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Use of ComBase data to develop an artificial neural network model for nonthermal inactivation of *Campylobacter jejuni* in milk and beef and evaluation of model performance and data completeness using the acceptable prediction zones method

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Abstract

ComBase is a widely used microbial modeling database. ComBase data can be used to develop and validate models and to test novel modeling methods like artificial neural networks (ANN) and acceptable prediction zones (APZ), which have been shown to outperform traditional methods. Here, ComBase data were used to evaluate the ANN and APZ methods for modeling nonthermal inactivation of *Campylobacter jejuni* in milk and beef as a function of time, temperature (−20, 1, 10, 20, 30, and 40°C), and strain (18177, ATCC 29428). Four ANN were developed using Excel and NeuralTools, and the best-performing was a general regression neural network (GRNN) whose performance and data completeness were evaluated using the APZ method. Relative variable impacts in the GRNN model were 42.5%, 31.5%, 20.1%, and 5.9% for time, temperature, food, and strain, respectively. Nonthermal inactivation of *C. jejuni* was faster and greater at ambient than at cold temperatures and in milk than in beef except at 1°C where it was similar. The proportion of residuals in the APZ (pAPZ) ranged from 0.77 to 1 for individual nonthermal inactivation curves. Although the model had acceptable performance (pAPZ ≥0.7), it failed validation because of data gaps like one instead of four replicates per combination of independent variables and no data at −10°C. Thus, these and other data gaps identified need to be filled before the model can be used with confidence to predict behavior of *C. jejuni* in milk and beef. Nonetheless, results indicated that ANN and APZ methods can be used to model data for nonthermal inactivation of *C. jejuni* in food.

1 | INTRODUCTION

Campylobacter is a leading cause of foodborne illness (campylobacteriosis) that is often attributed to chicken and raw milk (Batz, Hoffmann, & Morris Jr., 2012; Tack et al., 2020). In fact, *Campylobacter* is a zoonotic pathogen and normal member of the gut microbiome of chickens and dairy cows (Indikova, Humphrey, & Hilbert, 2015; Rossler et al., 2019; Zbrun et al., 2020). Consequently, it has been isolated from chicken, raw milk, and beef where the most common species is *jejuni* (Del Collo et al., 2017; Nielsen, Engberg, & Madsen, 1997; Stern et al., 1985; Zbrun et al., 2020).

The primary route of zoonotic transmission of *C. jejuni* to humans is the fecal–oral route. This occurs from fecal contamination of milk during collection and of beef during processing followed by consumption of raw milk or undercooked beef (Troutt & Osburn, 1997). However, *C. jejuni* can also infect the udder and milk of dairy cows providing another route of zoonotic transmission (Hutchinson et al., 1985).

Although *C. jejuni* is sensitive to heat (Christopher, Smith, & Vanderzant, 1982; Whyte, Hudson, & Graham, 2006), consumption of raw milk and undercooked beef results in outbreaks and sporadic cases of campylobacteriosis involving bloody diarrhea, stomachache, fever, headache, and chronic conditions like Guillain–Barré syndrome (Heuvelink et al., 2009; Troutt & Osburn, 1997). In addition to heat, *C. jejuni* is sensitive to pH, drying, and oxygen leading to nonthermal inactivation in food stored under acidic, dry, or aerobic conditions (Al-Qadiri et al., 2015; Christopher et al., 1982; Doyle & Roman, 1982b; Kim et al., 2017). In general, nonthermal inactivation of *C. jejuni* is faster and greater at ambient than at cold temperatures (Olofsson, Berglund, Olsen, Ellstrom, & Axelsson-Olsson, 2015; Yoon, Burnette, & Oscar, 2004).

Models that predict nonthermal inactivation of *C. jejuni* in food (Burnette & Yoon, 2004; Oyarzabal, Oscar, Speegle, & Nyati, 2010; Ritz et al., 2007) are valuable tools for food safety (Elliott, 1996; Notermans, Gallhoff, Zwietering, & Mead, 1995). However, because of its sensitivity to oxygen and other stresses (Al-Qadiri et al., 2015; Christopher et al., 1982; Doyle & Roman, 1982b; Kim et al., 2017), *C. jejuni* is difficult to cultivate and enumerate (Eideh & Al-Qadiri, 2011), which makes it hard to collect the large amount of data needed for model development and validation. Consequently, there are few models for *C. jejuni*.

Validation of models for foodborne pathogens is important because it provides users with confidence that predictions are reliable (Zwietering, Cuppers, deWit, & van 'T Riet, 1994). Criteria for test data, model performance, and model validation help ensure that the validation is objective, complete, not confounded, accurate, and unbiased (Oscar, 2005b, 2020b). However, few studies use such criteria to validate models.

Validation of models is also important because it can identify data gaps and prediction problems that can be repaired by additional research (Oscar, 2005b). The goal of validation is not to reject models but, rather, to develop better models.

The acceptable prediction zones (APZ) method was developed to address limitations of traditional methods, it has been shown to outperform traditional methods, and is the only method that has criteria for test data, model performance, and model validation (Oscar, 2005a, 2005b, 2020b;). Although it is not used by all predictive microbiologists, it is used by some (Desriac, Vergos, Achberger, Coroller, & Couvert, 2018; Jayeola et al., 2019; Luo, Hong, & Oh, 2015; Min & Yoon, 2010; Mohr et al., 2015).

The APZ method has criteria for three types of data: (a) dependent data; (b) independent data for interpolation; and (c) independent data for extrapolation. These criteria require validation for dependent data before validation for interpolation and validation for interpolation before validation for extrapolation (Oscar, 2005b, 2020b), which is optional but important because it can save time and money by identifying independent variables for which new models are not needed (Oscar, 2007, 2013, 2015, 2018a).

The APZ method has performance criteria for three types of models: (a) secondary models for lag time (Oscar, 2005b); (b) secondary models for growth rate (Oscar, 2005b); and (c) primary, secondary, and tertiary models for log number (Oscar, 2005a, 2020b). Criteria for test data and validation are the same for all three types of models. However, prediction errors and APZ differ among model types (Oscar, 2005a, 2005b).

Not all criteria of the APZ method are used by modelers. Therefore, the APZ method was incorporated into the Validation Software Tool (ValT; Oscar, 2020b), which contains formula, decision trees, and pivot tables that perform the APZ method after data entry. Thus, it is now easy to use all criteria of the APZ method.

Artificial intelligence (AI), which involves machine learning of patterns in data to perform human behaviors like driving a car or medical diagnosis is changing the world we live in. Artificial neural network (ANN) modeling, a method of AI, can learn patterns in complex sets of microbial data and make predictions of pathogen behavior in food (Kuroda, Okuda, Ishida, & Koseki, 2019; Yolmeh, Habibi Najafi, & Salehi, 2014). In the past, ANN was difficult to use in predictive microbiology applications. However, with the arrival of commercial software programs, it is now easy to develop ANN models for foodborne pathogens (Oscar, 2009, 2014, 2017a, 2017b, 2018a, 2018b, 2021).

Advantages of ANN models over traditional regression models in predictive microbiology applications are: (a) more rapid development of models (one-step vs. three-steps); (b) easier development of models (less time and knowledge); (c) greater flexibility to predict sets of data with diverse patterns of pathogen behavior; and (d) same or better prediction (Hajmeer, Basheer, & Najjar, 1997; Palanichamy, Jayas, & Holley, 2008).

Disadvantages of ANN models versus traditional regression models in predictive microbiology applications are (a) parameters lack biological meaning and (b) over-training (poor interpolation). However, ANN learn patterns in data in a way that mimics the process used by the human brain and their output (lag time, growth rate, and log number) has biological meaning. Also, predictive microbiology is about prediction and when it comes to prediction, ANN models are the same or better than regression models (Hajmeer & Basheer, 2003; Schepers, Thibault, & Lacroix, 2000).

Finally, over-training can be avoided by following the criteria of the APZ method when developing and validating ANN (Oscar, 2017b, 2018a, 2018b, 2020a, 2021). Thus, there is no reason not to use ANN in predictive microbiology applications.

ComBase is an open access database for microbial modeling that is widely used (Baranyi & Tamplin, 2004). Data in ComBase can be used to test novel modeling methods like ANN and APZ without collecting new data. Data in ComBase are from peer-reviewed publications. Thus, it is generally assumed by ComBase users that models developed with data from ComBase provide reliable predictions. However, this assumption has never been tested. Therefore, data from ComBase for nonthermal inactivation of *C. jejuni* in milk and beef (Christopher et al., 1982) were used to (a) develop an ANN model; (b) evaluate model performance and data completeness using the APZ method; and (c) to identify future research needs.

2 | MATERIALS AND METHODS

2.1 | Data source and description

Log number data ($n = 328$) for nonthermal inactivation of *C. jejuni* in milk and beef were mined from ComBase (www.portal.errc.ars.usda.

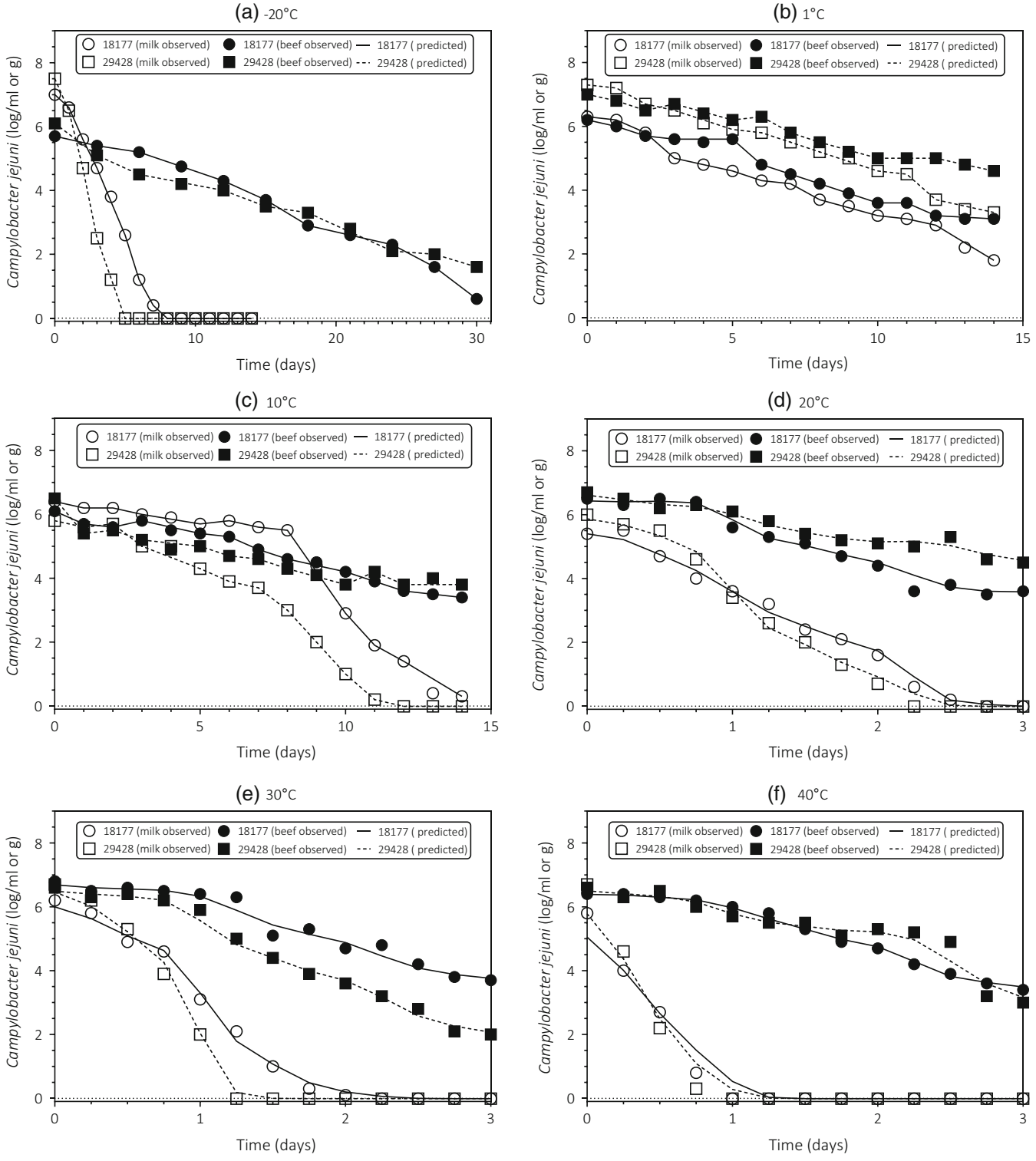

FIGURE 1 Nonthermal inactivation of two strains (18177 or ATCC 29428) of *Campylobacter jejuni* in milk and beef incubated at (a) −20°C; (b) 1°C; (c) 10°C; (d) 20°C; (e) 30°C; or (f) 40°C. Symbols are observed values and lines are predicted values

gov). These data were collected as described in the original publication (Christopher et al., 1982).

In brief, inoculums of *C. jejuni* were prepared by growing them for 3 days at 37°C in Brucella broth supplemented with 0.15% agar, 0.02% sodium metabisulfite, 0.05% sodium pyruvate, and 0.02% $FeSO_4$. After incubation, cultures were used to inoculate milk (10 ml, sterile, skim [0% fat]; pH = 6.6) and beef (10 g, surface sterile, not ground; pH = 6.0) with 6.4 ± 0.5 log per ml or g, respectively, of *C. jejuni* 18177 or ATCC 29428.

Inoculated samples were incubated at −20°C for 14 (milk) or 30 (beef) days, at 1 or 10°C for 14 days, or at 20, 30, or 40°C for

3 days. At evenly spaced time intervals, undiluted or diluted samples of milk and homogenized beef in Brucella broth were streaked onto Brucella agar plates supplemented with 10% defibrinated horse blood. Plates were incubated under microaerophilic (5% O_2, 10% CO_2, and 85% N_2) conditions for 3 days at 37°C before counting of colonies.

The lowest reported plate count was 0.1 log/ml or g. Also, it was reported that *C. jejuni* was not detected in some samples. When *C. jejuni* was not detected, a value of −0.01 log/ml or g was assigned. This was done to include these samples in model development and validation. In addition, it was based on the convention used in ComBase (Baranyi & Tamplin, 2004).

2.2 | Model development

Data were entered in an Excel (Office 365, MicroSoft Corporation, Redmond, WA) spreadsheet using six columns: (a) tag (train or test); (b) strain (independent categorical variable); (c) food (independent categorical variable); (d) temperature (°C; independent numerical variable); (e) time (days; independent numerical variable); and (f) log number (per ml or g; dependent numerical variable). There was one replicate per combination of independent variables.

Based on previous studies (Oscar, 2009, 2014, 2017a, 2017b, 2018a, 2018b, 2021), data were tagged for testing as follows: (a) for a time course of 30 days at 9 and 21 days; (b) for a time course of 14 days at 4, 9, and 13 days; and (c) for a time course of 3 days at 1 and 2.25 days. This resulted in 270 (82%) data for training and 58 (18%) data for testing the ANN during training.

Tagging was done for two reasons. First, a portion of the data were needed for testing the ANN during training. Second, to avoid local prediction problems from data gaps caused by random tagging of data for training and testing (Najjar, Basheer, & Hajmeer, 1997). In fact, in the present study, when data were randomly tagged, local prediction problems were observed (results not shown).

The BestNet Search option of NeuralTools (version 7.6, Palisade Corporation, Ithaca, NY) was used to find the best-performing ANN or to develop the best model. Four ANN were evaluated: general regression neural network (GRNN) and multiple-layer feedforward neural networks (MLFNN) with one hidden layer of two, three, or four nodes. Performance was based on the root mean squared error (RMSE) for testing data. Other details (diagram, formula, and training algorithms) of the ANN methods used in NeuralTools can be found in previous publications (Oscar, 2009, 2015).

For proprietary reasons, NeuralTools does not provide ANN parameters. However, the predict function of NeuralTools can be used to develop a standalone version of the model (Oscar, 2017c). This can be accomplished by creating an array of predictions and then using the CONCATENATE and VLOOKUP functions of Excel to generate a predicted nonthermal inactivation curve. Once published, the model will be available at: www.ars.usda.gov/nea/errc/PoultryFARM.

2.3 | Model performance and data completeness

Model performance and data completeness were evaluated using the APZ method in the Validation Software Tool (ValT) (Oscar, 2020b). Decision trees in ValT were used to combine criteria for test data, model performance, and model validation into an objective decision about model performance and data completeness using a series of "yes" or "no" questions (Q) where answers of "yes" led to model validation and a single answer of "no" resulted in failure of the validation process. An answer of "no" indicated that the model had a data gap or prediction problem that needed to be repaired by additional research before the model could be used with confidence to make predictions. Model repair can be accomplished by collection of new data, use of a different model, or both.

The data used for testing the ANN during training were evaluated as dependent data even though they were independent data for interpolation. This was done because there were too few data to evaluate

TABLE 1 Acceptable prediction zone analysis of model performance for dependent data

Question	Answer	Decision tree
1	Yes	Were the data used to develop the model?
2	No	Were the independent variables evenly spaced?
3	No	Was there a minimum of four prediction cases per combination of independent variables?
4	No	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	No	Was the model validated for dependent data?

pAPZ[a]	Temperature (°C)						
Other	−20	1	10	20	30	40	Average
Milk	**1.00**	**1.00**	**1.00**	**0.85**	**0.88**	**0.78**	**0.92**
18177[b]	1.00	1.00	1.00	0.85	0.92	0.79	0.93
29428	1.00	1.00	1.00	0.85	0.85	0.77	0.92
Beef	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**0.99**	**1.00**
18177	1.00	1.00	1.00	1.00	1.00	1.00	1.00
29428	1.00	1.00	1.00	1.00	1.00	0.98	1.00
Average	**1.00**	**1.00**	**1.00**	**0.92**	**0.94**	**0.89**	**0.96**

Note: The bold values in the rows for milk and beef are the average APZ values for the indicated temperature (column label) when the data for both strains are combined. The bold values in the row for average is the average APZ value for the indicated temperature (column label) when the data for both strains and both food matrices are combined.
[a]Proportion of residuals in the acceptable prediction zones.
[b]*Campylobacter jejuni* strain 18177 or ATCC 29428.

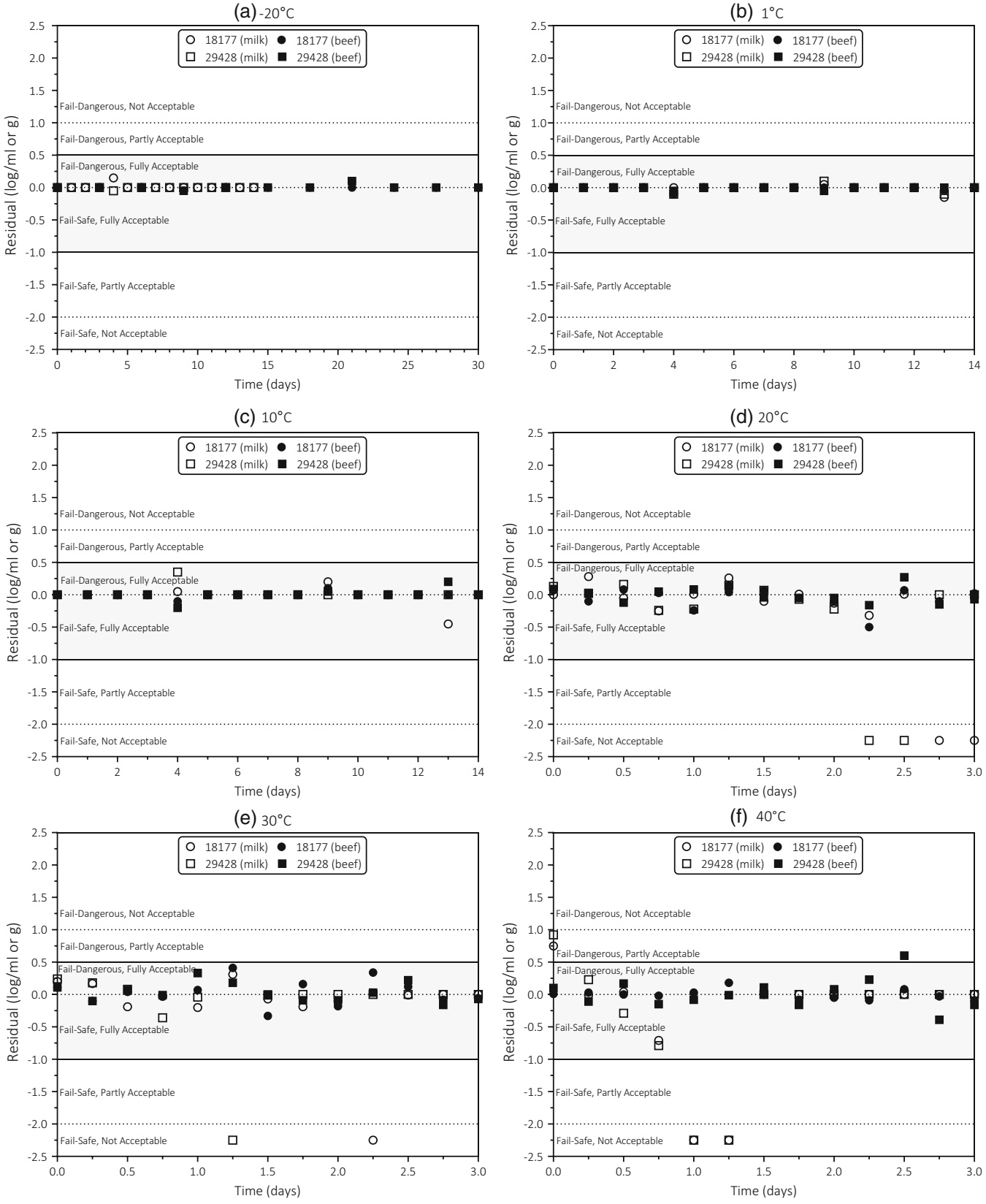

FIGURE 2 Residual (observed – predicted) plots of dependent data and acceptable prediction zones for evaluating performance of a General Regression Neural Network model for predicting nonthermal inactivation of two strains (18177 or ATCC 29428) of *Campylobacter jejuni* in milk or beef as a function of time and temperatures of (a) −20°C; (b) 1°C; (c) 10°C; (d) 20°C; (e) 30°C; or (f) 40°C

and validate the model for interpolation, as explained below, and because it simplified presentation of results without changing conclusions. Thus, evaluation of model performance was only done for dependent data and for extrapolation to the other food matrix.

Global and local model performance were evaluated. Proportion of residuals (observed -predicted log number per ml or g) in fully acceptable and partly acceptable prediction zones (pAPZ) were calculated by ValT (see below). A pAPZ ≥0.7 was considered acceptable model performance (acceptable prediction accuracy and bias). A local prediction problem occurred when pAPZ were <0.7 for a single level of an independent variable or for three consecutive combinations of independent variables.

The pAPZ were calculated using four APZ: (a) 0 to −1 log/ml or g (fail-safe and fully acceptable); (b) <−1 to >−2 log/ml or g (fail-safe and partly acceptable); (c) 0–0.5 log/ml or g (fail-dangerous and fully acceptable); and (d) >0.5 and <1 log/ml or g (fail-dangerous and partly acceptable). For calculation of pAPZ, residuals in fully acceptable APZ were assigned a value of one, residuals in partly acceptable APZ were assigned values from >0 to <1 depending on their linear distance from the corresponding fully acceptable APZ, and residuals outside the APZ were assigned a value of zero. The pAPZ were calculated in ValT for individual levels and combinations of independent variables and overall. This was accomplished using the pivot table feature of Excel.

Special prediction cases occurred when observed or predicted values were −0.01 log/ml or g. In other words, when survival of *C. jejuni* was not observed or predicted. When the ANN model predicted survival (≥0 log/ml or g), but no survival (−0.01 log/ml or g) was observed, a residual outside the fail-safe APZ (≤−2 log) was assigned. When the ANN predicted no survival (−0.01 log/ml or g), but survival (≥0 log/ml or g) was observed, a residual outside the failure dangerous APZ (≥1 log) was assigned. Finally, when the ANN model predicted no survival (−0.01 log/ml or g) and no survival (−0.01 log/ml or g) was observed, a residual of 0 log/ml or g was assigned. For clarity of presentation in the residual plots, assigned residuals for special prediction cases were equal to the maximum observed residual in the set of residuals being plotted.

For calculation of pAPZ, a value of one was assigned to special prediction cases with a residual of zero, whereas a value of zero was assigned to special prediction cases with an assigned residual outside the APZ. Inclusion of special prediction cases was done so that the evaluation of model performance was complete, accurate, and unbiased (Oscar, 2020b).

3 | RESULTS AND DISCUSSION

3.1 | Nonthermal inactivation curves

Log number of both strains of *C. jejuni* declined in milk and beef as a function of time and temperature (Figure 1). This decline was similar among strains, displayed diverse patterns, and was faster and greater at ambient (20, 30, and 40°C) than at cold (−20, 1, and 10°C) temperatures and in milk than in beef except at 1°C where it was similar.

3.2 | Model development

The RMSE (log/ml or g) for test data were 0.18 for GRNN and 1.49, 0.89, and 0.86 for MLFNN with 2, 3, and 4 nodes in the hidden layer, respectively. Thus, the GRNN was the best-performing ANN because it had the lowest RMSE for test data. The RMSE for the training data used to develop the GRNN model was 0.14 log/ml or g. Relative variable impacts in the GRNN model were 42.5% for time, 31.5% for temperature, 20.1% for food matrix, and 5.9% for strain. Thus, time had

TABLE 2 Acceptable prediction zones analysis of model performance for extrapolation to the other food matrix

Question	Answer	Decision tree					
1	No	Was the model validated for interpolation?					
2	Yes	Were data independent?					
3	Yes	Were data collected with the same methods as dependent data except for the new independent variable being evaluated?					
4	No	Were the independent variables at the same values as those used in model development?					
5	No	Was there a minimum of two prediction cases per combination of independent variables?					
6	No	Did all combinations of the independent variables have the same number of prediction cases?					
7	No	Was the overall pAPZ ≥0.70?					
8	No	Was pAPZ for all individual levels of independent variables ≥0.70?					
9	No	Was a single pAPZ ≥0.70 for every three consecutive combinations of the independent variables?					
10	No	Was the model validated for extrapolation?					
pAPZ[a]	Temperature (°C)						
Other	−20	1	10	20	30	40	Average
Milk	**0.15**	**0.96**	**0.60**	**0.22**	**0.21**	**0.08**	**0.38**
18177[b]	0.17	0.98	0.62	0.17	0.19	0.08	0.38
29428	0.13	0.93	0.58	0.26	0.23	0.09	0.38
Beef	**0.19**	**0.80**	**0.53**	**0.04**	**0.10**	**0.01**	**0.31**
18177	0.26	0.79	0.67	0.00	0.04	0.00	0.32
29428	0.12	0.80	0.39	0.07	0.15	0.01	0.29
Average	**0.16**	**0.88**	**0.56**	**0.13**	**0.15**	**0.04**	**0.35**

Note: The bold values in the rows for milk and beef are the average APZ values for the indicated temperature (column label) when the data for both strains are combined. The bold values in the row for average is the average APZ value for the indicated temperature (column label) when the data for both strains and both food matrices are combined.
[a]Proportion of residuals in the acceptable prediction zones.
[b]*Campylobacter jejuni* strain 18177 or ATCC 29428.

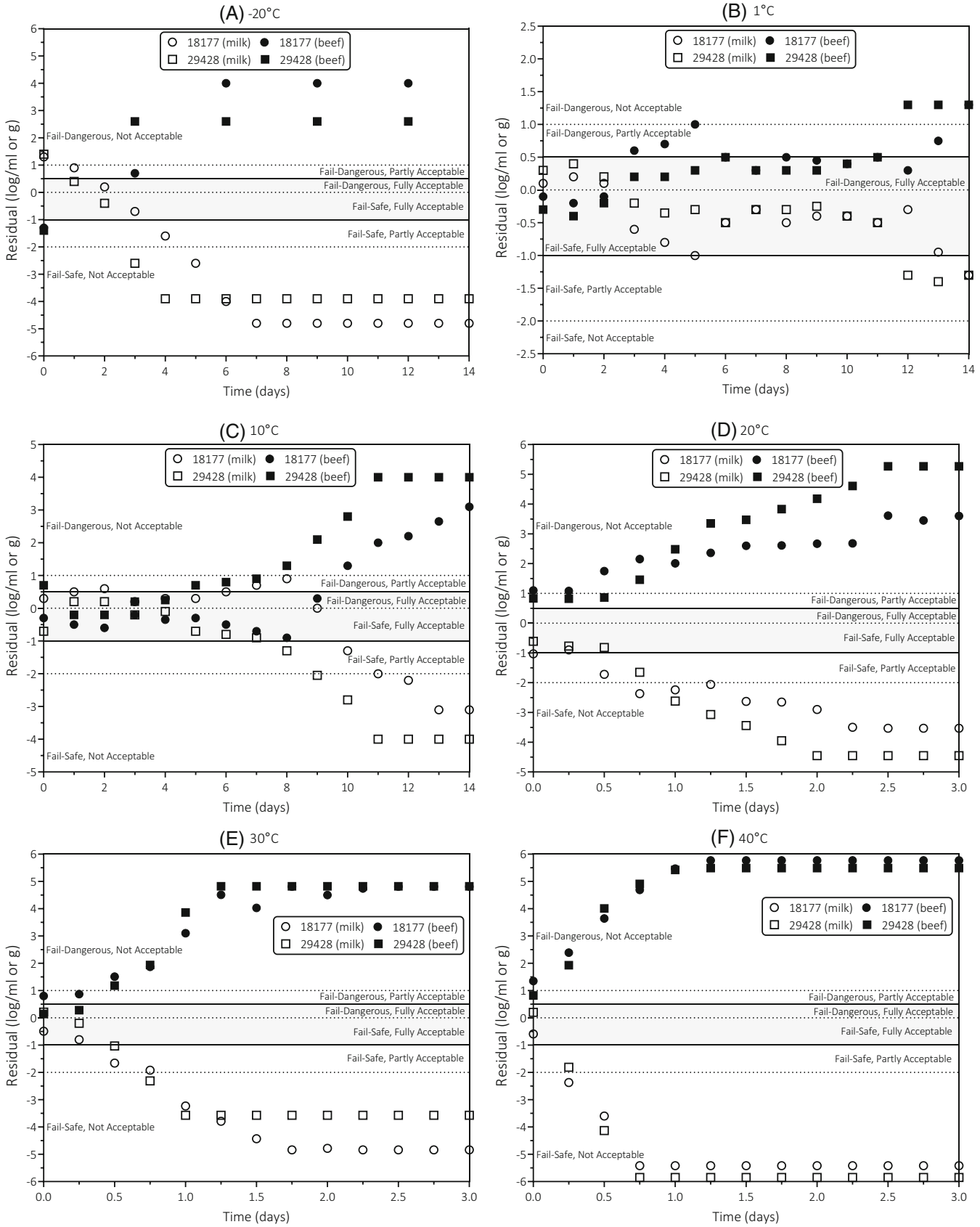

FIGURE 3 Residual (observed – predicted) plots and acceptable prediction zones for evaluation of milk predictions to beef (solid symbols) and beef predictions to milk (open symbols) for a General Regression Neural Network model that predicts nonthermal inactivation of two strains (18177 or ATCC 29428) of *Campylobacter jejuni* in milk and beef as a function of time and temperatures of (a) −20°C; (b) 1°C; (c) 10°C; (d) 20°C; (e) 30°C; or (f) 40°C

the most impact on the model output (log number of *C. jejuni*), whereas strain had the least impact on the model output.

3.3 | Model prediction

The predict function of NeuralTools was used to create the final GRNN model, which predicted nonthermal inactivation of *C. jejuni* in milk or beef as a function of strain, temperature, and time. For reasons presented below, pull down menus were used to restrict predictions to the strains (18177, ATCC 29428), food matrices (milk, beef), and temperatures (−20, 1, 10, 20, 30, and 40°C) used in model development and formula were used to restrict predictions to the time courses used in model development. After selection of a strain, food, and temperature from the pull-down menus, a nonthermal inactivation curve was predicted by the GRNN model for the range of times investigated. One of 24 possible nonthermal inactivation curves could be predicted by the final GRNN model, one for each combination of independent variables (strain, food, temperature, and time) investigated (Figure 1). The final GRNN model could interpolate within the nonthermal inactivation curves to provide predictions for times that were not investigated.

3.4 | Model performance and data completeness

3.4.1 | Dependent data

Overall pAPZ of the GRNN model for dependent data was 0.96 (Table 1), whereas pAPZ for individual nonthermal inactivation curves ($n = 24$) ranged from 0.77 to 1 for milk and from 0.98 to 1 for beef (Table 1). Thus, there were no global ("yes" to Question (Q) 5) or local ("yes" to Q6 and Q7) prediction problems (Table 1) and model performance was considered acceptable (pAPZ ≥0.7). In general, agreement between observed and predicted values was better at cold (−20, 1, 10°C) than at ambient (20, 30, and 40°C) temperatures (Figure 2).

Although the model provided acceptable predictions (pAPZ ≥0.7) of dependent data, it failed validation for dependent data ("no" to Q8) for three reasons (Table 1). First, values for temperature were not evenly spaced ("no" to Q2) because there were no data at −10°C and data were collected at 1°C instead of 0°C. Second, there was one instead of four replicates per combination of independent variables ("no" to Q3). Third, not all combinations of independent variables had the same number of replicates because sampling times at −20°C were different for milk and beef ("no" to Q4). Thus, there were gaps in the ComBase data that prevented model validation for dependent data. For these reasons, predictions of the final GRNN model were restricted to combinations of times, temperature, food, and strain investigated because these predictions were considered the least unreliable.

3.4.2 | Extrapolation

Although the GRNN model failed validation for dependent data and was not validated for interpolation because of too few data, it was evaluated for extrapolation to the other food. This was done to demonstrate how the APZ method can be used to examine the observed difference (relative variable impact of 20.1% for food) in nonthermal inactivation of *C. jejuni* in milk and beef (Table 2). Therefore, within a combination of strain and temperature and over time, predictions for milk were compared to observed data for beef and predictions for beef were compared to observed data for milk (Figure 3).

The only acceptable predictions (pAPZ ≥0.7) in these comparisons were at 1°C where the pAPZ for milk to beef predictions was 0.8 and the pAPZ for beef to milk predictions was 0.96 (Table 2). However, for strain ATCC 29428 at 12, 13, and 14 days of storage at 1°C, the residuals for both milk to beef and beef to milk predictions were outside the APZ (Figure 3b). Thus, there were local prediction problems.

In general, beef to milk predictions were overly fail-safe and milk to beef predictions were overly fail-dangerous (Figure 3) indicating that nonthermal inactivation of *C. jejuni* was faster and greater in milk than in beef. Also, in general, disagreement between observed and predicted values (size of residuals) for both beef to milk and milk to beef predictions increased as a function of time and temperature.

Per criteria of the APZ method (Table 2), the GRNN model failed validation for extrapolation to the other food ("no" to Q10) for five reasons. First, the model was not validated for interpolation ("no" to Q1) because of too few data. Second, there was one rather than two replicates per combination of independent variables ("no" to Q5). Third, the number of replicates per combination of independent variables was not the same because sampling times at −20°C were different for milk and beef ("no" to Q6). Fourth, there was a global ("no" to Q7) prediction problem. Fifth, there were local ("no" to Q8 and Q9) prediction problems.

4 | DISCUSSION

Compared to other major foodborne pathogens (*Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella*), there are fewer models for predicting behavior of *C. jejuni* in food. Thus, published data (Christopher et al., 1982), which had not been modeled, but that were archived in ComBase were used to develop an ANN model for predicting nonthermal inactivation of *C. jejuni* in milk and beef as a function of time, temperature (−20, 1, 10, 20, 30, and 40°C), and strain (18177, ATCC 29428).

Although the data were peer-reviewed, they were too few data to validate the ANN model using the APZ method (Oscar, 2020b). In fact, three data gaps were identified. First, data were missing at −10 and 0°C for model development. Second, three replicates per combination of independent variables were missing for model development. Third, two replicates per combination of independent variables were missing at intermediate times and temperatures to those used in model development. These data were needed to validate the model for interpolation.

To fill these data gaps, new data would need to be collected with the same methods as used to collect the dependent data or additional data gaps would be introduced. For example, if data for nonthermal

inactivation of a third strain of *C. jejuni* on chicken at −10 and 0°C were added, these data would not fill the first data gap. Rather, they would create more data gaps by introducing four new independent variable levels (third strain, third food, and two new temperatures) to the model. Thus, the model would be further away rather than closer to validation.

A valuable feature of predictive models for foodborne pathogens is the ability to interpolate or make predictions for combinations of independent variables that were not investigated but that are within ranges of independent variables used in model development. Validation for interpolation is crucial for ANN models because it shows they are not over-trained. Unfortunately, in the modeled study (Christopher et al., 1982), there were too few data to perform this validation. In addition, there was evidence that the GRNN developed from these data was over-trained. Specifically, some of the predicted nonthermal inactivation curves had wavy appearances as they seemed to follow the data used to develop the model. This was most evident at cold (−20, 1, and 10°C) temperatures where residuals were often zero.

The over-training likely occurred because of data gaps. First, there was only one rather than four replicates of dependent data per combination of independent variables. Second, there were no data at −10 and 0°C. Third, there were no data at times and temperatures that were intermediate to those used in model development. Fourth, sampling times differed between cold and ambient temperatures and between milk and beef at −20°C. These data gaps likely prevented proper training of the ANN and were identified using the APZ method.

Although sampling times in the modeled study (Christopher et al., 1982) were evenly spaced and appropriate within a nonthermal inactivation curve, they were not the same for all curves and thus, did not completely follow the preferred experimental design (full factorial with even spacing of independent variables) of the APZ method (Oscar, 2020b). In fact, using the test data criteria of the APZ method as a guide for model development and validation, the following approach could be used in the future to produce better models for *C. jejuni*.

Instead of developing a global and complex model like the one developed in the present study, which had a performance problem (over-trained) and data gaps, separate and simple models for milk and beef could be developed and validated for different time and temperature scenarios in the food production chain. For example, frozen storage (−20 to 0°C for 0–12 months), refrigerated storage (1–18°C for 14 days), and meal preparation (16–40°C for 0–8 hr). This would allow use of the same sampling times within each model, which would simplify model development and validation (Oscar, 2009, 2021).

Nonthermal inactivation of *C. jejuni* in milk and beef (Christopher et al., 1982) was faster and greater at ambient (20, 30, and 40°C) than at cold (−20, 1, and 10°C) temperatures. In comparison, Yoon et al. (2004) reported that the rate of nonthermal inactivation of *C. jejuni* in laboratory broth and on cooked chicken was constant and slowest from 4 to 14°C, increased linearly from 14 to 20°C, and was constant and fastest from 20 to 30°C. The faster nonthermal inactivation of *C. jejuni* at ambient temperatures than at cold temperatures was also observed in other studies conducted with chicken skin (Chantarapanont, Berrang, & Frank, 2003), milk (Olofsson et al., 2015), and beef (Kim et al., 2017).

Milk and beef samples used in the modeled study (Christopher et al., 1982) were pretreated with heat to eliminate or reduce native microflora. However, Doyle and Roman (1982a) reported that nonthermal inactivation of *C. jejuni* in raw milk with native microflora is faster than nonthermal inactivation in sterile milk without native microflora. In part, this was attributed to presence of lactoperoxidase in raw milk resulting in production of reactive oxygen species (H_2O_2) that inactivate *C. jejuni*.

On the other hand, in milk stored under aerobic conditions, nonthermal inactivation of *C. jejuni* is slower in the presence of the amoeba, *Acanthamoeba polyphaga* (Olofsson et al., 2015). Likewise, under aerobic conditions, nonthermal inactivation of *C. jejuni* is slower in co-culture with *Pseudomonas putida* (Hilbert, Scherwitzel, Paulsen, & Szostak, 2010). Scanning electron microscopy revealed that *P. putida* and *C. jejuni* live in close association within an extracellular matrix where *P. putida* is believed to create a more favorable (low oxygen tension) environment for *C. jejuni*. Therefore, in the future, it may be important to collect data in food with native microflora to develop models that provide better predictions of *C. jejuni* behavior in food.

Initial number of *C. jejuni* was variable (5.4–7.5 log/ml or g) among nonthermal inactivation curves in the modeled study (Christopher et al., 1982). This could be from variation of *C. jejuni* growth in inoculum cultures among individual challenge trials. This would not be surprising considering the many sensitivities of *C. jejuni* to environmental factors and resulting difficulties in cultivating this organism.

Although the effect of inoculum size on nonthermal inactivation of *C. jejuni* has not been investigated, with one exception where results were not conclusive (Eideh & Al-Qadiri, 2011), it could be an important variable to investigate and model in the future. Also, in future studies, it may be important to better control this variable and perhaps include it as an independent variable in predictive models for *C. jejuni*.

In addition to variability, initial number of *C. jejuni* in the modeled study (Christopher et al., 1982) was higher than typical levels found in raw milk (0–1.5 log/ml; Jaakkonen, Kivisto, Aarnio, Kalekivi, & Hakkinen, 2020) and beef liver (1–2 log/g; Enokimoto, Kubo, Bozono, Mieno, & Misawa, 2007). Quantitative data for *C. jejuni* in beef muscle were not found in the scientific literature. Nonetheless, use of lower inoculum sizes in future studies would likely result in better predictive models for *C. jejuni* behavior in food (Grigoriadis, Koidis, Vareltizis, & Batzios, 1997).

Although *C. jejuni* strain 18177 is more heat resistant than strain ATCC 29428 (Christopher et al., 1982), there was no consistent difference in nonthermal inactivation of these two strains in milk or beef (relative variable impact of 5.9%). However, other studies have found significant variation of inactivation rates among strains of *C. jeuni* in raw milk (Doyle & Roman, 1982a) and on agar media (Garenaux et al., 2008) over a range of temperatures. Thus, strain variation will be an important variable to further investigate in future studies and perhaps include as an independent variable in predictive models for *C. jejuni*.

Another important variable to consider is the physiological state or previous history of *C. jejuni*. In fact, when *C. jejuni* is stressed by starvation, it can enter a viable but nonculturable (VBNC) state (Federighi, Tholozan, Cappelier, Tissier, & Jouve, 1998; Painter et al., 2013). In addition, frozen storage of food can injure *C. jejuni* (Ritz et al., 2007). These altered physiological states due to previous history combined with an enumeration method that uses selective media ingredients could result in overestimation of inactivation and development of models that make fail-dangerous predictions of *C. jejuni* survival. However, a method like viability qPCR, which uses intercalation dyes to block quantification of DNA from dead cells, could be used to enumerate VBNC and injured cells in modeling studies (Wulsten, Galeev, & Stingl, 2020). It is also possible that a most probable number method could be designed to allow resuscitation and enumeration of VBNC and injured cells (Ritz et al., 2007). Regardless, in future studies, it will be important to use enumeration methods that detect and quantify VBNC and injured cells so that resulting models make accurate and unbiased predictions of *C. jejuni* survival in food.

Kim et al. (2017) investigated and modeled nonthermal inactivation of *C. jejuni* on beef tartare stored under aerobic conditions at 4, 10, 15, 25, and 30°C. Log number data within a temperature were graphed as a function of time and were fitted to a primary (Weibull) model that had two parameters: (a) the time to the first log reduction (δ) and (b) shape of the curve (ρ). A secondary (Davey) model was developed for predicting δ as a function of temperature but the data at 4°C were excluded. Although ρ was affected by temperature, a secondary model was not developed. Consequently, a full (tertiary) model that predicts nonthermal inactivation of *C. jejuni* on beef tartare as a function of time and temperature was not developed and validated.

Like the study of Kim et al. (2017), a three-step, traditional regression method for model development was tried in the current study (results not shown). Log number data for a single combination of independent variables (food, strain, and temperature) were graphed as a function of time and the resulting nonthermal inactivation curve was fitted to a three-phase (lag, log-linear inactivation, and bottom plateau) linear model that had four parameters: (a) initial log number; (b) lag time; (c) time to the bottom plateau; and (d) log number at the bottom plateau. However, because of the diverse patterns of nonthermal inactivation in the dataset (Figure 1), not all curve-fits provided results for all primary model parameters. Thus, because of these data gaps, it was not possible to develop secondary models for the primary model parameters or a full (tertiary) model for predicting nonthermal inactivation of *C. jejuni* in milk and beef as a function of time, temperature, and strain. However, it was possible to develop a full (tertiary) model in one-step using ANN modeling methods.

Kim et al. (2017) evaluated performance of their secondary model for δ. They used the coefficient of determination (R^2) to evaluate how well the model predicted the dependent data. An R^2 of 0.927 was obtained and it was concluded that the model was appropriate. Next, the secondary model for δ was evaluated for prediction of independent data obtained at 12 and 23°C. Observed and predicted values for δ were compared using RMSE, which was 0.475. Based on this result it was concluded that the model provides reliable prediction of *C. jejuni* behavior in beef tartare. However, there was no stated criterion for RMSE, which evaluates the model for prediction accuracy but not for prediction bias. In addition, no data were obtained between 25 and 30°C and the δ model only predicts part of the nonthermal inactivation curve. Thus, model development was incomplete and model validation was subjective, incomplete, inaccurate, and biased.

Like the δ model of Kim et al. (2017) there were too few data to validate the current ANN model. However, unlike the δ model Kim et al. (2017), the current model predicted the entire nonthermal inactivation curve and the evaluation of model performance was based on stated criteria. Also, unlike Kim et al. (2017), it was concluded that the current model did not provide reliable predictions. This conclusion was based on data gaps and prediction problems identified by the APZ method. A similar conclusion is reached when the APZ method is applied to the δ model of Kim et al. (2017).

A special prediction case occurred when the ANN model predicted survival, but no survival was observed or when the ANN model predicted no survival, but survival was observed. Quantitative comparison of differences in observed and predicted numbers is not possible in this situation because there is not a log number for zero. However, it is possible to make qualitative comparisons. This was accomplished by assigning a residual of that was outside the APZ to a no survival observation or prediction and then assigning an APZ value of zero to the prediction case, which was used in the calculation of pAPZ. In this way, special prediction cases could be included in model validation. This was important because if special prediction cases had been excluded, the evaluation of model performance would have been incomplete, inaccurate, and biased.

In the modeled study (Christopher et al., 1982), *C. jejuni* was not detected in some milk samples incubated for extended times, which resulted in a number of special prediction cases. Fortunately, these data were included in model development and validation because of the ANN and APZ methods used. In contrast, these data and special prediction cases would have been excluded if traditional regression and statistical methods had been used to develop and validate the model, as explained next.

A common practice in the secondary modeling step of the traditional regression method is to log transform the dependent variable (lag time, growth rate, and number) before curve-fitting (Buchanan & Phillips, 1990; Zwietering et al., 1994). However, dependent variable values of zero are excluded when this method is used because there is no log value for zero. Exclusion of these data during secondary modeling can result in a local prediction problem (Oscar, 2005b).

Exclusion of zero values from calculation of some model performance statistics is also necessary. For example, when lag time is not observed, it is not possible to calculate the relative error (residual/observed value) for the prediction case because it is not possible to divide by zero or into zero (Delignette-Muller, Rosso, & Flandrois, 1995). Moreover, any model performance statistic that uses a ratio, such as the bias and accuracy factors (Ross, 1996), would have to exclude values of zero. The result is a model validation process that is

incomplete, inaccurate, and biased. Thus, it is important to use modeling methods like those (ANN and APZ) used in the present study that include pathogen-free samples and special prediction cases in model development and validation.

In the modeled study (Christopher et al., 1982), milk and beef samples were heated before they were inoculated with *C. jejuni*. This heat treatment was done to inactive or reduce native microflora, but it also inactivated enzymes and other proteins that could affect the behavior of *C. jejuni* in these foods. Moreover, in contrast to milk, which was heated throughout, beef was only heated on its surface. Thus, native microflora, enzymes, and other proteins below the surface heating zone may have persisted and affected the results. Especially since *C. jejuni* was inoculated into the interior center of the beef samples.

In the modeled study (Christopher et al., 1982), nonthermal inactivation of *C. jejuni* was faster and greater in milk than in beef. Differences in pH, water activity, oxygen tension, nutrient composition (sulfur and iron), form (liquid and solid), native microflora (number and type), and enzyme activity (lactoperoxidase and superoxide dismutase) could explain the faster and greater nonthermal inactivation of *C. jejuni* in milk than beef, but the exact reason is not known. Thus, additional research is needed. Nonetheless, these results show the importance of the food matrix as an independent variable in predictive models for *C. jejuni*.

In the modeled study (Christopher et al., 1982), nonthermal inactivation of *C. jejuni* was faster and greater or the same at frozen than refrigeration temperatures. In part, this can be explained by formation of ice crystals during freezing of milk and beef that could damage cellular membranes and kill or injure *C. jejuni*. Skim milk (0% fat) was used. Thus, upon freezing, the milk was a one-phase system.

Although reduced metabolic activity during frozen storage would reduce generation of reactive oxygen species (ROS), it would also reduce activity of enzymes that protect *C. jejuni* from ROS. Thus, if the balance is in favor of damage from ROS during frozen storage, this could help to explain the faster and greater nonthermal inactivation of *C. jejuni* in milk and in beef under some circumstances during frozen versus refrigerated storage. Also, if an enumeration method is used with selective ingredients, injured cells of *C. jejuni* might be missed and counted as dead resulting in an underestimation of survival, which could help explain the observed results. Regardless, more research is needed to better understand why nonthermal inactivation of *C. jejuni* is faster and greater in some cases during frozen than refrigerated storage.

In the modeled study (Christopher et al., 1982), nonthermal inactivation of *C. jejuni* was faster and greater at ambient than at refrigeration temperatures. It is believed that a general response to cold stress involving synthesis of cold shock proteins like superoxide dismutase at refrigeration temperatures may offer cross-protection from oxidative stress (Garenaux et al., 2008; Stintzi & Whitworth, 2003). Also, lower metabolic activity at refrigeration temperatures is believed to reduce activity and production of ROS that kill or injure *C. jejuni* (Garenaux et al., 2008; Hazeleger, Wouters, Rombouts, & Abee, 1998). Thus, cold shock response and lower metabolic activity are plausible explanations for the observed results in Christopher et al. (1982). However, other factors and mechanisms may help explain the observed findings. Therefore, additional research is needed to better understand why nonthermal inactivation of *C. jejuni* is faster and greater at ambient than at refrigeration temperatures. This knowledge may facilitate design of better processes to control this foodborne pathogen and mitigate the risk it poses to public health. However, it is not being suggested that milk and beef should be held at ambient temperatures to reduce risk of campylobacteriosis as this mitigation measure would be risky because of other pathogens (*Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella*) in milk and beef that could grow under these conditions to high and dangerous levels.

5 | CONCLUSIONS

An ANN model was developed using data from ComBase for nonthermal inactivation of *C. jejuni* in milk and beef. Although the model failed validation because of data gaps, results were encouraging for two reasons. First, model performance was acceptable (pAPZ ≥ 0.7). This indicated that ANN has potential for modeling complex sets of data for nonthermal inactivation of *C. jejuni* in food; especially when the same data could not be completely modeled using traditional regression methods. Second, the APZ method was found to be effective at identifying data gaps and prediction problems in the model. Thus, together, ANN and APZ showed promise as novel methods for modeling *C. jejuni* behavior in food. However, in the future, it may be important to consider additional independent variables like inoculum size, native microflora, and previous history (physiological state) when developing predictive models for *C. jejuni* in food. Also, when developing models with data from ComBase, it should not be assumed that model predictions will be reliable. Rather, model performance and data completeness should be carefully evaluated using an objective set of criteria like those in the APZ method.

AUTHOR CONTRIBUTIONS

Bethany L. Boleratz mined data from ComBase, wrote the draft of the methods used in the modeled study, and reviewed and edited the manuscript before submission. Thomas P. Oscar conceived the study, analyzed, and modeled the data, performed the model validation, wrote the balance of the initial draft of the manuscript, and reviewed and edited the manuscript before submission.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest to report.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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