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Original article

Development and validation of a neural network model for predicting growth of *Salmonella* Newport on diced Roma tomatoes during simulated salad preparation and serving: extrapolation to other serotypes

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Summary A study was undertaken to model growth of *Salmonella* on tomatoes for developing and validating a predictive model for use in risk assessment. Cylindrical portions (0.14 g) of Roma tomato pulp were inoculated with a low dose (0.89 log MPN) of *Salmonella* Newport. The inoculated tomato portions were incubated for 0–8 h at 16–40 °C in 2 °C increments to obtain most probable number (MPN) data for model development and validation. A multiple-layer feedforward neural network model with two hidden layers of two nodes each was developed. The proportion of residuals in an acceptable prediction zone (pAPZ) from −1 (fail-safe) to 0.5 log (fail-dangerous) was 0.93 (194/209) for dependent data and 0.96 (86/90) for independent data for interpolation. A pAPZ ≥0.7 indicated that the model provided acceptable predictions. Thus, the model was successfully validated. It was also validated for extrapolation to seven other *Salmonella* serotypes.

Keywords Acceptable prediction zone method, growth, neural network model, risk assessment, Roma tomatoes, *Salmonella* Newport, validation.

Introduction

Salmonella is a leading cause of foodborne illness. In the United States, it is estimated that *Salmonella* causes 1 027 561 illnesses, 19 336 hospitalisations, and 378 deaths per year (Scallan *et al*., 2011). There are many types of food that have been identified as sources of human cases of salmonellosis. Of relevance to the present study, tomatoes have been responsible for several outbreaks of salmonellosis (Bennett *et al*., 2015). For example, eighty-six people living in eight different states of the United States became ill between December 8, 1998 and February 2, 1999 after consuming raw tomatoes prepared in restaurants. The tomatoes were contaminated with the same strain of *Salmonella* Baildon (Cummings *et al*., 2001). Three of the people died. Thus, tomatoes are an important source of *Salmonella* infections in humans.

A common food handling mistake made in the kitchen of consumers is the failure to separate raw and ready-to-eat food (Zhu *et al*., 2017). For example, on occasion, the unwashed utensils (cutting board, knife and hands) used to process raw chicken for cooking are used to prepare salads consisting of leafy greens and other ingredients, such as tomatoes, peppers, carrots, cucumbers and onions. Once prepared, the salad may be held at room temperature for an extended period of time before serving and consumption and thus, provide an opportunity for a human bacterial pathogen (HBP), like *Salmonella*, to grow from a low to a high and dangerous level.

Tomatoes are an acidic food with a pH of 3.90–4.37 (Asplund & Nurmi, 1991). Thus, one would not expect them to support rapid growth of *Salmonella* since the minimum pH for growth of *Salmonella* is 4.05 ± 0.05 (Chung & Goepfert, 1970). However, growth of *Salmonella* at low pH is affected by the acidulant. The minimum pH for growth of *Salmonella* in laboratory broth is 4.05, 4.30, 4.40 and 5.40 when citric acid,

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malic acid, lactic acid or acetic acid is used as acidulant, respectively (Chung & Goepfert, 1970). The main acids in tomato are citric (80%) and malic (10%) (Asplund & Nurmi, 1991). Consequently, growth of *Salmonella* on tomatoes held at room temperatures is observed (Asplund & Nurmi, 1991; Pan & Schaffner, 2010).

Models that predict growth of HBP are valuable tools for food safety. For example, a common practical application of predictive models for growth of HBP is in Hazard Analysis and Critical Control Point (HACCP) programs (Baker, 1995; Elliott, 1996; Panisello & Quantick, 1998). In HACCP, predictive models are used to verify that a Critical Control Point (CCP) is under control. For example, if there is a mechanical failure that results in the temporary shutdown of a chicken processing line, a predictive model can be used to assess how much growth of *Salmonella* could occur. The amount of growth that indicates that the CCP is out of control is specified in the HACCP plan and this value, usually one log cycle, is used to verify the CCP. If the CCP is found to be out of control then corrective action would be taken to mitigate this risk to public health. In this context, consumers could use a model to assess whether it is safe to eat diced Roma tomatoes that may have been cross-contaminated with *Salmonella* from raw chicken due to improper handling of the raw chicken during meal preparation. If the time and temperature between dicing the tomatoes and consumption of the salad is such that it would result in greater than a one log cycle growth of *Salmonella* then the consumer would conclude that the salad is not safe to eat and thus, take a correction action such as discarding the salad.

Although models for growth of HBP are usually developed in laboratory broth, there is one model for growth of *Salmonella* on tomatoes; it is a secondary model that predicts growth rate of a mixture of *Salmonella* serotypes (Typhimurium, Newport, Javianna, Braenderup) on red round tomatoes as a function of temperature from 10 to 35 °C (Pan & Schaffner, 2010). However, the aforementioned model only predicts growth during the linear phase and it was not properly validated against an independent set of data using established criteria for test data and model performance. Therefore, in the present study, a model that predicts growth of *Salmonella* Newport on Roma tomatoes as a function of time (0–8 h) and temperature (16–40 °C) will be developed and properly validated for interpolation against an independent set of data using the established test data and model performance criteria of the acceptable prediction zone (APZ) method (Oscar, 2005a,b). In addition, it will be evaluated by the APZ method for its ability to predict growth of ten other serotypes of *Salmonella* on Roma tomatoes.

Materials and methods

Organism

A single strain of *Salmonella* Newport was used for model development and validation. The strain was isolated on March 18, 2014 from the left wing of a whole broiler chicken sold in a flow pack wrapper and obtained at a local retail store (Princess Anne, MD, USA). The organism was stored at −80 °C in brain heart infusion broth (Difco™, Becton, Dickinson and Co., Sparks, MD, USA) that contained 15% (v/v) glycerol (Sigma Aldrich Co., St. Louis, MO, USA).

Inoculation culture

The frozen stock culture of *Salmonella* Newport was thawed for 30 min at 40 °C and then suspended by gentle shaking of the storage vial. Five μL of the suspended stock culture was added to 0.9 mL of buffered peptone water (BPW, Difco™, Becton, Dickinson and Co.) in a 1.5-mL polypropylene microcentrifuge tube. The BPW culture was incubated without shaking for 96 h at 22 °C to obtain stationary phase cells of *Salmonella* Newport for inoculation. Tomato portions (0.14 ± 0.02 g; mean ± SD) were inoculated with 5 μL of a 10^{-6} serial dilution (1:10) in BPW. The initial inoculum size was 0.89 ± 0.23 log most probable number (MPN) per portion.

Preparation of tomato portions

Roma tomatoes were obtained from local retail stores (Princess Anne, MD, USA and Fruitland, MD, USA) on a weekly basis. The ends were cut off and the remaining centre portion was cut in half (Fig. 1a). The core was removed and the remaining rings with skin and pulp were cut into flat sections. A Kimwipe tissue (Kimberly-Clark, Roswell, GA, USA) was used to remove adhering seeds and jelly. A #3 cork-borer was used to cut small portions (Fig. 1b), which were transferred to 1.5-mL polypropylene microcentrifuge tubes (Fig. 1c) with the pulp up and the skin down (Fig. 1d). Twenty or twenty-four portions were prepared per weekly storage trial, which consisted of four temperatures and five or six sampling times per temperature. After preparation, tomato portions were stored at 6 °C until the next day when the storage trial was conducted.

Experimental designs

A 7 by 5 or 6 full factorial design of temperature (16, 20, 24, 28, 32, 36, 40 °C) and time (0, 1 and/or 2, 3 or 4, 5 or 6, 7 or 8 h) was used to collect data for model development with four replicate storage trials per

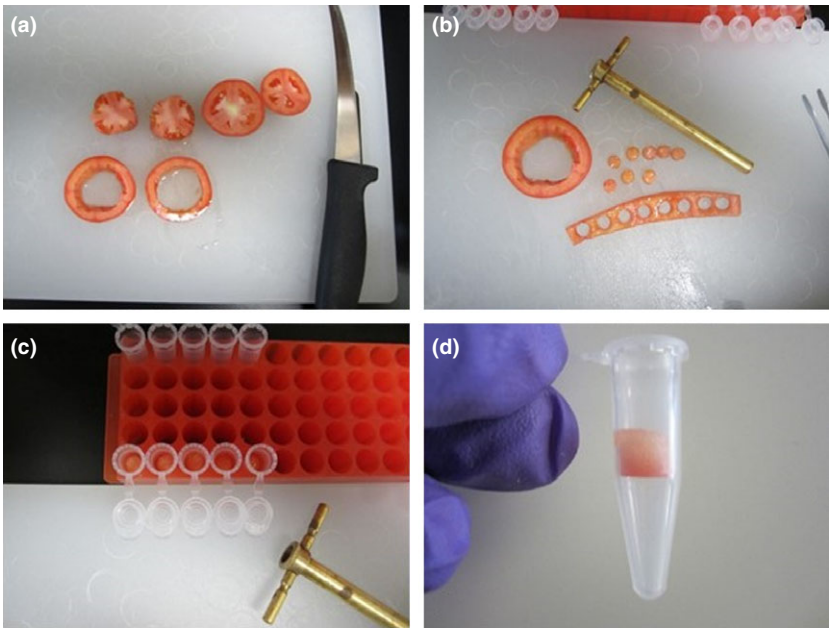


Figure 1 Preparation of Roma tomato portions for use in storage trials: (a) cut-up to yield rings; (b) removal of portions with a cork borer; (c) placement of portions in 1.5-mL tubes; and (d) view of portion with pulp end up and skin end down. [Colour figure can be viewed at wileyonlinelibrary.com]

temperature. To evaluate the model for its ability to interpolate, a 6 by 5 or 6 full factorial design of temperature (18, 22, 26, 30, 34, 38 °C) and time (0, 1 and/or 2, 3 or 4, 5 or 6, 7 or 8 h) with two or three replicate storage trials per temperature was used. To evaluate the model for extrapolation to other serotypes of *Salmonella*, a 4 by 5 or 6 by 10 full factorial design of temperature (22, 28, 34, 40 °C), time (0, 1 and/or 2, 3 or 4, 5 or 6, 7 or 8 h), and serotype (Montevideo, Hadar, Enteritidis, Typhimurium var 5-, 4,5,12:Nonmotile, 8,20:-:z_6, Typhimurium, Thompson, Kentucky, Heidelberg) with two replicate storage trials per temperature was used.

Data collection

Most probable number data for evaluating the model for interpolation were collected using the same methods as those used to collect the MPN data for model development. This was done so that comparisons of observed and predicted values were not confounded. After the model was validated for interpolation, MPN data for evaluating the model for extrapolation to ten other serotypes of *Salmonella* were collected using the same methods as used to collect the MPN data for model development except for the independent variable being evaluated for extrapolation; in this case, the serotype of *Salmonella*. Again, this was done to provide a valid comparison of observed and predicted MPN values. In addition, to obtain an unbiased and complete evaluation of model performance, MPN data for evaluating the model for its ability to interpolate and extrapolate were collected in a way that provided uniform and complete coverage of model predictions. Thus, the experiments were designed and data were collected in a manner to provide test data that met the criteria of the acceptable prediction zone (APZ) method (Oscar, 2005a,b).

Storage trial

After inoculation with the appropriate serotype of *Salmonella*, the tomato samples were placed in a heating and cooling block (ThermoStat Plus, Eppendorf, Hamburg, Germany, or Grant-bio PCH1, Grant Instruments [Cambridge] Ltd., Shepreth, Cambridgeshire, UK) that was warmed to the test temperature. At each sampling time, four samples were removed, one per test temperature, and then 0.7 mL of BPW at 4 °C was added to stop the growth of *Salmonella* on the tomato portion. Salmonella survive and do not grow or die for 10 days of storage at 4 °C on ground chicken with native microflora (Oscar, 2011). Thus, 4 °C is a good temperature for stopping the growth of *Salmonella* for accurate enumeration of this pathogen on or in a food portion at the time of sampling.

In the present study, the storage trials simulated the scenario where the consumer removes a Roma tomato from a domestic refrigerator, dices the Roma tomato with unwashed utensils used to prepare

raw chicken for cooking and in the process cross-contaminates the diced Roma tomato portion with a low dose (0.89 log MPN) of *Salmonella* and then the diced Roma tomato portion is added to a salad and held for 0–8 h at 16–40 °C before serving and consumption.

Pathogen enumeration

To determine the number of *Salmonella* on the tomato portion at each sampling time, tomato portions in 0.7 mL of BPW were vortexed (Digital Disruptor Genie, Scientific Industries Inc., Bohemia, NY, USA) for 1 min at 3000 r.p.m. to recover *Salmonella* into BPW for enumeration by a six replicate by four dilution most probable number (MPN) assay that was conducted in a 2-mL, 96-well deep-well plate (Axygen Scientific, Union City, CA, USA). This was accomplished by adding six 0.1 mL samples of BPW from the vortexed sample to the appropriate wells in the deep-well plate. Each well was prefilled with 0.9 mL of BPW. For some samples, the BPW from the vortexed sample was serially diluted (1:10) in BPW before addition to the deep-well plate. A robotic pipettor (SoloPlus, Hudson Robotics, Springfield, NJ, USA) was used to perform the final three serial dilutions (1:10). After serial dilution, the BPW deep-well plate was incubated for 24 h at 40 °C.

To the tomato portion and BPW (0.1 mL) remaining in the microcentrifuge tube of samples that were not serially diluted for setting up the MPN assay, 0.7 mL of BPW was added. For samples that were serially diluted before setting up the MPN assay, no additional BPW was added because there was sufficient BPW (0.6 mL) remaining in the microcentrifuge tube to cover the tomato portion. The microcentrifuge tubes with tomato portions were incubated for 24 h at 40 °C. This step completed the whole sample enrichment (WSE) component of the protocol so that a single cell of *Salmonella* on the tomato portion could be detected and enumerated in the MPN assay. Thus, the lower limit of detection of the WSE-miniature (m) MPN assay was one cell of *Salmonella* per tomato portion.

After incubation in BPW, the MPN assay was transferred to Rappaport Vassiliadis R10 broth (RVB; Difco™, Becton, Dickinson and Co.). The RVB was supplemented with novobiocin (N; Alfa Aesar, Ward Hill, MA, USA) by adding 150 μL of a sterilised solution (20 mg mL^{-1}) to 120 mL of sterile RVB in a 125-mL bottle with cap. Novobiocin (25 μg mL^{-1}) made the RVB more selective for *Salmonella*. In some of the early storage trials of the current study (results not shown) and in contrast with previous studies (Oscar, 2015, 2017c) with ground chicken with native microflora, growth of competitors that interfered with enumeration on selective agar (i.e. XLT4) was observed. Addition of novobiocin to the RVB was found to suppress the growth of these competitors while not affecting the growth of *Salmonella* Newport and the other 10 serotypes investigated; this made it easier to enumerate the *Salmonella*.

A robotic multi-channel dispenser (Micro10X, Hudson Robotics) was used to fill a 2-mL, 96-well, deep-well plate with 1 mL of RVB-N per well. The robotic pipettor was used to transfer 10 μL of the BPW incubation to corresponding wells of the RVB-N deep-well plate. For the BPW incubation in microcentrifuge tubes, 7 μL were transferred with a hand-held micropipettor to a 1.5-mL polypropylene microcentrifuge tube that contained 0.7 mL of RVB-N. After transfer, the RVB-N deep-well plate and microcentrifuge tubes were incubated for 24 h at 42 °C.

After selective enrichment, 2 μL of the RVB-N incubation from the deep-well plate or the microcentrifuge tubes were drop plated onto xylose lysine tergitol 4 (XLT4; Difco™, Becton, Dickinson and Co.) agar plates. Drop plating was performed by the robotic pipettor for the deep-well plate and with a hand-held micropipettor for the microcentrifuge tubes. After drop plating, the XLT4 plates were incubated for 24 h at 40 °C. After incubation, the number of drops per dilution that were positive (black growth) for *Salmonella* were recorded. These results were used to calculate the MPN (Thomas, 1942; Peeler *et al.*, 1992) as follows:

$$\mathrm{MPN} = \frac{P}{\sqrt{NT}}$$

where P was the number of positive wells, N was the total amount of sample in all the negative wells, and T was the total amount of sample or 0.7 mL. The resulting MPN was log base 10 transformed and expressed per tomato portion.

Model development

To develop the neural network model, a dataset ($n = 209$ MPN values) was created in a computer spreadsheet (Excel 2013, MicroSoft Corporation, Redmond, WA, USA). It had three columns: (i) temperature (independent numerical variable); (ii) time (independent numerical variable); and (iii) log MPN per portion (dependent numerical variable). A spreadsheet add-in program (NeuralTools version 6.3.1, Palisade Corporation, Ithaca, NY, USA) was used to develop a multiple-layer feedforward (MLF) neural network model with an input layer of two inputs, two hidden layers with two nodes each, and an output layer with one output (Fig. 2). Seventy percent of the data were used to train the MLF, whereas 30% of the

data, which were randomly selected by the spreadsheet add-in program using a seed of 1, were used to test the MLF.

Each connection (arrow in Fig. 2) in the model was assigned a weight (w) and each neuron was assigned a bias (b) term. Each hidden neuron computed a weighted sum (z_i) of its inputs:

$$z_i^l = \sum_{j=1}^{n} w_{ij}^l x_j + b_i^l$$

where l was the layer (input or hidden), j was the unit in layer l from 1 to n, i was the unit in $l + 1$, w were the connection weights, x were the outputs of the neurons in the previous layer, and b was the bias associated with unit i in layer $l + 1$. The output for the ith hidden neuron was obtained by applying an activation function to the weighted sum of its inputs. The activation function used in the hidden layers was the hyperbolic tangent (tanh) function:

$$f(z_i^l) = \tanh(z_i^l) = \frac{e^z - e^{-z}}{e^z + e^{-z}}$$

whereas the activation function used in the output layer was the identity function:

$$f(z) = \text{iden}(z) = z$$

For proprietary reasons, the spreadsheet add-in program does not provide weights and bias values for the neural networks it produces. Thus, a requirement to run the current model is the purchase and maintenance of the spreadsheet add-in program. Nonetheless, once published, the present model and the data used to develop and validate it will be made available for no charge at: www.ars.usda.gov/nea/errc/PoultryFARM. This approach to model development and distribution is commonly used to develop risk assessment models, which are developed in a computer spreadsheet (Excel) and require the purchase and maintenance of a spreadsheet add-in program (@Risk, Palisade Corp.) to run them.

Model validation

Performance of the model was evaluated using the test data and model performance criteria of the acceptable prediction zone (APZ) method (Oscar, 2005a,b, 2014). The test data criteria are: (i) that the test data for validation for interpolation are collected using the same

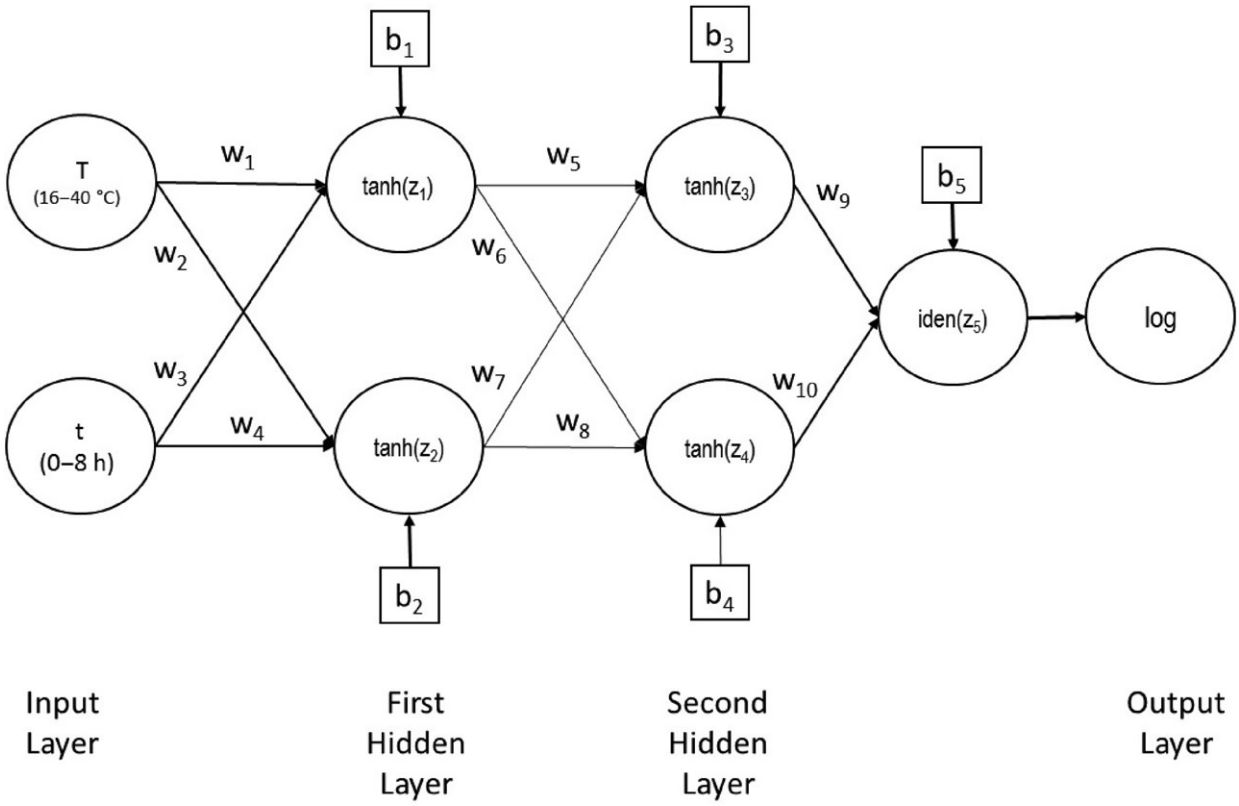


Figure 2 Structure of the multiple-layer feedforward neural network model for predicting growth of *Salmonella* Newport on diced Roma tomatoes. T, temperature; t, time; w, weight; b, bias; tanh, hyperbolic tangent function; iden, identity function.

methods as those used to collect the data used to develop the model so as to provide a valid comparison of observed and predicted values; (ii) that the test data for validation for extrapolation are collected using the same methods as those used to collect the data used to develop the model with the exception of the independent variable being evaluated; and (iii) that the test data for interpolation or extrapolation provide uniform and complete coverage of model predictions so as to provide a complete and unbiased evaluation of model performance.

The model performance criteria of the APZ method for a model that predicts log number are: (i) that a prediction is acceptable when the residual (observed value – predicted value) is in an APZ from −1 log (fail-safe) to 0.5 log (fail-dangerous); (ii) that the model provides predictions with acceptable accuracy and bias when the proportion of residuals in the APZ (pAPZ) for the test data is ≥0.7; (iii) that there are no local prediction problems where pAPZ is <0.7 for a growth curve or a significant portion of a growth curve; (iv) that it is only possible to validate a model for interpolation when the pAPZ for data used in model development is ≥0.7; and (v) that it is only possible to validate a model for extrapolation when the model has been validated for interpolation.

To look for local prediction problems, the observed MPN data were graphed as a function of dataset, time and temperature. In addition, these graphs (n = 53) included the predicted growth curve and the APZ so that the agreement between observed and predicted values for all combinations of time and temperature within a dataset could be visually assessed and if needed, a pAPZ for a subset of the data could be calculated.

Results

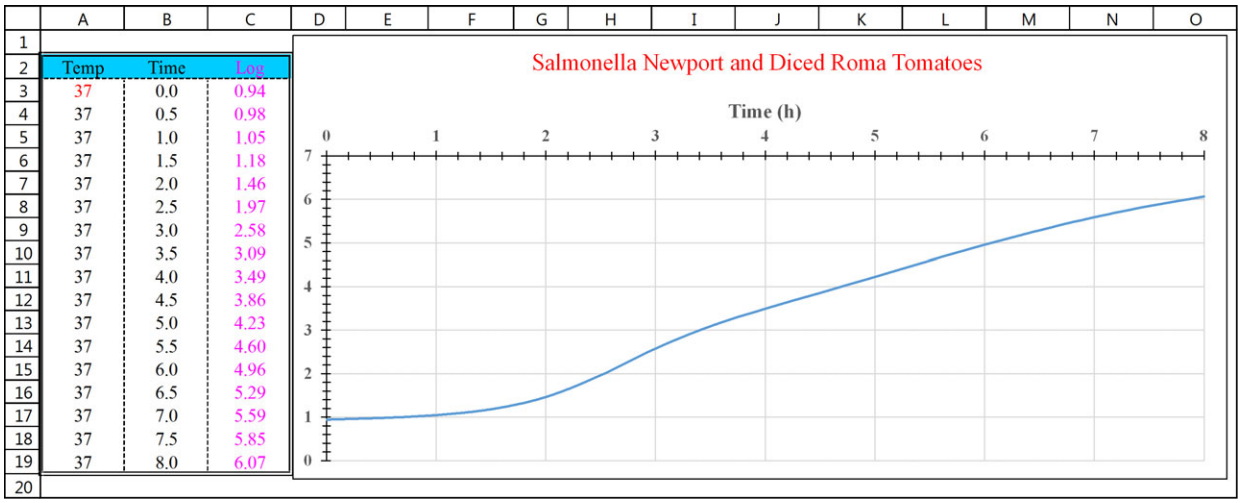
Model development

The MLF 2-2-2-1 model was developed based on past experience (Oscar, 2017a,b) and trial and error. In addition to various MLF architectures from 2-2-0-1 to 2-6-0-1, a General Regression Neural Network (GRNN) was also tested. Many of the MLF and GRNN models tested had pAPZ that were acceptable for dependent data as well as independent data for interpolation but some did not provide reliable predictions of the initial number of *Salmonella* Newport as a function of temperature, some did not predict the lag phase well and some provided predictions of the growth phase that were wavy, which was interpreted as an indication of over-training of the neural network. Once the MLF 2-2-2-1 was identified as a desirable neural network structure, it was refined by conducting a sensitivity analysis to identify the best amount of data to use to train the MLF and to test the MLF for generalisation. More specifically, a sensitivity analysis with 20%, 30% or 40% of the data used for testing was performed for the MLF 2-2-2-1 model. The lowest range of the Root Mean Squared Error (RMSE) among simulations of the MLF 2-2-2-1 model was obtained for the 30% test data level. Thus, the final MLF 2-2-2-1 model was developed using 70% of the MPN data for training and 30% of the MPN data for testing and a seed of 1 was used so that a unique solution of the model was obtained. The final model (Fig. 3) was able to predict the growth curve for *Salmonella* Newport on diced Roma tomatoes during storage for 0–8 h at 16–40 °C including times and temperatures that were not investigated but that were within the ranges of times and temperatures used to develop the model (e.g. 1.5 h at 37 °C).

Model validation (dependent data)

Before the model could be used with confidence to make important predictions and food safety decisions, the performance of the model had to be properly evaluated using the test data and model performance criteria of the APZ method. The first step was to evaluate how well the model predicted the dependent data or the data used to develop it. Here, the results for the training and testing data used to develop the model were analysed separately and the pAPZ values were determined for individual growth curves (Fig. 4). In addition, the observed MPN data were graphed as a function of time and temperature and with the predicted growth curves and APZ to look for local prediction problems.

The pAPZ for the training data for individual growth curves ranged from 0.89 at 20 °C to 1.00 at 24 and 36 °C (Fig. 4). Overall, the pAPZ for training data was 0.95 (138/146) and there were no signs of local prediction problems (Fig. 4). On the other hand, the pAPZ for the testing data for individual growth curves ranged from 0.67 at 40 °C to 1.00 at 16, 20 and 28 °C (Fig. 4). Overall, the pAPZ for testing data was 0.89 (58/63) and there were no signs of local prediction problems except at 40 °C where a small problem was observed. However, this local prediction problem seemed to be an unfortunate result of the random selection of data for testing by the spreadsheet add-in program rather than a true prediction problem as the majority of the training and testing data at 40 °C were in the APZ (Fig. 4g) with a pAPZ of 0.86 (25/29). The pAPZ for the combined training and testing data for all of the growth curves was 0.93 (194/209), which was ≥0.7. Thus, it was concluded that the model provided predictions with acceptable accuracy and bias for the data used in model development.



Temp	Time	Log
37	0.0	0.94
37	0.5	0.98
37	1.0	1.05
37	1.5	1.18
37	2.0	1.46
37	2.5	1.97
37	3.0	2.58
37	3.5	3.09
37	4.0	3.49
37	4.5	3.86
37	5.0	4.23
37	5.5	4.60
37	6.0	4.96
37	6.5	5.29
37	7.0	5.59
37	7.5	5.85
37	8.0	6.07

Figure 3 Neural network model for predicting growth of *Salmonella* Newport on diced Roma tomatoes. The model was created in a computer spreadsheet (Excel) and was simulated with a spreadsheet add-in program (NeuralTools). [Colour figure can be viewed at wileyonlinelibrary.com]

Model validation (independent data for interpolation)

The ability of the model to interpolate or provide predictions of the growth of *Salmonella* Newport on diced Roma tomatoes at times and temperatures not investigated but that fall within the ranges of times and temperatures used to develop the model required the collection of an independent set of data that provided uniform and complete coverage of model predictions. This was accomplished by collecting MPN data at temperatures intermediate to those used in model development and at times from 0–8 h. These MPN data were then compared to predictions made by the model using the APZ method. The pAPZ for individual growth curves for these data, which were not used in model development, ranged from 0.87 at 38 °C to 1.00 at 22, 26 and 30 °C (Fig. 5). The overall pAPZ for the independent data for interpolation was 0.96 (86/90) and there were no signs of local prediction problems (Fig. 5). As the model had acceptable performance for the dependent data and as the pAPZ for interpolation for the individual growth curves and all growth curves combined were ≥0.7 and there were no local prediction problems, it was concluded that the model provided predictions with acceptable accuracy and bias for the independent data for interpolation. Thus, it was concluded that the model was successfully validated for interpolation.

Model validation (extrapolation)

Once a model has been validated for interpolation, it can be tested for its ability to extrapolate to other independent variables. This is a valuable activity because it can save time and money by identifying independent variables for which new models are not needed. In the present study, the validated model was evaluated for its ability to predict the growth of ten other serotypes of *Salmonella* on diced Roma tomatoes stored for 0–8 h at 22, 28, 34, or 40 °C (Table 1). The MPN data for this evaluation were collected using the same methods as used to collect the data for model development except for one variable, the serotype of *Salmonella*. Thus, the MPN data met the criteria of the APZ method for proper testing of a validated model for extrapolation to a new independent variable.

The model was found to provide predictions with acceptable accuracy and bias for seven (Montevideo, Hadar, Typhimurium var 5-, 4,5,12:Nonmotile, Kentucky, Thompson, Heidelberg) of the ten serotypes with pAPZ for individual growth curves and all growth curves combined that were ≥0.7 (Table 1) and that upon graphical analysis presented no signs of local prediction problems (results not shown). However, three serotypes (8,20:-:z_6, Typhimurium, Enteritidis) failed the evaluation for extrapolation.

Serotype 8:20:-:z_6 had pAPZ for individual growth curves that ranged from 0.31 at 40 °C to 0.62 at 22 °C with an overall pAPZ of 0.48 for the four temperatures investigated (Table 1). Graphs of the observed MPN data for serotype 8:20:-:z_6 vs. the predicted growth curves (Fig. 6) revealed that it grew less on diced Roma tomatoes than serotype Newport, which was used to develop the model.

The results for serotype Typhimurium were mixed with the model providing acceptable predictions (pAPZ ≥0.7) at 34 and 40 °C and unacceptable predictions (pAPZ <0.7) at 22 and 28 °C (Table 1). Overall

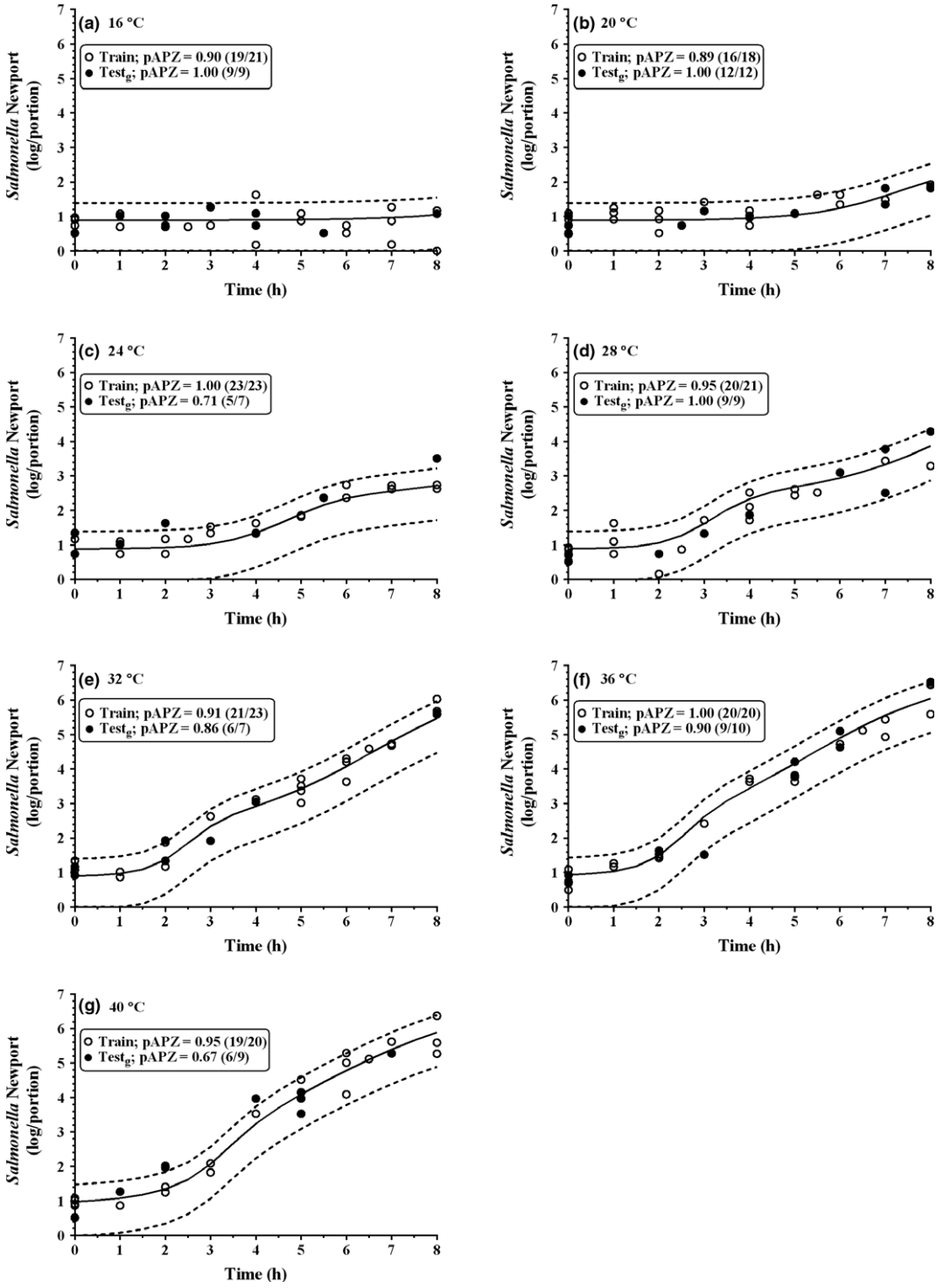


Figure 4 Growth of *Salmonella* Newport on diced Roma tomato portions incubated at (a) 16 °C; (b) 20 °C; (c) 24 °C; (d) 28 °C; (e) 32 °C; (f) 36 °C; and (g) 40 °C. Open symbols are observed values used to train the neural network, closed symbols are observed values used to test the neural network for generalisation (g), the solid line is the predicted growth curve, and the dashed lines are the boundaries of the acceptable prediction zone (APZ), which extends 1 log in the fail-safe direction and 0.5 log in the fail-dangerous direction and where pAPZ is the proportion of observed values in the APZ.

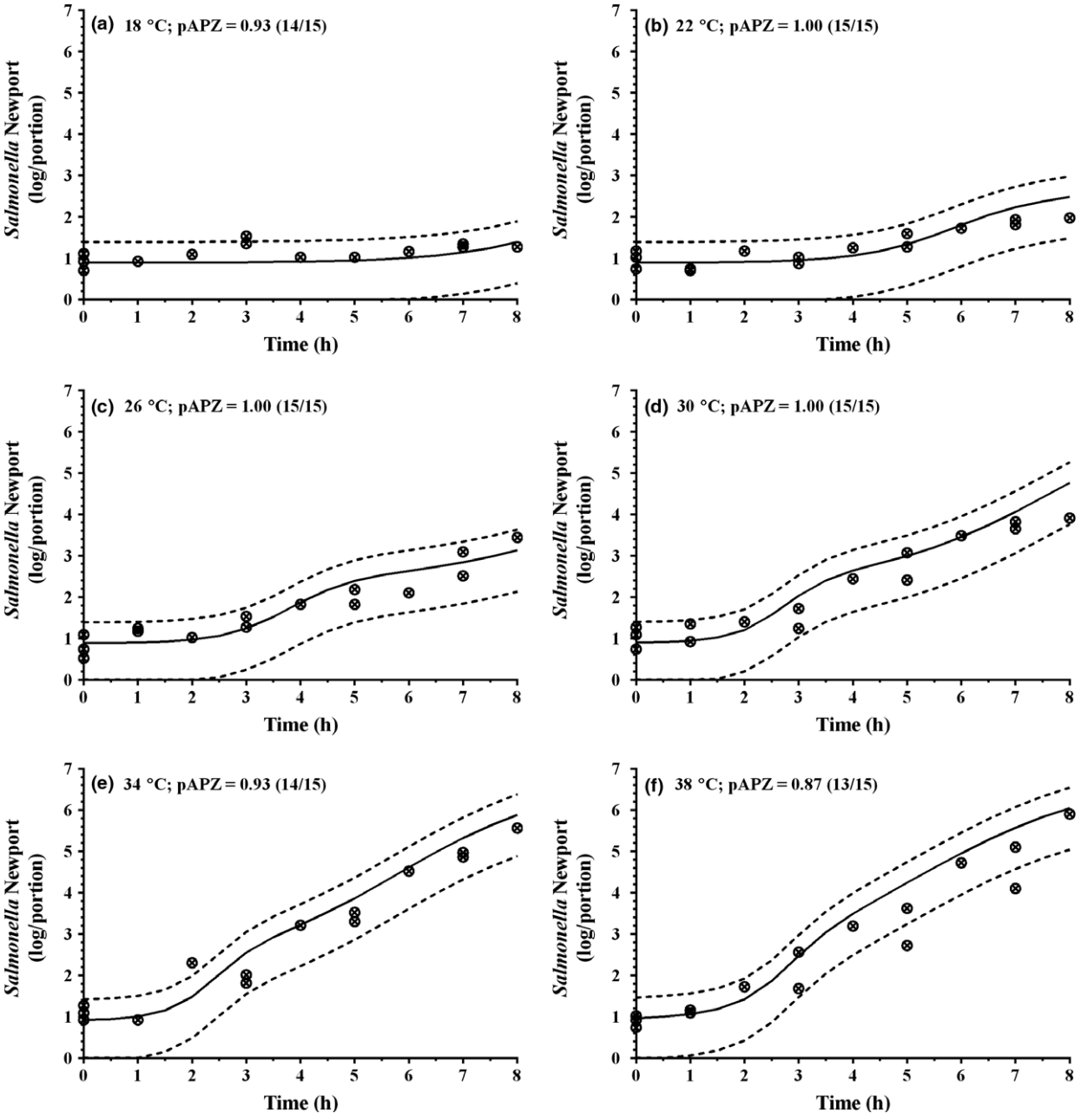


Figure 5 Growth of *Salmonella* Newport on diced Roma tomato portions incubated at (a) 18 °C; (b) 22 °C; (c) 26 °C; (d) 30 °C; (e) 34 °C; and (f) 38 °C. Symbols are observed values used to evaluate the neural network model for interpolation, the solid line is the predicted growth curve, and the dashed lines are the boundaries of the acceptable prediction zone (APZ), which extends 1 log in the fail-safe direction and 0.5 log in the fail-dangerous direction and where pAPZ is the proportion of observed values in the APZ.

(i.e. data from all four temperatures combined), the model provided unacceptable predictions (pAPZ = 0.66) for the growth of serotype Typhimurium on Roma tomatoes.

The results for serotype Enteritidis were also mixed (Table 1) with the model providing acceptable predictions at all temperatures except at 22 °C (pAPZ = 0.64). When the data for all four temperatures were combined, the pAPZ was ≥0.7 indicating that a local prediction problem caused the model to fail validation for extrapolation to serotype Enteritidis.

Table 1 Extrapolation of a model for growth of *Salmonella* Newport on diced Roma tomatoes to other serotypes of *Salmonella*

		Temperature				
Serotype	**Source[a]**	**22°C**	**28°C**	**34°C**	**40°C**	**All**
Montevideo	FSIS via NVSL	0.86 (14) [b]	1.00 (14)	0.86 (14)	0.86 (14)	0.89 (56)
Hadar	Poultry company	0.86 (14)	0.77 (13)	0.86 (14)	0.75 (12)	0.81 (53)
8,20:-:z_6	Broiler chicken wing	**0.62 (13)**	**0.57 (14)**	**0.43 (14)**	**0.31 (13)**	**0.48 (54)**
Typhimurium var 5-	Broiler chicken breast	0.82 (11)	0.73 (11)	0.82 (11)	0.91 (11)	0.82 (44)
4,5,12:Nonmotile	Broiler chicken wing	0.90 (10)	0.91 (11)	0.91 (11)	0.70 (10)	0.86 (42)
Kentucky	Broiler chicken thigh	0.73 (11)	0.91 (11)	0.78 (9)	0.70 (10)	0.78 (41)
Typhimurium	Broiler chicken breast	**0.64 (11)**	**0.45 (11)**	0.73 (11)	0.82 (11)	**0.66 (44)**
Thompson	Broiler chicken wing	0.90 (10)	0.91 (11)	0.90 (10)	0.88 (8)	0.90 (39)
Enteritidis	Broiler chicken drumstick	**0.64 (11)**	0.73 (11)	0.91 (11)	0.91 (11)	0.80 (44)
Heidelberg	Broiler chicken breast	0.70 (10)	0.90 (10)	1.00 (11)	0.82 (11)	0.86 (42)

The number in parentheses is the total number of residuals. Numbers in bold indicate results where performance of the model was not acceptable (pAPZ <0.7).

[a]FSIS via NVSL = Food Safety Inspection Service via National Veterinary Services Laboratory; the rest of the isolates were from chicken parts that were harvested from whole broiler chickens sold in flow pack wrappers and obtained at a local retail store in Princess Anne, MD and maintained in the author's culture collection.

[b]Proportion of residuals (observed - predicted) in an acceptable prediction zone (pAPZ) from −1 log (fail-safe) to 0.5 log (fail-dangerous).

Discussion

Models that predict growth of human bacterial pathogens (HBP) in food are valuable tools for achieving greater food safety (McMeekin & Ross, 2002). Historically, predictive models have been developed in pure culture with a high initial number of HBP growing alone in laboratory broth (Gibson *et al*., 1988; Buchanan & Phillips, 1990). This approach is used because it makes it easier to obtain the large amount of enumeration data needed to develop a model. Two common methods of enumeration used in these studies are viable counts and optical density. The time needed to perform both these methods can be reduced by automation (i.e. spiral plating and automated plate counting for viable counts and microplate reading for optical density).

The main concerns with models developed with pure cultures of HBP growing in laboratory broth are: (i) the high initial number of HBP used; (ii) the absence of microbial competition; and (iii) the absence of HBP attachment. In other words, there are concerns about how well a model developed with a pure culture of HBP growing in suspension in laboratory broth predicts the growth of a lower number of HBP growing on a solid food surface with other microorganisms. However, development of models with a low initial number of HBP growing on a solid food surface with other microorganisms is difficult because it requires an enumeration method with high sensitivity and specificity.

A high initial number of HBP is used to develop models in laboratory broth because of the low sensitivity of the enumeration methods. The lower limit of detection (LLD) of the viable count method is 3 log per mL whereas the LLD of the optical density method is 6 log per mL. Thus, a high initial number of HBP is needed for enumeration. The problem is that in nature, the number of HBP in food is usually lower than the aforementioned LLD. Thus, to develop models in food with an ecological number of HBP, an enumeration method is needed that is more sensitive than the viable count and optical density methods. One method that is more sensitive than these methods is the most probable number (MPN) method, which with the proper experimental design has an LLD of 0 log per mL (Oscar, 2017a). However, the MPN method requires a large amount of media and is labour intensive unless it is miniaturised and automated as was done in this and previous studies (Oscar, 2015, 2017b).

The enumeration method for HBP in food with native microflora needs to be specific for the HBP. Optical density is not specific for HBP and thus, is not an option. In addition, optical density does not work with food samples. On the other hand, viable counts can be specific for the HBP if a chromogenic media is used or if a marker strain of the HBP is used. However, other problems occur with chromogenic media and marker strains that limit their use for enumerating HBP in food with native microflora. First, chromogenic media are selective and thus, do not allow injured and stressed cells of the HBP to grow resulting in an under-estimation of their number. Second, when the HBP is a minority member of the native microflora, which is the typical situation in nature, another microorganism in the food may predominate in the food and on the chromogenic media, mask the HBP, and make it difficult or impossible to enumerate.

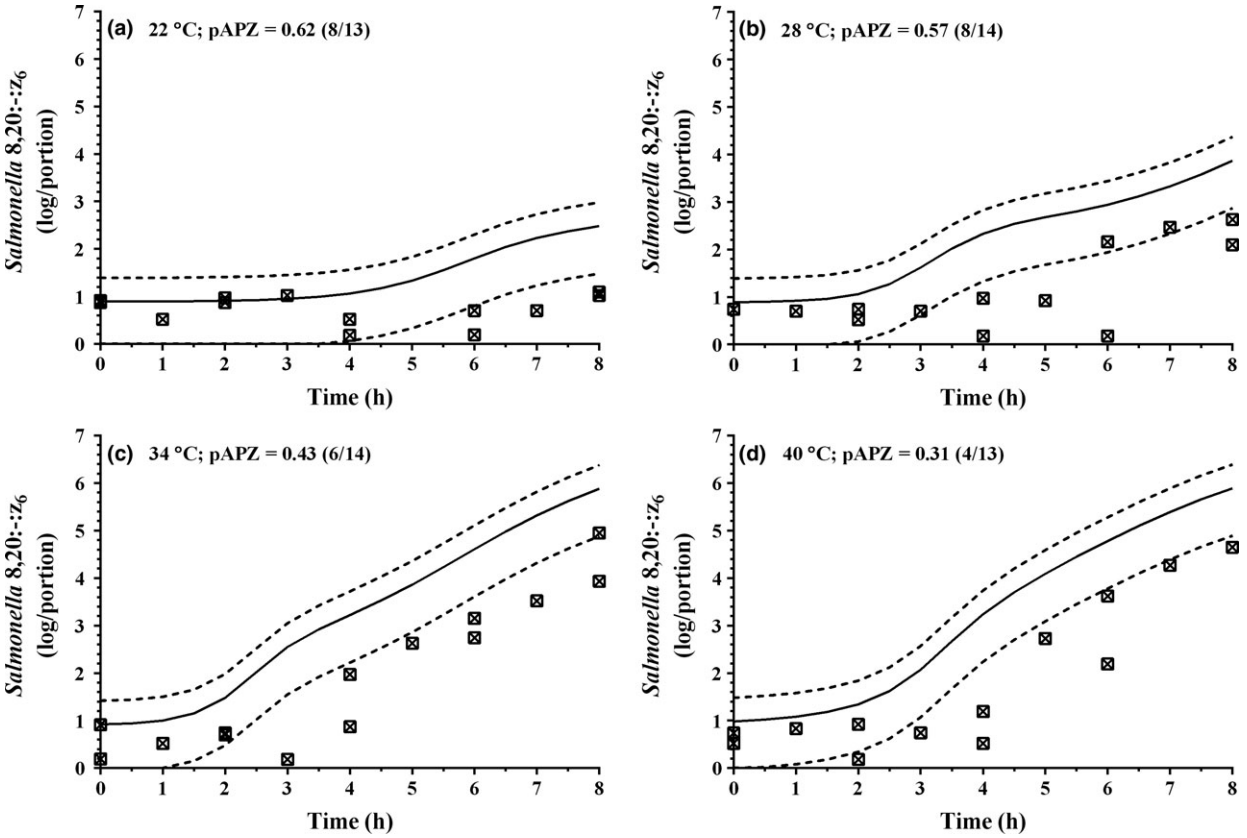


Figure 6 Growth of *Salmonella* 8,20:-:z_6 on diced Roma tomato portions incubated at (a) 22 °C; (b) 28 °C; (c) 34 °C; and (d) 40 °C. Symbols are observed values used to evaluate the neural network model for extrapolation, the solid line is the predicted growth curve, and the dashed lines are the boundaries of the acceptable prediction zone (APZ), which extends 1 log in the fail-safe direction and 0.5 log in the fail-dangerous direction and where pAPZ is the proportion of observed values in the APZ.

Two types of marker strains of HBP are used to develop models: (i) genetically modified organisms (GMO) that express foreign genes and are luminescent (Karsi *et al*., 2008) or fluorescent (Oscar, 2003); and (ii) antibiotic resistant (MDR) strains produced in the laboratory (Blackburn & Davies, 1994) or found in nature (Oscar, 2006). The GMO strains provide a phenotype that can be enumerated on agar plates in the presence of other microorganisms, whereas the MDR strains provide a phenotype that can be enumerated on agar plates with antibiotics that inhibit or suppress the growth of other microorganisms in the food. A potential problem with GMO and MDR strains produced in the laboratory is that they may not behave the same as the parent strain and thus, may not be valid strains for model development (Oscar, 2003). A problem with MDR strains found in nature is that the models developed with them cannot be validated for extrapolation to non-MDR strains because the enumeration method that requires antibiotics would not work with non-MDR strains that are sensitive to antibiotics. Thus, an enumeration method is needed that is specific for all possible strains of a HBP found in the food of interest.

The MPN method, as shown in this and previous studies (Oscar, 2017a,c) with *Salmonella*, is one such method as it can enumerate all examined strains of this HBP in chicken without using antibiotics or genetic markers. In addition, because it uses a non-selective media in the first step, it can enumerate injured and stressed cells of *Salmonella* in the food sample, which, as mentioned above, is not true for chromogenic media, which are specific but may contain selective agents that prevent growth of injured and stressed cells.

In previous studies (Oscar, 2015, 2017b), an automated WSE-mMPN method with an LLD of 0 log per portion was developed and used to enumerate a low number of *Salmonella* in ground chicken with native microflora. During the transition of this automated WSE-mMPN method from ground chicken to Roma tomatoes in the current study, it was found that

addition of novobiocin to the selective enrichment broth helped to inhibit growth of microbial competitors found in Roma tomato samples making it easier to detect and enumerate the inoculated *Salmonella* serotypes on the selective agar media. Although novobiocin is an antibiotic, most *Salmonella* are not sensitive to it. In fact, none of the eleven serotypes of *Salmonella* used in the present study were affected by its presence in the selective enrichment broth.

In the present study, the model was developed using a single strain of *Salmonella* Newport that was isolated in a previous study (Oscar, 2017d) from the left wing of a whole broiler chicken sold in a flow pack wrapper and obtained from a local retail store in the Delmarva region of the United States. This isolate of *Salmonella* Newport was selected for model development for two reasons. First, *Salmonella* Newport has been linked to outbreaks of salmonellosis from tomatoes (Greene *et al*., 2008) and cucumbers (Angelo *et al*., 2015) grown and harvested in the Delmarva region with a possible link to chicken manure, which is used as a fertiliser. Second, the strain of *Salmonella* Newport used in the present study for model development was isolated from chicken produced in the Delmarva region.

The experimental design used in the present study was such that the developed model simulated the scenario whereby the diced Roma tomatoes were cross-contaminated with *Salmonella* Newport from raw chicken during salad preparation and then the diced Roma tomatoes in the salad were "temperature abused" before serving and consumption. To determine how broadly the model could be applied to other serotypes of *Salmonella*, most of which were isolated from chicken, the model was evaluated for its ability to predict the growth of ten other serotypes of *Salmonella* on diced Roma tomatoes held for 0–8 h at 22, 28, 34 or 40 °C. Using the test data and model performance criteria of the APZ method, the model was successfully validated for seven of the ten serotypes. Three serotypes had growth on diced Roma tomatoes that was different from that of *Salmonella* Newport. The finding that growth among serotypes of *Salmonella* is mostly similar but on occasion differs is consistent with previous studies conducted in laboratory broth (Oscar, 1998), on cooked chicken without native microflora (Oscar, 2000, 2003), and on ground chicken with native microflora (Oscar, 2009, 2015).

Predictive models for growth of HBP are usually developed with a cocktail of strains with the assumption that the fastest growing strain in the cocktail will predominate and lead to a fail-safe model. Interestingly, researchers usually do not characterise the growth kinetics of the individual strains before combining them into a cocktail. Thus, it is possible that all of the strains in a cocktail may actually grow in a similar manner. If this is true, then the only real outcome from using a cocktail is to unnecessarily complicate the storage trial. An alternative approach is that used in the present study. Namely, develop the model with one strain and then validate it for extrapolation to other strains. If needed, the model can be expanded to include strain as an independent variable. An additional benefit of this approach is that information is obtained about the growth kinetics of individual strains, which is valuable for risk assessment. Moreover, the model could be designed to predict the growth of the HBP as a function of strain prevalence and growth variation as was done in a previous study for *Salmonella* serotypes and chicken skin (Oscar, 2009).

The standard approach for developing predictive models for HBP has been a three-step regression approach that involves primary, secondary and tertiary modelling (Oscar, 2005a). First, the enumeration data for growth of the HBP over time for a single combination of the independent variables is fitted to a primary model to obtain growth parameters such as lag time, growth rate and maximum population density. Next, a database is created that contains all the combinations of the independent variables investigated and their corresponding growth parameter values obtained from primary modelling. This database is then fitted to a secondary model (e.g. quadratic polynomial model) that predicts the primary model parameter as a function of the independent variables. Finally, the secondary models for lag time, growth rate, and maximum population density are inserted into the primary model to obtain the tertiary model that predicts the growth curve as a function of the independent variables (Oscar, 2005a).

Conclusion

In the current study, a different approach was used instead of the standard three-step regression approach to produce a tertiary model. Here, the database was used to train a neural network in one step that in essence resulted in a tertiary model that predicts the growth curve as function of the independent variables. The neural network model developed was successfully validated against an independent set of data that met the test data and model performance criteria of the APZ method. Thus, although the modelling approach used in the present study was not the conventional regression-based approach used in the field of predictive microbiology, it was properly validated against a properly collected set of independent data that met a set of established criteria for test data and model performance. Thus, users of the model can have confidence that its predictions are reliable.

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