

ORIGINAL ARTICLE

Predictive model for growth of *Salmonella* Newport on Romaine lettuce

Thomas P. Oscar 

United States Department of Agriculture,
Agricultural Research Service, Chemical
Residue and Predictive Microbiology Research
Unit, Princess Anne, Maryland

Correspondence

Thomas P. Oscar, United States Department of
Agriculture, Agricultural Research Service,
Chemical Residue and Predictive Microbiology
Research Unit, Princess Anne, MD.
Email: thomas.oscar@usda.gov

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Abstract

Cross-contamination of ready-to-eat (RTE) salad vegetables with *Salmonella* from raw chicken followed by growth during meal preparation are important risk factors for human salmonellosis. To better predict and manage this risk, a model (general regression neural network) for growth of a chicken isolate of *Salmonella* Newport (0.91 log) on Romaine lettuce (0.18 g) at times (0–8 hr) and temperatures (16–40°C) observed during meal preparation was developed with Excel, NeuralTools, and @Risk. Model performance was evaluated using the acceptable prediction zones (APZ) method. The proportion of residuals in the APZ (pAPZ) was 0.93 for dependent data ($n = 210$) and 0.93 for independent data ($n = 72$) for interpolation. A pAPZ ≥ 0.70 indicates acceptable model performance. Thus, the model was successfully validated for interpolation and can be used with confidence to predict and manage this important risk to public health.

1 | INTRODUCTION

Chicken is an important source of human salmonellosis cases in the United States and throughout the world (Majowicz et al., 2010). Important risk factors are undercooking of raw chicken, cross-contamination of ready-to-eat (RTE) food with *Salmonella* from raw chicken, and growth of *Salmonella* following cross-contamination during meal preparation (Luber, 2009). Salad consisting of fruit (e.g., tomatoes) and vegetables (e.g., lettuce) is an RTE food that is often prepared and served with chicken. On occasion, consumers prepare RTE salad fruit and vegetables with unwashed utensils (e.g., cutting board) used to prepare raw chicken for cooking leading to cross-contamination, growth, and exposure to and illness from *Salmonella* of chicken origin (Zhu et al., 2017).

Models that predict growth of *Salmonella* on salad vegetables (e.g., leafy greens) are valuable tools for helping assess and manage this risk to public health (Koseki & Isobe, 2005). However, it is important to validate these models so users are confident that predictions are reliable (Ross, 1996). Validation involves comparison of model predictions to data used to develop the model (dependent data) and data not used to develop the model (independent data). In addition, validation requires application of criteria that ensure comparisons of

observed and predicted values are accurate and unbiased, and that ensure the evaluation of model performance is objective (Oscar, 2005b). Thus, the present study was undertaken to develop and validate a model for growth of a chicken isolate of *Salmonella* Newport on Romaine lettuce at times and temperatures observed during meal preparation. The data and model address an important data and model gap in risk assessments for *Salmonella* and chicken that are used as the scientific basis for new food safety regulations aimed at protecting public health.

2 | MATERIALS AND METHODS

2.1 | Materials

The chicken isolate of *Salmonella* Newport that was used to develop and validate the model was obtained from the author's culture collection (Princess Anne, MD). Buffered peptone water (BPW) was from Microbiology International (Frederick, MD), Rappaport Vassiliadis (RV) broth and xylose lysine tergitol four (XLT4) agar were from Becton Dickinson (Sparks, MD), and novobiocin (N) was from Alfa Aesar (Ward Hill, MA).

Romaine lettuce was from a local retail store (Salisbury, MD). Lettuce pH was measured with a pH spear (Oakton Instruments, Vernon Hills, IL) and was 6.10 ± 0.20 (mean $\pm$ standard deviation; $n = 72$).

Software applications used to graph, model, and analyze data were Excel 2016 (Microsoft Corp., Redmond, WA), NeuralTools 7.6 and @Risk 7.6 (Palisade Corp., Ithaca, NY), and Prism 8.3 (GraphPad Software, San Diego, CA).

2.2 | Experimental designs

A 5×7 full factorial design of time (0, 2, 4, 6, and 8 hr) and temperature (16, 20, 24, 28, 32, 36, and 40°C) was used for model development where each combination ($n = 35$) was replicated once in six separate storage trials for a total of 210 most probable number (MPN) values for model development. A 4×6 full factorial design of time (1, 3, 5, and 7 hr) and temperature (18, 22, 26, 30, 34, and 38°C) was used for model validation (interpolation) where each combination ($n = 24$) was replicated once in three separate storage trials for a total of 72 MPN values for model validation.

2.3 | Inoculation culture

Five microliters of a frozen (-80°C), thawed, and resuspended stock culture of *Salmonella* Newport was added to 0.7 ml of BPW in a 1.5 ml polystyrene, micro-centrifuge tube. Stationary phase cells, as determined by a predictive model (Oscar, 2018b), were obtained by incubating the culture for 96 hr at 22°C.

2.4 | Lettuce preparation and inoculation

A cork borer (#4) was used to cut circular portions (0.18 ± 0.04 g) of Romaine lettuce from a detached leaf. Portions were placed in 1.5 ml tubes and stored overnight at 4°C before inoculation with *Salmonella* Newport.

The stationary phase culture of *Salmonella* Newport was serially diluted (1:10) in BPW. Lettuce portions (4°C) were spot inoculated on their surface with 5 μl of the 10^{-6} dilution for an initial MPN of 0.91 ± 0.35 log per portion.

2.5 | Storage trials

Inoculated samples were incubated in a heating and cooling block (ThermoStat Plus, Eppendorf, Hamburg, Germany) equilibrated to the test temperature. A single storage trial consisted of four temperatures and five (model development) or four (model validation) sampling times. One storage trial was conducted per week. One lettuce sample per temperature was removed at each sampling time and 0.7 ml of cold (4°C) BPW was added to stop growth of *Salmonella* Newport.

Samples were vortexed for 1 min at 3,000 rpm (Digital Disruptor Genie, Scientific Industries, Bohemia, NY) to recover *Salmonella* Newport into BPW for enumeration.

2.6 | Most probable number

The MPN of *Salmonella* Newport on lettuce portions at each sampling time was determined using a 6 (replicate) $\times$ 16 (dilution) assay with an enumeration range from 0 to 16 log per portion (Oscar, 2018a). The assay had three steps: (a) serial dilution (1:10) followed by nonselective growth in 0.9 ml BPW (24 hr, 40°C); (b) transfer (10 μL) followed by selective growth in 1 ml RVN broth (24 hr, 42°C); and (c) drop plating (2 μl) followed by selective growth on XLT4 agar (24 hr, 40°C). All steps were performed by a robotic pipettor (SoloPlus, Hudson Robotics, Springfield, NJ). The first two steps were in 96-well, deep-well (2 ml) plates. *Salmonella*-positive wells produced black colonies on XLT4 agar. The MPN was calculated by the method of Thomas (1942).

2.7 | Model development and simulation

Missing data ($n = 13$) were interpolated within individual growth curves using the two-phase linear model (Buchanan, Whiting, & Damert, 1997). Next, the dependent variable (MPN) and independent variables (time and temperature) were arranged into four columns of an Excel spreadsheet: (a) tag (train or test); (b) temperature (°C); (c) time (hr); and (d) MPN (log). NeuralTools was then used to develop a general regression neural network (GRNN) model as described in a previous study (Oscar, 2009). Dependent data ($n = 210$) were used to develop (train) the model, whereas independent data ($n = 72$) were used to validate (test) the model for interpolation.

The predict function of NeuralTools was used to predict MPN (log) as a function of time (0–8 hr) and temperature (16–40°C). The distribution function of @Risk was used to define pert distributions (minimum, most likely, maximum) for time and temperature, which were used to make stochastic predictions of growth (log increase). The model was simulated with @Risk settings of Latin Hypercube sampling, Mersenne Twister, 1,000 iterations, and a seed of one. Simulation results were filtered to remove no growth (log increase <0.3) events and the Best Fit option of @Risk was used to identify the best fitting distribution to the filtered results using Akaike's Information Criterion.

2.8 | Model performance and validation

Model performance was evaluated using the test data and model performance criteria of the acceptable prediction zones (APZ) method (Oscar, 2005a, 2018a). A prediction was considered fully acceptable ($Y\text{-value} = 1$) when the residual (observed – predicted) was in an APZ from -1 log (fail-safe) to 0.5 log (fail-dangerous), whereas a prediction was considered partially acceptable ($Y\text{-value}$

>0 but <1) when the residual was in an APZ from > -2 to < -1 log (fail-safe) or from >0.5 to <1 log (fail-dangerous). The value (Y) assigned to a residual (X) was:

$$Y = 0 \text{ if } X \leq -2$$

$$Y = X + 2 \text{ if } X > -2 \text{ to } < -1$$

$$Y = 1 \text{ if } X \geq -1 \text{ to } \leq 0.5$$

$$Y = -2X + 2 \text{ if } X > 0.5 \text{ to } < 1$$

$$Y = 0 \text{ if } X \geq 1.$$

For example, if $X = -2.2$ then $Y = 0$, if $X = -1.6$ then $Y = -1.6 + 2 = 0.4$, if $X = -0.6$ then $Y = 1$, if $X = 0.3$ then $Y = 1$, if $X = 0.8$ then $Y = -2 * 0.8 + 2 = 0.4$, and if $X = 1.2$ then $Y = 0$ and the proportion of residuals in the APZ (pAPZ) = $\Sigma Y/n = (0 + 0.4 + 1 + 1 + 0.4 + 0) \div 6 = 0.47$.

A model provides predictions with acceptable bias and accuracy when the overall pAPZ ≥ 0.70 and there are no local prediction problems. A local prediction problem occurs when pAPZ < 0.70 for an individual level of an independent variable (e.g., 6 hr) or when pAPZ < 0.70 for three consecutive combinations of independent variables. A model was classified as validated when it satisfied all criteria for

dependent data (Figure 1) and all criteria for independent data for interpolation (Figure 2).

3 | RESULTS

3.1 | Growth of *Salmonella*

Growth of *Salmonella* Newport on Romaine lettuce was observed and increased as a function of time and temperature for dependent data (Figure 3) and for independent data (Figure 4) for interpolation. During 8 hr of incubation, growth (≥ 0.3 log increase) was observed at temperatures of 20°C (Figure 3b), 22°C (Figure 4b), 24°C (Figure 3c), 26°C (Figure 4c), 28°C (Figure 3d), 30°C (Figure 4d), 32°C (Figure 3e), 34°C (Figure 4e), 36°C (Figure 3f), 38°C (Figure 4f), and 40°C (Figure 3g) but not at temperatures of 16°C (Figure 3a) or 18°C (Figure 4a). The time for 0.3 log of growth or lag time ranged from 7.1 hr at 20°C (Figure 3b) to 1.2 hr at 40°C (Figure 3g). Total growth during 8 hr of incubation ranged from 0.42 log at 20°C (Figure 3b) to 4.42 log at 40°C (Figure 3g).

3.2 | Model simulation

Growth curves in Figures 3 and 4 were obtained using the model shown in Figure 5. The model also made stochastic predictions of

Q	A	Decision Tree for Dependent Data												
1	yes	Were the data used to develop the model?												
2	yes	Were the independent variables evenly spaced?												
3	yes	Was there a minimum of four prediction cases per combination of independent variables?												
4	yes	Did all combinations of independent variables have the same number of prediction cases?												
5	yes	Was the overall pAPZ ≥ 0.70 ?												
6	yes	Was pAPZ for all individual levels of independent variables ≥ 0.70 ?												
7	yes	Was a single pAPZ ≥ 0.70 for every three consecutive combinations of the independent variables?												
8	yes	Was the model validated for dependent data?												
Pivot Table for Test Data (Prediction Case Count)								Pivot Table for Model Performance (pAPZ)						
	t								t					
T	0	2	4	6	8	Total		T	0	2	4	6	8	Total
16	6	6	6	6	6	30		16	1.00	0.97	1.00	1.00	1.00	0.99
20	6	6	6	6	6	30		20	0.94	0.99	1.00	1.00	1.00	0.99
24	6	6	6	6	6	30		24	1.00	0.93	1.00	0.86	0.93	0.94
28	6	6	6	6	6	30		28	0.95	1.00	1.00	0.94	0.83	0.94
32	6	6	6	6	6	30		32	0.97	0.95	0.89	0.91	1.00	0.94
36	6	6	6	6	6	30		36	1.00	0.93	0.79	0.83	0.67	0.84
40	6	6	6	6	6	30		40	1.00	0.83	0.83	0.89	0.71	0.85
Total	42	42	42	42	42	210	Total	0.98	0.94	0.93	0.92	0.88	0.93	

FIGURE 1 Screenshot of the decision tree (dependent data) and acceptable prediction zones (APZ) analysis for development of a model (general regression neural network) for growth of *Salmonella* Newport on Romaine lettuce as a function of time (t) and temperature (T) where pAPZ is the proportion of residuals in the APZ

Q	A	Decision Tree for Interpolation									
1	yes	Was the model validated for dependent data?									
2	yes	Were the data independent?									
3	yes	Were the data collected using the same methods as dependent data?									
4	yes	Were the independent variables at values intermediate to those used in model development?									
5	yes	Was there a minimum of two prediction cases per combination of independent variables?									
6	yes	Did all combinations of independent variables have the same number of prediction cases?									
7	yes	Was the overall pAPZ ≥ 0.70 ?									
8	yes	Was pAPZ for all individual levels of independent variables ≥ 0.70 ?									
9	yes	Was a single pAPZ ≥ 0.70 for every three consecutive combinations of the independent variables?									
10	yes	Was the model validated for interpolation?									
Pivot Table for Test Data (Prediction Case Count)						Pivot Table for Model Performance (pAPZ)					
	t						t				
T	1	3	5	7	Total	T	1	3	5	7	Total
18	3	3	3	3	12	18	1.00	1.00	1.00	1.00	1.00
22	3	3	3	3	12	22	1.00	1.00	0.96	1.00	0.99
26	3	3	3	3	12	26	1.00	1.00	1.00	1.00	1.00
30	3	3	3	3	12	30	1.00	1.00	0.67	0.86	0.88
34	3	3	3	3	12	34	1.00	0.67	0.46	0.98	0.78
38	3	3	3	3	12	38	1.00	1.00	0.96	0.85	0.95
Total	18	18	18	18	72	Total	1.00	0.94	0.84	0.95	0.93

FIGURE 2 Screenshot of the decision tree (independent data) and acceptable prediction zones (APZ) analysis for validation (interpolation) of a model for growth of *Salmonella* Newport on Romaine lettuce as a function of time (t) and temperature (T) where pAPZ is the proportion of residuals in the APZ

growth by random sampling of pert distributions for time (0, 2, and 8 hr) and temperature (16, 22, and 40°C; Figure 5). For the iteration shown in Figure 5, the model predicted the growth curve for the randomly selected temperature of 30.6°C and it predicted the amount of growth (0.73 log) for the randomly selected time of 3.9 hr.

After 1,000 model iterations, results were filtered to remove no growth events (<0.3 log increase) and incidence and extent of growth were determined. Incidence of growth was 18.7% (187/1,000), whereas extent of growth ranged from 0.3 to 2.34 log with a mean of 0.71 log. The best fitting distribution for extent of growth was the BetaGeneral (Figure 6), whereas a discrete distribution can be used to simulate incidence of growth events. These distributions can be used together in a risk assessment to simulate the variability and uncertainty of the incidence and extent of *Salmonella* growth on Romaine lettuce following cross-contamination from raw chicken during meal preparation (Oscar, 2004b).

3.3 | Model performance and validation

Fitness of data for evaluating model performance was evaluated using decision trees with a series of “yes” or “no” questions pertaining to criteria of the APZ method. An answer of “yes” indicated

that the data met the criterion, whereas as an answer of “no” indicated that the data did not meet the criterion. The dependent data met all criteria for test data as indicated by answers of “yes” to Questions 1–4 in Figure 1. Likewise, the independent data for interpolation met all criteria for test data as indicated by answers of “yes” to Questions 2–6 in Figure 2. Thus, both sets of data were found to provide an accurate and unbiased evaluation of model performance.

Model performance was evaluated using decision trees with three “yes” or “no” questions pertaining to criteria for model performance in the APZ method. One question was for overall performance and two questions were for local performance and they were the same for both sets of data. For dependent data ($n = 210$), overall pAPZ was 0.93, pAPZ for individual times ranged from 0.88 to 0.98, pAPZ for individual temperatures ranged from 0.84 to 0.99, and maximum number of pAPZ <0.70 for consecutive combinations of time and temperature was one (Figure 1). Thus, answers to Questions 5–7 in Figure 1 were “yes” indicating that model performance was acceptable for dependent data.

For independent data ($n = 72$) for interpolation, overall pAPZ was 0.93, pAPZ for individual times ranged from 0.84 to 1.00, pAPZ for individual temperatures ranged from 0.78 to 1.00, and maximum number of pAPZ <0.70 for consecutive combinations of time and temperature was two (Figure 2). Thus, answers to Questions 7–9 in Figure 2

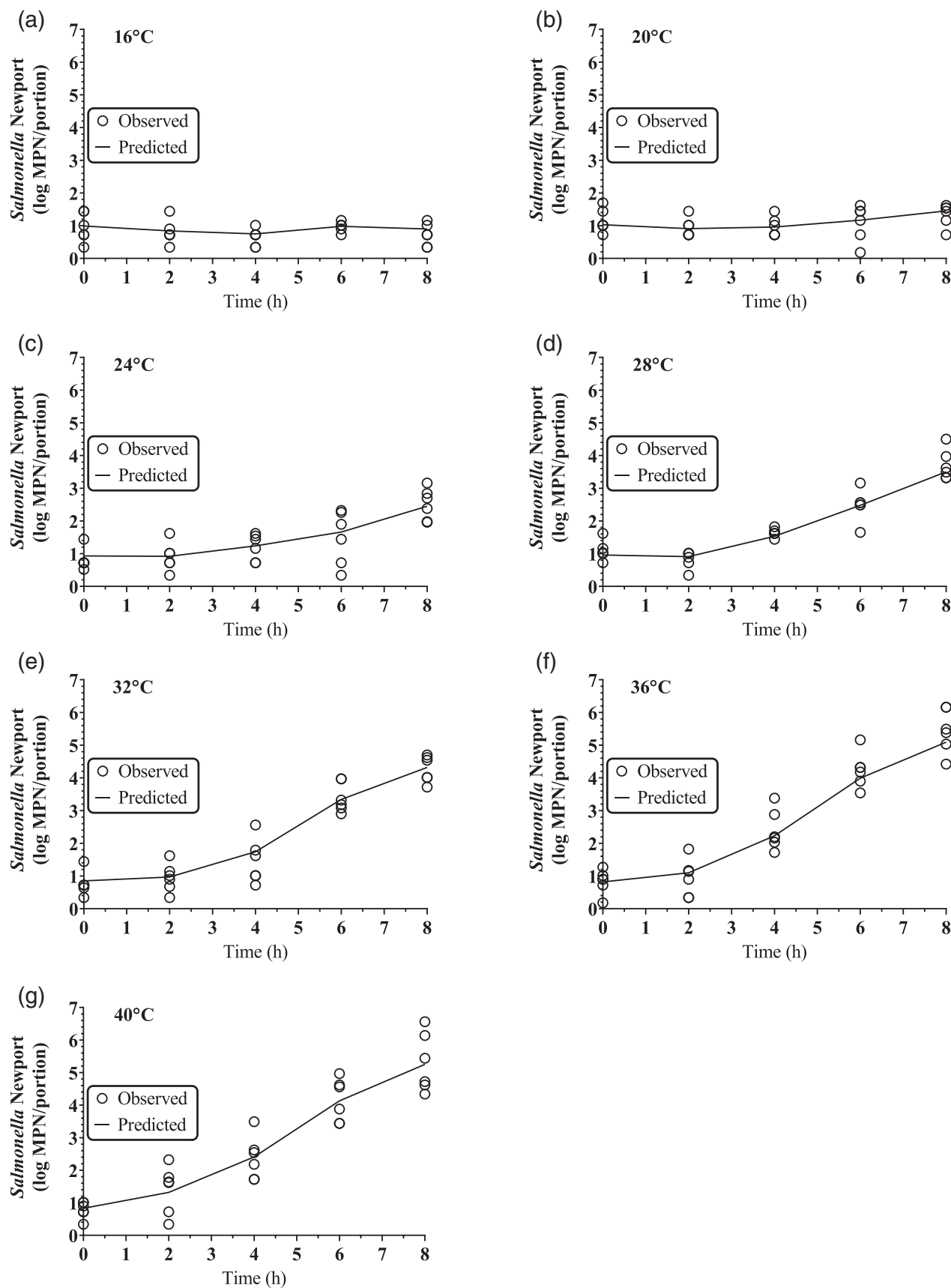


FIGURE 3 Growth of *Salmonella* Newport on Romaine lettuce as a function of time and temperature for dependent data where MPN is the most probable number

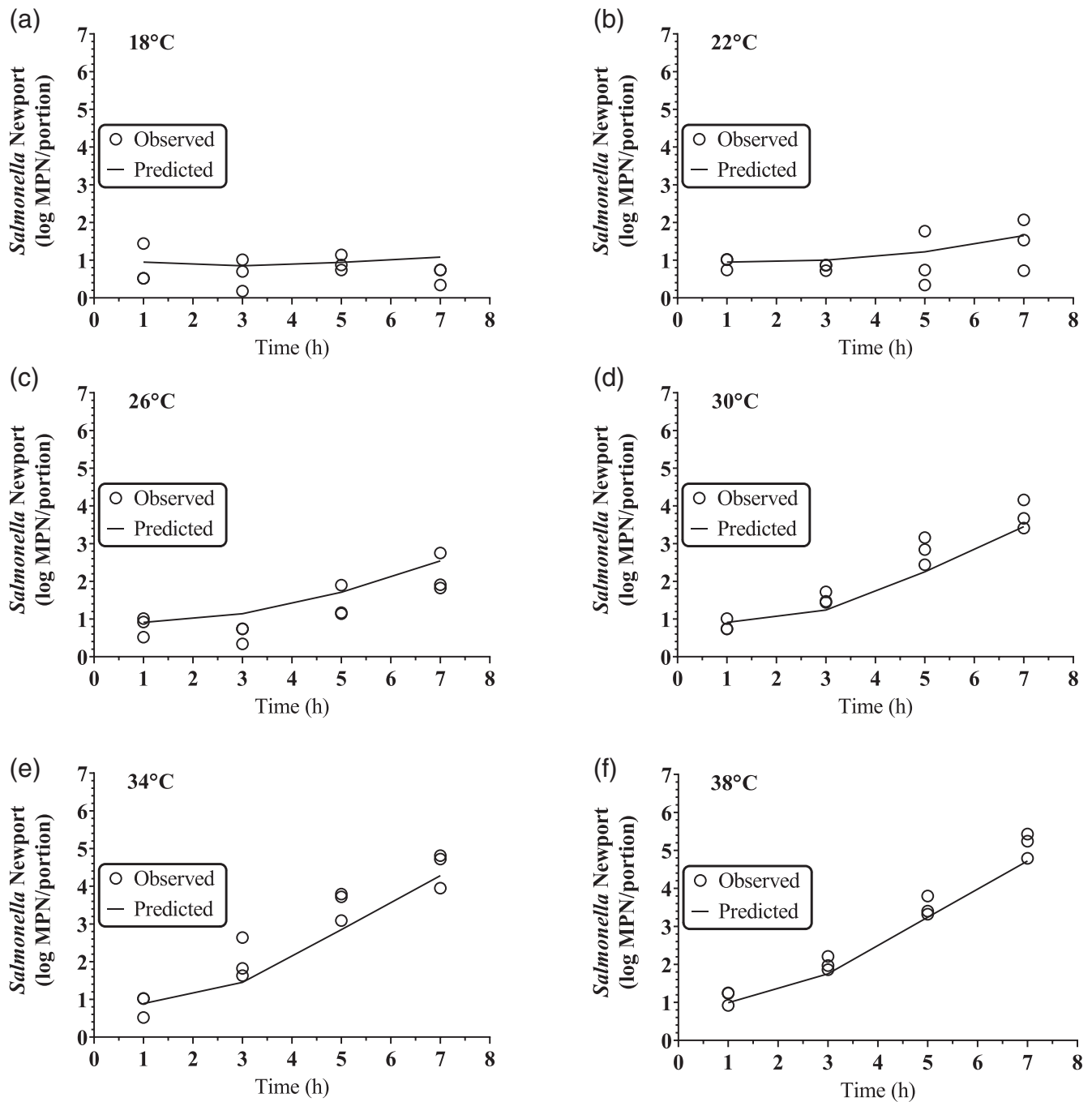


FIGURE 4 Growth of *Salmonella* Newport on Romaine lettuce as a function of time and temperature for independent data (interpolation) where MPN is the most probable number

were “yes” indicating that model performance for interpolation was acceptable.

Because all test data and model performance criteria for dependent data (Figure 1) and all test data and model performance criteria for independent data (Figure 2) were satisfied, the model was classified as validated for interpolation. Thus, it can be used with confidence to predict growth of a low initial number (0.91 log) of *Salmonella* Newport on Romaine lettuce as a function of time (0–8 hr) and temperature (16–40°C).

4 | DISCUSSION

Cross-contamination of RTE salad vegetables with *Salmonella* from raw chicken followed by growth during meal preparation are important risk factors for human salmonellosis. Oscar (2017) quantified natural cross-contamination of RTE cooked chicken with *Salmonella* from raw chicken during simulated meal preparation. When raw chicken was properly stored (6 hr at 4°C) before meal preparation, 10% of RTE cooked chicken portions were cross-contaminated with low

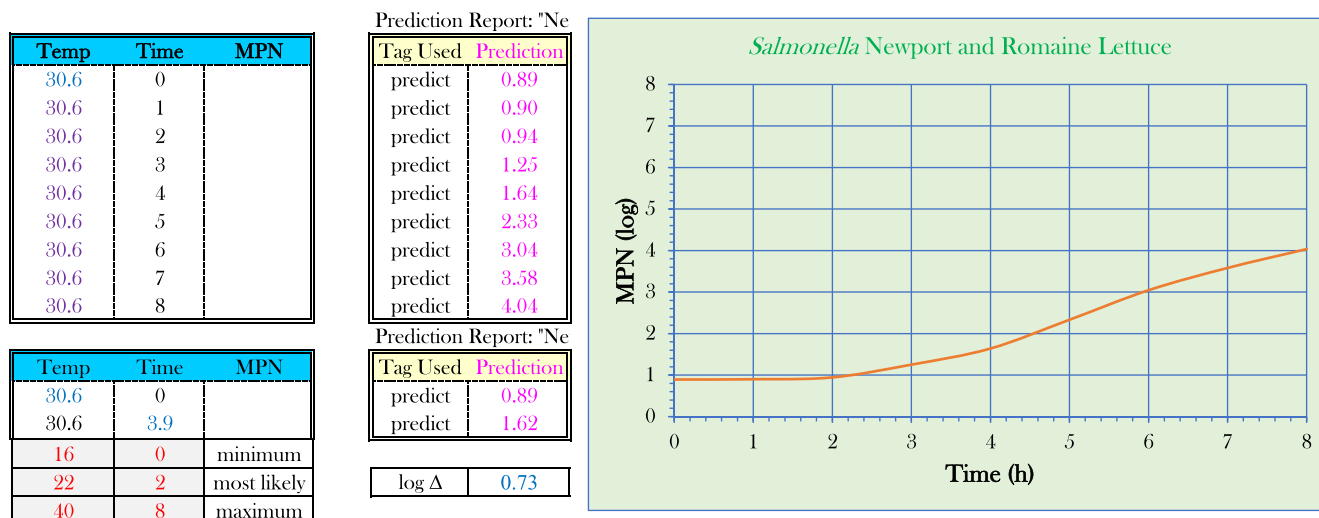
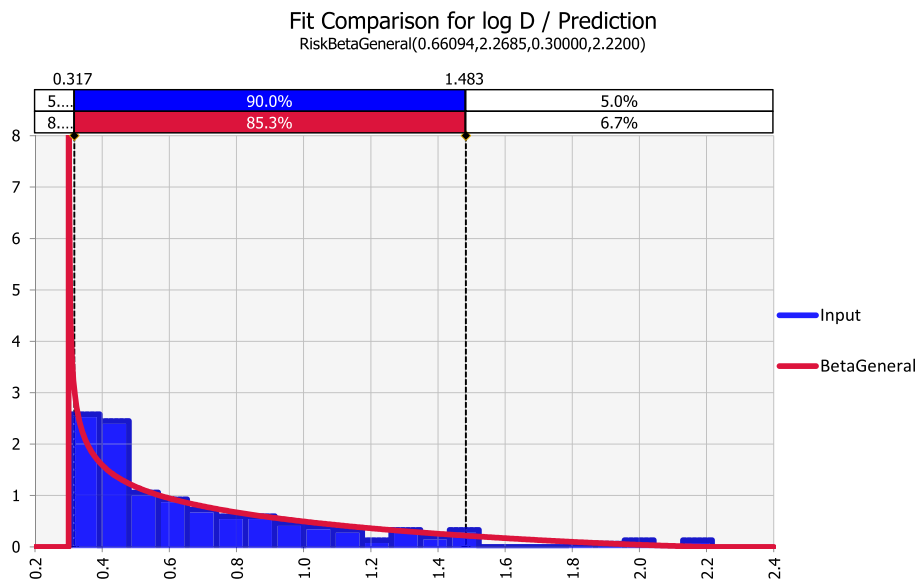


FIGURE 5 Screenshot of a model (general regression neural network) for growth of *Salmonella* Newport on Romaine lettuce as a function of time and temperature

FIGURE 6 Best-fit distribution for growth (≥ 0.3 log increase) of *Salmonella* Newport on Romaine lettuce for the time and temperature scenario shown in Figure 5



(0–0.2 log) numbers of *Salmonella* from unwashed utensils (i.e., cutting board, knife, and hands) used to prepare raw chicken for cooking. Consequently, based on these results, the current model was developed with a low initial number (0.91 log) of a single chicken isolate of *Salmonella* Newport.

Oscar (2017) also quantified natural cross-contamination of RTE cooked chicken with *Salmonella* from raw chicken that was improperly stored (72 hr at 15°C) before meal preparation. He found that 52% of RTE cooked chicken portions were cross-contaminated with 0.3–6.2 log of *Salmonella* from unwashed utensils used to prepare raw chicken for cooking. How well the current model predicts growth of higher initial numbers of *Salmonella* on Romaine lettuce was not investigated but could be (Oscar, 2007) because the APZ method has criteria for validation of models for extrapolation (Oscar, 2013, 2018a). Validation for extrapolation is important because it saves time and money by

identifying independent variables (e.g., initial number) for which new models are not needed (Oscar, 2005b). In addition, it provides model users with confidence that predictions made outside the range of independent variables used to develop the model are reliable.

Recently, a model (multiple-layer feed forward neural network) for growth of the same strain of *Salmonella* Newport (0.85 log) on Roma tomato portions (0.14 g) incubated at times (0–8 hr) and temperatures (16–40°C) observed during meal preparation was developed and validated (interpolation) using the APZ method (Oscar, 2018a). This model was also validated for extrapolation to other serotypes of *Salmonella* (e.g., Montevideo, Thompson, Hadar, Heidelberg, and Typhimurium var 5-) using the APZ method. Thus, the current model can be improved by validating it for extrapolation to other serotypes and if serotypes are found that grow differently than *Salmonella* Newport, the model could be expanded to include these serotypes as was done

in a previous study (Oscar, 2009). In general, most serotypes of *Salmonella* have similar growth kinetics (Oscar, 1998, 2000) but a few, such as Enteritidis (Oscar, 2003), Kentucky (Oscar, 2009), and 8.20:–:z₆ (Oscar, 2018a), grow slower

The APZ method is being used by other researchers to evaluate model performance. Mishra, Guo, Buchanan, Schaffner, and Pradhan (2016) used it to evaluate performance of models for growth of *Salmonella* and *Listeria monocytogenes* on leafy greens. Luo, Hong, and Oh (2015) used it to evaluate performance of models for growth of *Listeria monocytogenes* on RTE ham and sausages. Li et al. (2011) used it to evaluate performance of models for thermal inactivation of *Listeria innocua* in poultry products. Min and Yoon (2010) used it to evaluate performance of models for growth of *Salmonella* Typhimurium and *Staphylococcus aureus* on cooked pork. Mohr et al. (2015) used it to evaluate performance of models for growth of *Clostridium perfringens* during the cooling of cooked, uncured and cured poultry and meat products. Although the model performance criteria of the APZ are based on established performance standards (Oscar, 2005b) and a statistical analysis of experimental error associated with bacterial enumeration (Oscar, 2005a), these studies are important because they confirm that the APZ method is now an accepted validation method in the field of predictive microbiology.

Predictive models for pathogen growth are often developed with a mixture of strains (Buchanan & Phillips, 1990; Gibson, Bratchell, & Roberts, 1988) with the assumption that the fastest-growing strain will predominate and result in a “fail-safe” model. However, without information about growth of individual strains in the cocktail it is not possible to validate this assumption. Thus, it is possible that the model could be “fail-dangerous” instead. A way to avoid this issue is to develop a model with a single strain, like was done in this study, and then validate the model for extrapolation to other strains as was done in previous studies (Oscar, 2015, 2018a). In addition, a model developed with a single strain can be expanded to include other strains that growth differently. In fact, models that predict pathogen growth (Oscar, 2009) or dose–response (Oscar, 2004a) as a function of strain prevalence and variation are valuable tools for risk assessment.

The previous history of the strain used to develop a model (Buchanan & Klawitter, 1991; Hawkins et al., 2019) is important for accurate prediction of pathogen behavior in the next step of the farm-to-table chain. Thus, it is important to use a previous history that is relevant to the step in the risk pathway that is being simulated by the model. In the current study, the strain used for model development was grown to stationary phase at 22°C, which is a temperature that is commonly observed in the kitchen or meal preparation environment. In addition, stationary phase cells were used to simulate the likely physiological state of cells transferred from raw chicken to utensils during meal preparation. Thus, the previous history used in the current study was designed to simulate a scenario that is likely to occur in the real-world during meal preparation. Although a previous study (Oscar, 1999) showed that previous temperature (22–34°C) does not alter subsequent growth of *Salmonella*

Typhimurium on RTE cooked chicken over a range of temperatures (22–34°C), the current model could still be improved by evaluating it for extrapolation to other previous life cycle phases and temperatures and then expanding it to include other life cycle phases and previous temperatures if needed.

Growth of *Salmonella* on lettuce has been investigated and modeled by other researchers. Veys, de Oliveira Elias, Sampers, and Tondo (2016) investigated growth of a five-strain mixture of *Salmonella* on lettuce (10-g portions) stored at 5, 10, 25, and 37°C. They developed secondary models for lag time and growth rate but did not evaluate model performance against an independent set of data or determine growth kinetics for individual strains in the cocktail. Sant'Ana, Franco, and Schaffner (2012) developed secondary models for lag time and growth rate for a mixture of *Salmonella* serotypes on lettuce stored at 7–30°C but did not evaluate model performance against an independent set of data or report growth kinetics for individual strains in the cocktail. Thus, both models could be improved by validation against an independent set of data for interpolation and by validation against independent data for extrapolation to individual strains.

5 | CONCLUSIONS

Validation of models is important because it provides users of models with confidence that predictions are reliable. This is especially important when models are used to inform decisions about food safety and public health. Criteria that ensure comparison of observed and predicted values is accurate and unbiased, and that provide an objective evaluation of model performance are important to the validation process. In the current study, a model for growth of a chicken isolate of *Salmonella* Newport on Romaine lettuce as a function of times and temperatures observed during meal preparation was successfully validated using criteria that ensure model validation was accurate, unbiased, and objective. Thus, the model can be used with confidence to predict and manage this important risk to public health; namely, the growth of *Salmonella* on an RTE salad vegetable following cross-contamination from raw chicken during meal preparation.

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ORCID

Thomas P. Oscar  <https://orcid.org/0000-0001-6253-1286>

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