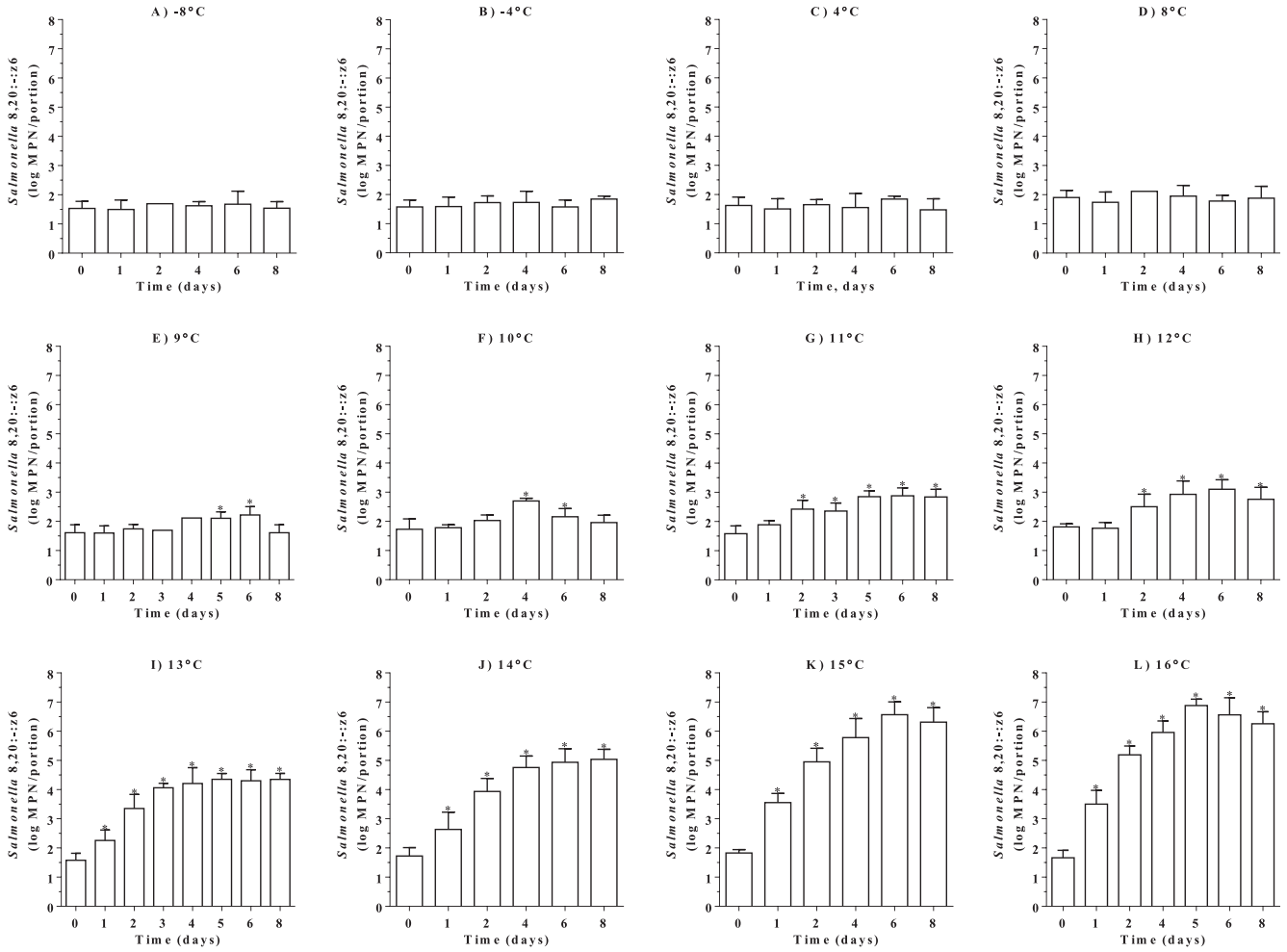

FIGURE 2. *Survival and growth of* Salmonella *8,20:–:z*$_6$ *in ground chicken thigh meat stored for 0 to 8 days at (A) –8°C, (B) –4°C, (C) 4°C, (D) 8°C, (E) 9°C, (F) 10°C, (G) 11°C, (H) 12°C, (I) 13°C, (J) 14°C, (K) 15°C, or (L) 16°C. Results for 0°C are not shown. Bars and error bars show means and standard deviations, respectively. Bars with an asterisk represent means that are significantly (*$P < 0.05$*) different from the mean at zero days of storage as determined by one-way ANOVA followed by Dunnett's multiple comparison test.*

cold storage were compared with the mean *Salmonella* number for 0 days of cold storage using Dunnett's multiple comparison test ($P < 0.05$).

To provide an objective assessment of the effect of serotype ($n = 5$) on the behavior of *Salmonella,* one-way ANOVA was applied within combinations of time (0, 1, 2, 4, 6, or 8 days) and temperature (–4, 4, 12, or 16°C) of cold storage to evaluate the effect of serotype on *Salmonella* numbers. When serotype had a significant effect ($P < 0.05$), all pairwise comparisons ($n = 9$) of mean *Salmonella* numbers were made among serotypes using Tukey's multiple comparison test ($P < 0.05$). Statistical analyses were performed using a commercial software program (Prism, version 6, GraphPad Software, San Diego, CA).

RESULTS

Effects of time and temperature on the behavior of *Salmonella* in ground chicken thigh meat during cold storage. The numbers of *Salmonella* 8,20:–:z$_6$ in ground chicken thigh meat stayed the same ($P > 0.05$) during storage for 0 to 8 days at –8 to 8°C but increased ($P < 0.05$) during storage for 0 to 8 days at 9 to 16°C (Fig. 2). Similar results were obtained for the four other serotypes of *Salmonella* investigated (results not shown). Thus, during 8 days of cold storage in ground chicken thigh meat with native microflora, *Salmonella* survived at temperatures from –8 to 8°C and grew at temperatures from 9 to 16°C.

Effect of serotype on numbers of *Salmonella* in ground chicken thigh meat during cold storage. The numbers of *Salmonella* in ground chicken thigh meat were not affected ($P > 0.05$) by serotype during storage for 0 to 8 days at –4, 4, or 12°C (results not shown). However, at 16°C, serotype affected ($P < 0.05$) the numbers of *Salmonella* in ground chicken thigh meat at 4, 6, and 8 days of storage but not at 0, 1, or 2 days of storage (Fig. 3). The mean numbers of *Salmonella* 8,20:–:z$_6$ and Kentucky in ground chicken thigh meat during extended storage (4, 6, or 8 days) at 16°C were slightly lower (<1 log) than the mean numbers of *Salmonella* Typhimurium and Thompson.

Development of a model for the behavior of *Salmonella* 8,20:–:z$_6$ in ground chicken thigh meat during cold storage. Enumeration data ($n = 426$ log MPN values) for *Salmonella* 8,20:–:z$_6$ with planned tagging were used to develop the GRNN, MLF2, MLF3, and MLF4

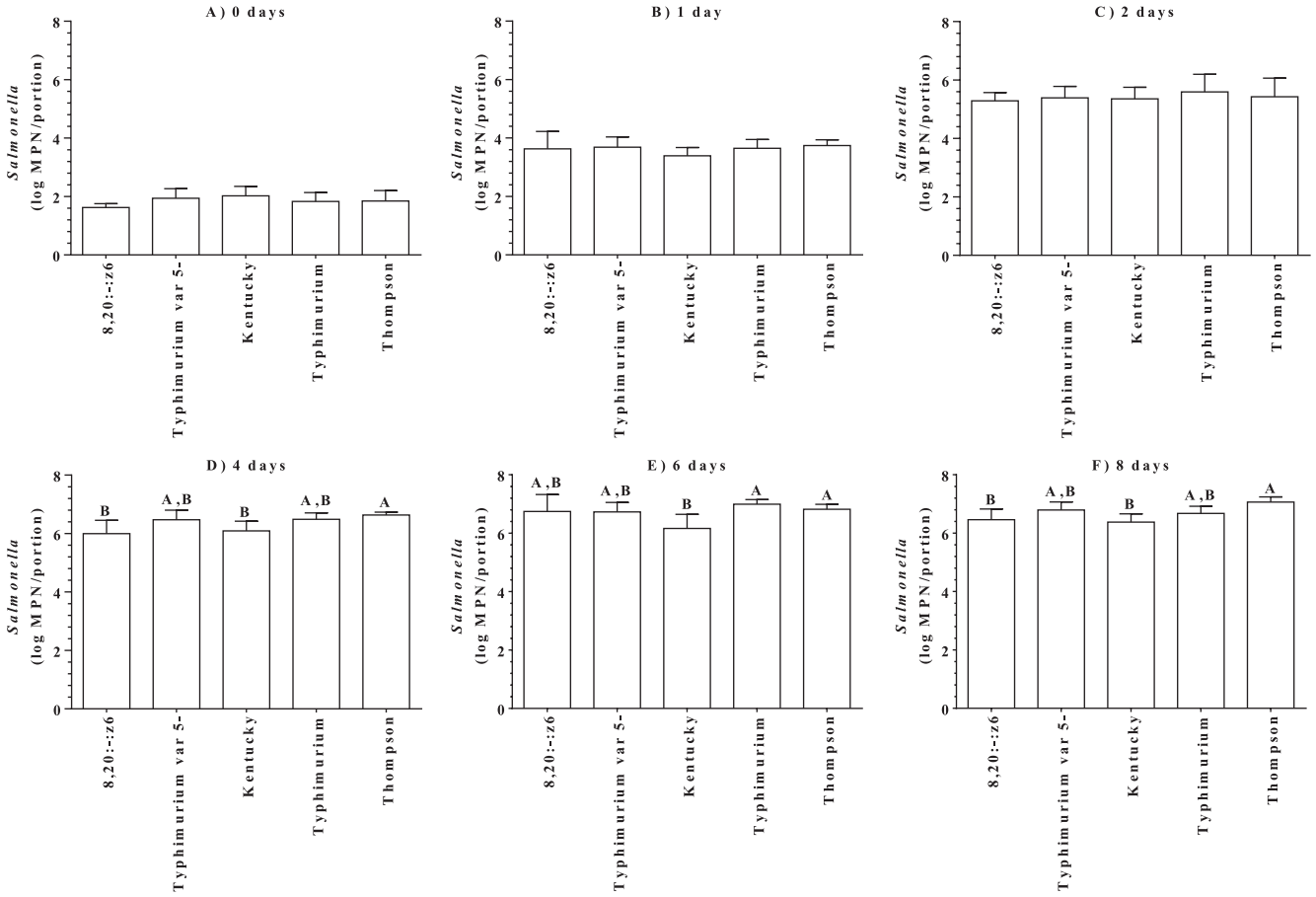

FIGURE 3. *Effect of serotype on survival and growth of* Salmonella *in ground chicken thigh meat stored at 16°C for (A) 0 days, (B) 1 day, (C) 2 days, (D) 4 days, (E) 6 days, or (F) 8 days. Bars and error bars show means and standard deviations, respectively. Means within a day of storage with different letters are significantly (*$P < 0.05$*) different as determined by one-way ANOVA followed by Tukey's multiple comparison test.*

models. All four models had acceptable pAPZs (Table 1), and plots of the residual values indicated no local prediction problems or systematic prediction bias (results not shown). The rank order from best to worst for the complete data set based on the pAPZs was GRNN, then MLF4, then MLF3, and finally, MLF2. However, the predicted survival and growth curves for GRNN, MLF3, and MLF4 were wavy compared with the predicted survival and growth curves for MLF2 (Fig. 4), a sign of overtraining. Thus, MLF2 was selected as the best model for generalization.

Effect of tagging method on model performance. The effect of the tagging method (planned versus random) on the performance of the MLF2 model was evaluated. Both tagging methods produced MLF2 models with acceptable predictions (pAPZ $\geq$ 0.7) for individual survival and growth curves and for complete data sets, with the exception of the growth curve at 15°C for the random tagging method, where the pAPZ was 0.667 (Table 1). A plot of residual values for the MLF2 model developed by random tagging of data indicted no local prediction problems or systematic prediction bias except for 4, 6, and 8 days of storage at 15°C, where it provided overly fail-dangerous predictions. Similar to the MLF2 model developed by planned tagging, the MLF2 model developed by random tagging provided predicted survival and growth curves that were smoother in appearance than those provided by the GRNN, MLF3, and MLF4 models developed by planned tagging. Overall, the performance of the MLF2 model developed by planned tagging (pAPZ = 0.939) was better than that of the MLF2 model developed by random tagging (pAPZ = 0.883) of data (Table 1). As expected, the MLF2 model with planned tagging had an average bias (i.e., mean residual value) that was slightly fail-safe (–0.14 log), whereas the MLF2 model with random tagging had no average bias (0.00 log).

Performance of the MLF2 model for *Salmonella* 8,20:–:z_6 for extrapolation to other serotypes of *Salmonella*. The ability of the MLF2 model developed with planned tagging to be extrapolated to other serotypes of *Salmonella* was evaluated for test data collected during 0 to 8 days of storage at –4, 4, 12, or 16°C. For all four serotypes tested, the MLF2 model for *Salmonella* 8,20:–:z_6 provided acceptable predictions (pAPZ $\geq$ 0.70) of individual survival and growth curves and for complete data sets (Table 2), and there were no signs of local prediction problems or systematic prediction bias (results not shown).

TABLE 1. *Acceptable prediction zone analysis of neural network models for predicting the survival and growth of* Salmonella *8,20:–:z_6 in ground chicken thigh meat*[a]

Temp (°C)	No. of log MPN values	pAPZ for indicated model and data-tagging method				
		GRNN	MLF2		MLF3	MLF4
		Planned	Planned	Random	Planned	Planned
−8	21	1.000	1.000	1.000	1.000	1.000
−4	24	1.000	1.000	1.000	1.000	1.000
0	14	1.000	1.000	1.000	1.000	1.000
4	21	1.000	1.000	1.000	1.000	0.952
8	17	1.000	1.000	0.882	1.000	1.000
9	36	1.000	0.972	0.944	1.000	1.000
10	43	1.000	0.907	0.814	0.953	1.000
11	32	1.000	0.938	0.906	1.000	1.000
12	41	0.976	0.951	0.951	0.976	0.976
13	35	0.971	0.886	0.800	0.914	0.943
14	40	0.975	0.950	0.925	0.900	0.900
15	21	0.857	0.810	0.667	0.762	0.762
16	80	0.975	0.925	0.813	0.963	0.963
All	426	0.981	0.939	0.883	0.955	0.960

[a] Data for training ($n = 310$ log MPN values) and testing ($n = 116$ log MPN values) of a general regression neural network (GRNN) model or multilayer feedforward neural network models with a single hidden layer of two (MLF2), three (MLF3), or four (MLF4) nodes were manually selected (planned) or randomly selected (random) as described in "Materials and Methods." Data shown are the proportions of residual values (observed minus predicted values) in an acceptable prediction zone (pAPZ) from −1 log (fail-safe) to 0.5 log (fail-dangerous); training and testing data are combined.

DISCUSSION

Most servings of chicken meat are not contaminated with *Salmonella,* and those that are usually contain only a few cells *(12, 18)*. The types of *Salmonella* found in chicken meat vary and are affected by several factors, such as flock, chicken house, farm, processing plant, company, and geographic region *(1, 30)*. The ideal model for predicting the behavior of *Salmonella* in chicken meat is one that is developed with chicken meat and a low number of *Salmonella* cells isolated from that chicken meat. However, in the past, the main hurdle to developing this type of model has been the lack of a method to enumerate low to high levels of any type of *Salmonella* that might be found in chicken meat.

Prior to this study, the natural antibiotic resistance of four isolates of *Salmonella* and small portions of chicken (≤1 g) were used to acquire data needed to develop models for predicting the survival and growth of a low initial number (<1 log per portion) of *Salmonella* cells in chicken meat with native microflora *(15, 16)*. However, this approach requires knowledge of the antibiotic resistance profile of the isolate and the preparation of specialty enumeration media with a defined mixture of antibiotics. Moreover, it will not work with isolates that are susceptible to antibiotics. Nonetheless, it was realized during the course of previous studies that *Salmonella* cells could be enumerated without knowledge of their antibiotic resistance profile or the use of antibiotics in enumeration media by using small portions of chicken meat (≤1 g) and a three-step MPN method involving (i) preenrichment in BPW, (ii) selective enrichment in RVB, and (iii) selective drop plating onto XLT4 agar. However, in previous studies, this method was performed in large tubes with 10 ml of medium, which made it labor-intensive and expensive and limited the amount of data that could be collected in a given period of time.

The MPN method for enumeration of *Salmonella* can be made more time and cost-effective by miniaturizing and automating it. To accomplish this, the MPN assay used in previous studies was reduced in volume from 10 ml in large tubes to 1 ml in 2-ml, 96-well deep-well plates, and a robotic pipettor was designed to perform the serial dilution, transfer, and drop-plating steps of the method. The method was then deployed for the first time in the present study to efficiently acquire the large amount of data needed to develop and validate a model for the behavior of *Salmonella* in chicken meat during cold storage. This involved successfully applying the new method to five chicken meat isolates of *Salmonella* whose antibiotic resistance profiles were not known.

One of the most expensive aspects of the automated miniaturized MPN method was its consumption of pipet tips. The procedure required 4 1/4 boxes of pipet tips per storage trial with eight portions of chicken meat. However, it was observed that the MPN results obtained on XLT4 matched those obtained in RVB. Thus, the third step of the new method was found to be optional. Omission of the third step saves two boxes of pipet tips and eight XLT4 plates (actually, it eliminates the need for XLT4 plates) per storage trial without loss of data quality or quantity.

Four neural network models (i.e., GRNN, MLF2, MLF3, and MLF4) were developed in the present study. The predictions of the models were evaluated using the test data criteria and model performance criteria of the APZ method *(19)*. All of the models were found to provide acceptable predictions (pAPZ ≥ 0.7) of the data used to train and test them; thus, all had acceptable goodness of fit and were successfully validated for interpolation. However, some signs of overtraining were observed in three models (GRNN, MLF3, and MLF4). Namely, the predicted survival and growth curves were wavy rather than smooth in appearance, indicating that the predictions were following fluctuations in the training data rather than predicting the general response of the dependent variable as a function of the independent variables. The neural network that was found to provide acceptable predictions with the smoothest curves (i.e., the least overtraining) was the MLF2. Thus, it was selected as the best model for generalization and further evaluation.

To improve the performance and to create a slightly fail-safe model, MPN data were manually tagged into training and testing data. Within a combination of time and temperature, the highest 70 to 80% of MPN data were tagged for training, whereas the lowest 20 to 30% of MPN data were tagged for testing. Compared with an MLF2 model developed with a random allocation of MPN data to training and testing sets, the MLF2 model developed by manual tagging had more residual values in the APZ (i.e.,

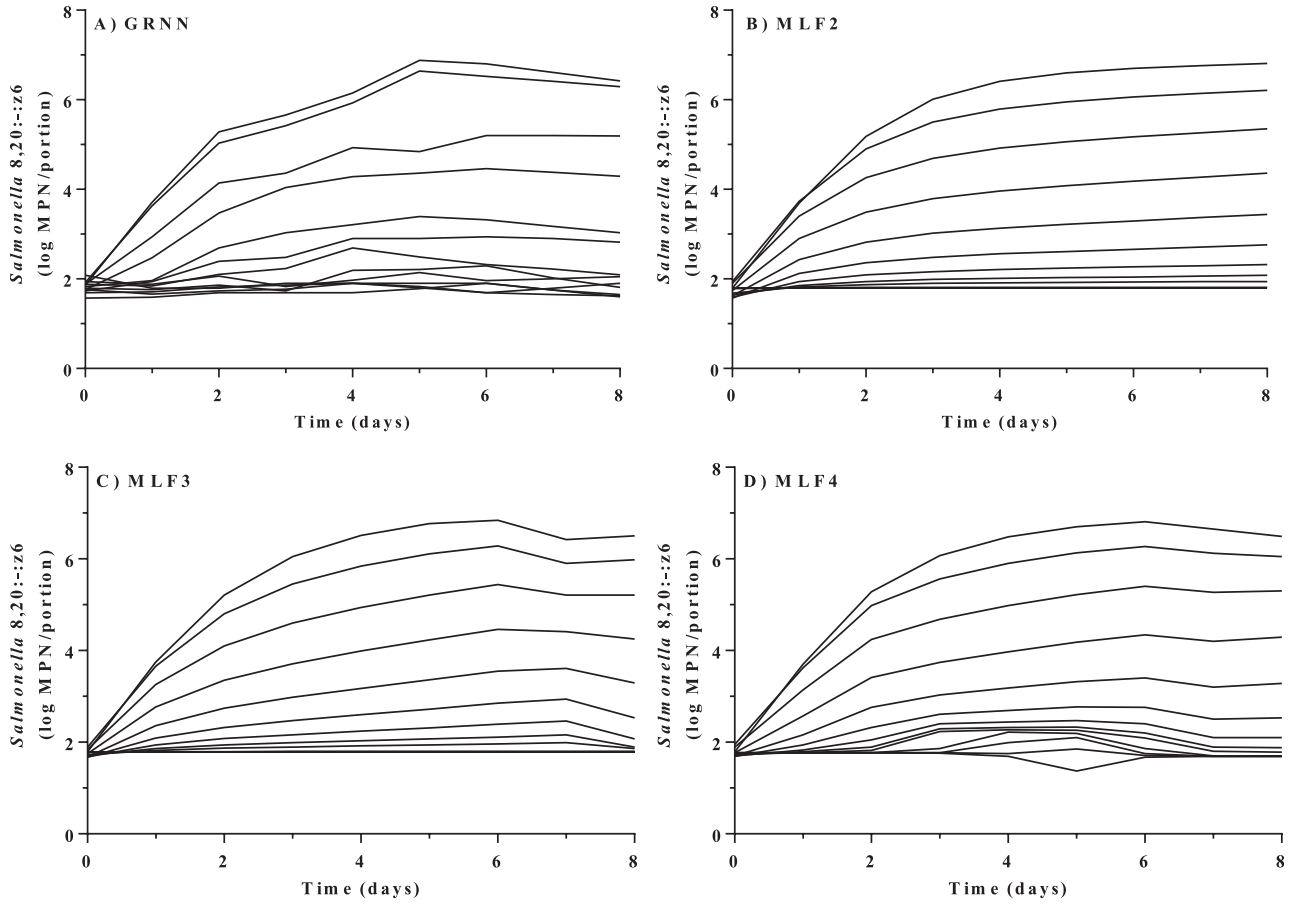

FIGURE 4. *Survival and growth curves for* Salmonella *8,20:–:z$_6$ in ground chicken thigh meat as predicted by (A) a general regression neural network (GRNN) model or multilayer feedforward neural network models with a single hidden layer of (B) two nodes (MLF2), (C) three nodes (MLF3), or (D) four nodes (MLF4). Each graph contains 13 lines corresponding to the 13 test temperatures: from top to bottom, 16, 15, 14, 13, 12, 11, 10, 9, 8, 4, 0, –4, and –8°C.*

pAPZ = 0.939 versus 0.883). This was expected because the APZ is twice as wide in the fail-safe direction (–1 log) than in the fail-dangerous (0.5 log) direction, and by purposely segregating the highest MPN values into the training data set, the resulting model tended to overpredict the number of *Salmonella* cells and drive the residual values in the direction of the wider part of the APZ, thus creating a fail-safe model with better performance for the dependent data.

TABLE 2. *Acceptable prediction zone analysis of the extrapolation of a multilayer feedforward neural network model with one hidden layer of two nodes for prediction of the survival and growth of* Salmonella *8,20:–:z$_6$ in ground chicken thigh meat to other serotypes of* Salmonella

Temp (°C)	No. of log MPN values	pAPZ for serotype[a]: Typhimurium var 5–	Kentucky	Typhimurium	Thompson
–4	24	0.958	0.958	0.958	0.958
4	24	1.000	1.000	0.958	1.000
12	24	0.917	0.958	0.958	0.958
16	36	0.917	0.917	0.917	0.917
All	108	0.944	0.954	0.944	0.954

[a] Proportion of residual values (observed minus predicted values) in an acceptable prediction zone (pAPZ) from –1 log (fail-safe) to 0.5 log (fail-dangerous).

The APZ is twice as wide in the fail-safe direction because, when using a model to predict food safety, it is desirable to allow a model to err more in the fail-safe direction to provide an added level of safety. However, it is not desirable to allow a predictive model to err too much in the fail-safe direction because this would result in an overestimation of risk and the unnecessary condemnation and destruction of safe food that could otherwise benefit public health. The MLF2 model developed in the present study was not overly fail-safe, as the mean residual was only –0.14 log. In contrast, and as expected, the MLF2 model developed by random tagging of data exhibited no average bias.

In the current study, the ability of the MLF2 model for *Salmonella* 8,20:–:z$_6$ to predict the survival and growth of other serotypes of *Salmonella* (i.e., Typhimurium var 5–, Kentucky, Typhimurium, or Thompson) in ground chicken thigh meat stored for 0 to 8 days at –4, 4, 12, or 16°C was evaluated using the APZ method. The data for model validation for extrapolation to these four serotypes were collected using the same methods as were used to acquire the data for model development, except for the test variable (i.e., serotype). Although serotypes 8,20:–:z$_6$ and Kentucky

were found by one-way ANOVA to grow slightly less (<1 log) than serotypes Typhimurium and Thompson in ground chicken thigh meat during extended (i.e., 4, 6, or 8 days) storage at 16°C, the results of this evaluation indicated that the MLF2 model for *Salmonella* 8,20:–:z_6 provided acceptable predictions of the survival and growth of all four test serotypes (Typhimurium var 5–, Kentucky, Typhimurium, and Thompson). Thus, the MLF2 model for *Salmonella* 8,20:–:z_6 provided valid predictions for the other four serotypes investigated, indicating that new models are not needed for these serotypes, which will save considerable time and money.

The data acquired in this study indicated that storage for 0 to 8 days at –8 to 0°C did not change the number of *Salmonella* cells in ground chicken thigh meat, regardless of the serotype examined. In contrast, Foster and Mead *(6)* reported that storage of minced chicken breast or leg meat for 0 to 8 days and up to 100 days at –5, –2, or 1°C resulted in the death of five different serotypes (Typhimurium, Agona, Cerro, Haardt, and Livingstone) of *Salmonella*. Slow formation of larger ice crystals at temperatures just below freezing is believed to create more cellular damage than quick freezing at lower temperatures (i.e., –20°C), where the ice crystals formed are smaller. Thus, we hoped to validate freezing at higher temperatures as a potential practical, home-based method for the reduction of the load of *Salmonella* in chicken meat. Our inability to replicate the previous findings of Foster and Mead *(6)* indicates an incomplete understanding of the effects of frozen storage on the behavior of *Salmonella* in chicken meat and the need for additional research.

According to data acquired in this study, the number of *Salmonella* cells will stay the same during 8 days of cold storage at temperatures from –8 to 8°C. However, some studies report significant growth of *Salmonella* in this temperature range. For example, Pradhan et al. *(22)* reported that *Salmonella* Typhimurium numbers increased by 1.2 log on raw chicken breasts stored for 7 days at 8°C. Zhou et al. *(32)* observed growth of *Salmonella* in chicken meat at both 4 and 8°C with lag times of 118 and 69 h and growth rates of 0.026 and 0.083 h^{-1}, respectively. In agreement with the results of this study, Sharma et al. *(25)* reported that the number of *Salmonella* Typhimurium (10^6/g) on chicken breast meat does not change during 7 days of storage at 4°C. Other studies *(3, 19)* report a small reduction (<1 log) in the number of *Salmonella* during refrigerated storage of chicken meat for 7 to 8 days. More research is needed to better understand these important differences in results among studies.

In summary, a new automated miniaturized MPN method was developed for the enumeration of low to high levels of *Salmonella* in chicken meat with native microflora. Data collected with this new method were used to develop and validate a model for predicting the survival and growth of a low initial number (1.7 log) of *Salmonella* 8,20:–:z_6 cells in ground chicken thigh meat stored for 0 to 8 days at –8 to 16°C. In addition, the model was successfully validated for extrapolation to four other serotypes (Typhimurium var 5–, Kentucky, Typhimurium, and Thompson) of *Salmonella* isolated from chicken meat. The new model will be a valuable tool for chicken producers, helping them to better predict and manage the impact of consumer cold-storage conditions and practices on the risk of salmonellosis from chicken obtained at retail. Additional research is needed to determine how the new miniaturized MPN method can be applied to the development and validation of models for other pathogen and food combinations.

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REFERENCES

1. Bailey, J. S., N. A. Cox, S. E. Craven, and D. E. Cosby. 2002. Serotype tracking of *Salmonella* through integrated broiler chicken operations. *J. Food Prot.* 65:742–745.
2. Batz, M. B., S. Hoffmann, and J. G. Morris, Jr. 2012. Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. *J. Food Prot.* 75:1278–1291.
3. Cosansu, S., and K. Ayhan. 2012. Effects of lactic and acetic acid on survival of *Salmonella enteritidis* during refrigerated and frozen storage of chicken meats. *Food Bioprocess Technol.* 5:372–377. doi:10.1007/s11947-009-0320-x.
4. Evans, J. 1992. Consumer handling of chilled foods: perceptions and practice. *Int. J. Refrig.* 15:290–298.
5. Flynn, O. M. J., I. Blair, and D. McDowell. 1992. The efficiency and consumer operation of domestic refrigerators. *Int. J. Refrig.* 15:307–312.
6. Foster, R. D., and G. C. Mead. 1976. Effect of temperature and added polyphosphate on the survival of salmonellae in poultry meat during cold storage. *J. Appl. Bacteriol.* 41:505–510.
7. James, S. J., and J. Evans. 1992. The temperature performance of domestic refrigerators. *Int. J. Refrig.* 15:313–319.
8. James, S. J., J. Evans, and C. James. 2008. A review of the performance of domestic refrigerators. *J. Food Eng.* 87:2–10.
9. Juneja, V. K., M. V. Melendres, L. Huang, V. Gumudavelli, J. Subbiah, and H. Thippareddi. 2007. Modeling the effect of temperature on growth of *Salmonella* in chicken. *Food Microbiol.* 24:328–335.
10. Kennedy, J., V. Jackson, I. S. Blair, D. A. McDowell, C. Cowan, and D. J. Bolton. 2005. Food safety knowledge of consumers and the microbiological and temperature status of their refrigerators. *J. Food Prot.* 68:1421–1430.
11. Masters, T. 1995. Advanced algorithms for neural networks: a C++ sourcebook. John Wiley and Sons, Inc., New York.
12. Mazengia, E., M. Samadpour, H. W. Hill, K. Greeson, K. Tenney, G. Liao, X. Huang, and J. S. Meschke. 2014. Prevalence, concentrations, and antibiotic sensitivities of *Salmonella* serovars in poultry from retail establishments in Seattle, Washington. *J. Food Prot.* 77:885–893.
13. Oscar, T. P. 2005. Development and validation of primary, secondary, and tertiary models for predicting growth of *Salmonella* Typhimurium on sterile chicken. *J. Food Prot.* 68:2606–2613.
14. Oscar, T. P. 2005. Validation of lag time and growth rate models for *Salmonella* Typhimurium: acceptable prediction zone method. *J. Food Sci.* 70:M129–M137.
15. Oscar, T. P. 2006. 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. *J. Food Prot.* 69:2048–2057.
16. Oscar, T. P. 2009. General regression neural network and Monte Carlo simulation model for survival and growth of *Salmonella* on raw chicken skin as a function of serotype, temperature, and time for use in risk assessment. *J. Food Prot.* 72:2078–2087.

17. Oscar, T. P. 2011. Development and validation of a predictive microbiology model for survival and growth of *Salmonella* on chicken stored at 4 to 12°C. *J. Food. Prot*. 74:279–284.
18. Oscar, T. P. 2013. Initial contamination of chicken parts with *Salmonella* at retail and cross-contamination of cooked chicken with *Salmonella* from raw chicken during meal preparation. *J. Food Prot*. 76:33–39.
19. Oscar, T. P. 2014. General regression neural network model for behavior of *Salmonella* on chicken meat during cold storage. *J. Food Sci*. 79:M978–M987.
20. Oscar, T. P. 2014. Use of enrichment real-time PCR to enumerate *Salmonella* on chicken parts. *J. Food Prot*. 77:1086–1092.
21. Pouillot, R., M. B. Lubran, S. C. Cates, and S. Dennis. 2010. Estimating parametric distributions of storage time and temperature of ready-to-eat foods for U.S. households. *J. Food Prot*. 73:312–321.
22. Pradhan, A. K., M. Li, Y. Li, L. C. Kelso, T. A. Costello, and M. G. Johnson. 2012. A modified Weibull model for growth and survival of *Listeria innocua* and *Salmonella* Typhimurium in chicken breasts during refrigerated and frozen storage. *Poult. Sci*. 91:1482–1488.
23. Roccato, A., M. Uyttendaele, V. Cibin, F. Barrucci, V. Cappa, P. Zavagnin, A. Longo, and A. Ricci. 2015. Survival of *Salmonella* Typhimurium in poultry-based meat preparations during grilling, frying and baking. *Int. J. Food Microbiol*. 197:1–8.
24. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M.-A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis*. 17:7–15.
25. Sharma, C. S., S. K. Williams, K. R. Schneider, R. H. Schmidt, and G. E. Rodrick. 2012. Sodium metasilicate affects growth of *Salmonella* Typhimurium in fresh, boneless, uncooked chicken breast fillets stored at 4°C for 7 days. *Poult. Sci*. 91:719–723.
26. Smadi, H., and J. M. Sargeant. 2013. Quantitative risk assessment of human salmonellosis in Canadian broiler chicken breast from retail to consumption. *Risk Anal*. 33:232–248.
27. Soleimanzadeh, B., L. Hemati, M. Yolmeh, and F. Salehi. 2015. GA-ANN and ANFIS models and *Salmonella* Enteritidis inactivation by ultrasound. *J. Food Saf*. 35:220–226. doi:10.1111/jfs.12174.
28. Straver, J. M., A. F. Janssen, A. R. Linnemann, M. A. van Boekel, R. R. Beumer, and M. H. Zwietering. 2007. Number of *Salmonella* on chicken breast filet at retail level and its implications for public health risk. *J. Food Prot*. 70:2045–2055.
29. Thomas, H. A. 1942. Bacterial densities from fermentation tube tests. *J. Am. Water Works Assoc*. 34:572–576.
30. van Asselt, E. D., J. T. Thissen, and H. J. van der Fels-Klerx. 2009. *Salmonella* serotype distribution in the Dutch broiler supply chain. *Poult. Sci*. 88:2695–2701.
31. Yang, B., Y. Cui, C. Shi, J. Wang, X. Xia, M. Xi, X. Wang, J. Meng, W. Q. Alali, I. Walls, and M. P. Doyle. 2014. Counts, serotypes, and antimicrobial resistance of *Salmonella* isolates on retail raw poultry in the People's Republic of China. *J. Food Prot*. 77:894–902.
32. Zhou, K., K. Zhong, C. Long, X. Han, and S. Liu. 2014. Development and validation of a predictive model for the growth of *Salmonella enterica* in chicken meat. *J. Food Saf*. 34:326–332.