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Response Surface Models for Effects of Temperature, pH, and Previous Growth pH on Growth Kinetics of *Salmonella* Typhimurium in Brain Heart Infusion Broth†

ABSTRACT

Response surface models were developed for effects of temperature (15 to 40°C), pH (5.2 to 7.4), and previous growth pH (5.7 to 8.6) on lag time (λ) and specific growth rate (μ) of *Salmonella* Typhimurium in brain heart infusion broth (BHIB). Seventy-five growth curves for model development and 30 growth curves for model validation were fit to a two-phase linear growth model to obtain direct estimates of λ and μ of *Salmonella* Typhimurium in BHIB. Response surface models for natural logarithm transformations of λ and μ as a function of temperature, pH, and previous growth pH were obtained by regression analysis. Previous growth pH did not alter ($P > 0.05$) or interact with temperature or pH to alter subsequent growth kinetics of *Salmonella* Typhimurium. However, λ and μ of *Salmonella* Typhimurium in BHIB were affected ($P < 0.05$) by linear and quadratic effects of temperature and pH. The models were validated against data not used in their development. Mean absolute relative error of predictions (model accuracy) was 7.8% for λ and 6.6% for μ. Median relative error of predictions (model bias) was −1.8% for λ and −2.8% for μ. Results of the current study indicated that the models developed accurately predicted growth kinetics of *Salmonella* Typhimurium in BHIB within the matrix of factors modeled and that the range of previous growth pH (5.7 to 8.6) investigated did not alter the subsequent growth kinetics of *Salmonella* Typhimurium in BHIB.

Mathematical models that predict growth of foodborne pathogens in laboratory medium as a function of temperature and food formulation factors (pH, water activity, nitrite) provide reasonable estimates of bacterial growth in food *(4, 20)*. Predictive models for effects of temperature, pH, and water activity on growth kinetics of *Salmonella* spp. in laboratory medium are available for strains grown under optimal conditions *(2, 3, 10, 19)*. However, *Salmonella* spp. that contaminate food may originate from environments (i.e., fecal material) in which their growth occurred under nonoptimal conditions. Effects of previous growth conditions on subsequent growth kinetics of *Salmonella* spp. have not been investigated and modeled. Development of models that consider previous growth conditions as variables may improve our ability to predict growth of *Salmonella* spp. in food. Consequently, the present study was undertaken to develop response surface models for effects of temperature (15 to 40°C), pH (5.2 to 7.4), and previous growth pH (5.7 to 8.6) on lag time (λ) and specific growth rate (μ) of *Salmonella* Typhimurium in brain heart infusion broth (BHIB).

MATERIALS AND METHODS

Stock cultures. *Salmonella* Typhimurium (American Type Culture Collection 14028, Rockville, Md.) at a concentration of 9.0 to 9.4 $\log_{10}$ CFU/ml were maintained at −20°C in BHIB supplemented with 15% glycerol.

Starter cultures. Stock cultures of *Salmonella* Typhimurium were thawed at room temperature and then 5 μl of the resuspended stock culture was added to 5 ml of BHIB in starter cultures resulting in an initial concentration of 6.0 to 6.4 $\log_{10}$ CFU/ml. BHIB used in starter cultures was adjusted to pH of 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 with 1 N HCl or 1 N NaOH before autoclaving. After autoclaving, the pHs of BHIB used in starter cultures were 5.7, 6.3, 6.7, 7.4, 7.8, 8.3, and 8.6, respectively.

Starter cultures were incubated for 47 h at 30°C and 150 rpm in 25-ml Erlenmeyer flasks sealed with foam plugs. Regardless of pH, viable counts of starter cultures at the end of the 47-h incubation were between 10.0 and 10.4 $\log_{10}$ CFU/ml. The maximum population density of *Salmonella* Typhimurium ATCC 14028 in BHIB is between 10.0 and 10.4 $\log_{10}$ CFU/ml *(13)*. Thus, the viable count data at 47 h of incubation indicated that the cultures were in stationary phase rather than the death phase of their life cycle.

Previous research *(12)* indicates that the growth kinetics of stationary phase cells of *Salmonella* Typhimurium ATCC 14028 in BHIB are similar at 24 and 48 h of incubation at 37°C. Thus, although stage of stationary phase was not standardized for *Salmonella* Typhimurium grown at different starter culture pHs in this experiment, it seems unlikely that growth kinetic results were confounded by a stage of stationary phase by starter culture pH interaction.

Experimental designs. The experimental design for development of response surface models was a full 6 × 3 × 4 factorial arrangement of temperature (15, 20, 25, 30, 35, 40°C), pH (5.2,

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6.3, 7.4), and previous growth pH (5.7, 6.7, 7.8, 8.6). Three conditions were conducted twice for a total of 75 growth curves.

The experimental design for validation of response surface models was a full 5 × 2 × 3 factorial arrangement of temperature (17.5, 22.5, 27.5, 32.5, 37.5°C), pH (5.7, 6.7), and previous growth pH (6.3, 7.4, 8.3) for a total of 30 growth curves.

Growth cultures. Cultures of *Salmonella* Typhimurium for growth kinetic determinations were conducted in 250-ml Erlenmeyer flasks sealed with foam plugs. Each culture contained 50 ml of BHIB adjusted to pH of 5.0, 5.5, 6.0, 6.5, or 7.0 with 1 N HCl before autoclaving. After autoclaving, the pHs of BHIB used in growth cultures were 5.2, 5.7, 6.3, 6.7, and 7.4, respectively.

Salmonella Typhimurium from starter cultures, which had a final concentration of 10.0 to 10.4 $\log_{10}$ CFU/ml, were serially diluted by 10^{-3} in buffered peptone water, and then 50 μl of the diluted starter culture was added to 50 ml of BHIB in the growth cultures to achieve an initial *Salmonella* Typhimurium concentration of 4.0 to 4.4 $\log_{10}$ CFU/ml of growth culture. Growth cultures were incubated at 15 to 40°C and 150 rpm for 0 to 70 h.

Determination of viable counts. At selected times postinoculation, depending on the temperature and pH of incubation, 50 μl of undiluted and diluted (10^{-1} to 10^{-5}) samples (1 or 4 ml) from growth cultures was spiral plated (Whitley Automatic Sprial Plater, Don Whitley Scientific Limited, West Yorkshire, UK) onto brain heart infusion agar (Difco Laboratories, Detroit, Mich.). Sampling times for each growth condition (i.e., combination of temperature and pH in the growth culture) were based on estimated λ and μ and were selected to produce a growth curve that accurately defined the lag phase and exponential growth phase over four log cycles of growth.

Spiral plates were inverted and incubated for 18 to 24 h at 30°C and colonies that formed on the brain heart infusion agar were counted using an automated colony counter (Protos Colony Counter, Synoptics, Cambridge, UK). Using this protocol, countable plates were obtained when the undiluted or diluted samples from growth cultures had a *Salmonella* Typhimurium concentration of 3.0 to 4.9 $\log_{10}$ CFU/ml.

A high initial concentration of *Salmonella* Typhimurium (4.0 to 4.4 $\log_{10}$ CFU/ml) were used in the growth cultures to facilitate automated colony counting. Results of the current study were probably not affected by use of a high initial concentration of *Salmonella* Typhimurium as it has been shown by others *(10)* that the initial level of *Salmonella* does not alter their growth kinetics in pure cultures conducted in laboratory medium.

Curve fitting. Growth curves of viable counts (Y, $\log_{10}$ CFU/ml) versus sample time (X, h) were iteratively fit using GraphPad PRIZM (GraphPad Software, San Diego, Calif.) to a two-phase linear growth model *(8, 12)*,

$$Y = \text{Baseline} + \text{Increase}$$

$$\begin{aligned} \text{Increase} &= 0 && \text{if } X \le (\lambda) \\ &= (\mu) \times \Delta X && \text{if } X > (\lambda) \end{aligned}$$

$$\Delta X = X - (\lambda)$$

where the viable count Y was equal to Baseline (initial viable count) plus Increase (increase of viable count). In turn, Increase was equal to zero if the sample time X was less than or equal to lag time (λ; h); otherwise Increase was equal to the specific growth rate (μ; $\log_{10}$ CFU/h) times ΔX (the sample time minus the λ).

Response surface modeling. A data set containing model variables (i.e., previous growth pH, temperature, and pH) and natural logarithm transformations (ln) of λ and μ from 75 growth curve fits was created. The data set was subjected to regression analysis *(18)* using the following response surface model *(10)*,

$$\ln \lambda \quad \text{or} \quad \ln \mu = b_0 + b_1A + b_2B + b_3C + b_4AB + b_5AC + b_6BC + b_7A^2 + b_8B^2 + b_9C^2 + \epsilon$$

where A was the initial pH of the starter cultures or previous growth pH, B was the incubation temperature of the growth cultures or temperature, C was the initial pH of the growth cultures or pH, b_0 to b_9 were regression coefficients, and ε was random error. Models were developed using pH values of BHIB after autoclaving.

Model validation. Models were validated against data not used in their development. Relative error (RE) of each prediction case was calculated using the following equation *(9)*,

$$\text{RE} = \frac{(X_p - X_o)}{X_o}$$

where X_p was the predicted λ or μ and X_o was the observed λ or μ. Median relative error (MRE) of model predictions was used as the measure of model prediction bias. Mean absolute relative error (MARE) of each model, the measure of model prediction accuracy, was calculated using the following equation *(9)*,

$$\text{MARE} = \frac{1}{n}\sum_{i=1}^{n} |\text{RE}|_i$$

where n was the number of prediction cases.

RESULTS AND DISCUSSION

Modeling experiments in the U.S. Department of Agriculture (USDA) have traditionally used the Gompertz equation to fit microbial growth curves *(1, 5, 6, 15)*. In the current study, a two-phase linear growth model was used to fit growth curves for *Salmonella* Typhimurium. This change was made because the two-phase linear growth model requires less kinetic data than the Gompertz equation to obtain a good curve fit for estimating λ and μ *(8)*. In fact, kinetic data in the current study showed a high degree of goodness-of-fit to the two-phase linear growth model (Fig. 1). The mean coefficient of determination (r^2), a measure of the proportion of total variation associated with the dependent variable (i.e., viable count) that is accounted for by the independent variables (i.e., λ, μ, and initial viable count), was 0.9916 ± 0.0011 (range: 0.9441 to 0.9984) for the 75 growth curves used in model development and 0.9928 ± 0.0018 (range: 0.9828 to 0.9975) for the 30 growth curves used in model validation.

The model development phase of this study involved 75 growth curves conducted under 72 combinations of temperature, pH, and previous growth pH in BHIB. Lag time and μ from these 75 growth curve fits were transformed to their natural logarithm (ln), to stabilize model variance *(10)*, and regressed against model variables (i.e., temperature, pH, and previous growth pH) to obtain response surface models. Both the ln λ and ln μ models had high r^2 (Table 1), indicating a high degree of goodness-of-fit between the models and the data. Analysis of variance indicated that none of the model variables interacted to affect λ or μ of

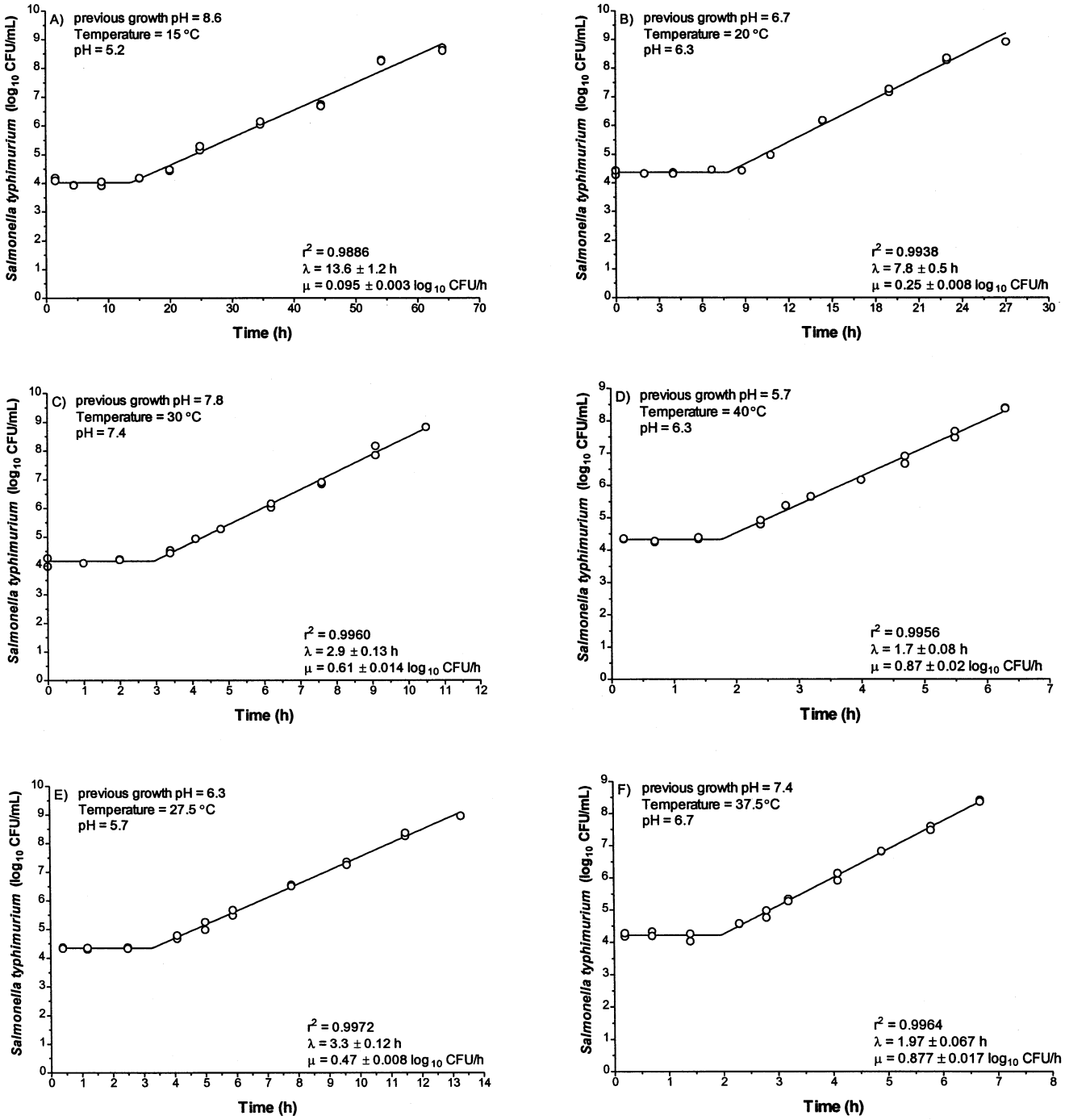

FIGURE 1. *Typical growth curve fits for* Salmonella *Typhimurium in brain heart infusion broth. Values for lag time (λ; h) and specific growth rate (μ; log_{10} colony forming units [CFU] per h) are mean ± standard error of the mean.*

Salmonella Typhimurium in BHIB. However, λ and μ of *Salmonella* Typhimurium in BHIB were affected by linear and quadratic effects of temperature and pH. In contrast, λ and μ of *Salmonella* Typhimurium in BHIB were not affected by linear or quadratic effects of previous growth pH (Table 1).

To the best of my knowledge, this study is the first to report on the effect of previous growth pH on the subsequent growth kinetics of a foodborne pathogen. The range of previous growth pH (5.7 to 8.6) investigated and modeled in this study was selected to mimic the range of pH found in fecal material, whereas the range of temperature (15 to 40°C) and pH (5.2 to 7.4) investigated and modeled were selected to mimic the growth of *Salmonella* Typhimurium on meat during temperature abuse. Thus, the current experiment was designed to simulate the fecal contamination of meat with *Salmonella* followed by growth of the contaminating *Salmonella* during temperature abuse. Overall, the results of this study indicated that a range of previous growth pH (i.e., 5.7 to 8.6) common to fecal material did not significantly alter the subsequent growth kinetics of *Salmonella* Typhimurium in BHIB. However, it appeared that λ was slightly affected by previous growth pH (P = 0.0626 for the linear effect and P = 0.1078 for the qua-

TABLE 1. *Response surface models for effects of previous growth pH (pgPH), temperature (T), and pH (PH) on lag time (λ) and specific growth rate (μ) of* Salmonella *Typhimurium in brain heart infusion broth*

Parameter	ln λ, h			ln μ, $\log_{10}$ CFU/h		
	Estimate	*F* value[a]	*P* value[b]	Estimate	*F* value[a]	*P* value[b]
Intercept	7.2961			−6.5403		
pgPH	0.5599	3.59	0.0626	−0.2246	1.23	0.2721
T	−0.2043	69.67	0.0001	0.2371	199.40	0.0001
PH	−1.3230	10.59	0.0018	0.5943	4.54	0.0369
pgPH × T	0.0009	0.27	0.6077	−0.0004	0.09	0.7699
pgPH × PH	−0.0168	0.93	0.3384	−0.0046	0.15	0.6998
T × PH	−0.0012	0.32	0.5724	0.0010	0.50	0.4826
pgPH × pgPH	−0.0309	2.66	0.1078	0.0182	1.96	0.1658
T × T	0.0023	75.76	0.0001	−0.0030	256.22	0.0001
PT × PH	0.1116	13.76	0.0004	−0.0358	3.01	0.0874
r^2	0.9626			0.9834		

[a] The *F* value is the test statistic for the analysis of variance of the regression model and is equal to the mean square residuals (MSR) for the corresponding model parameter divided by the mean square error (MSE) for the model. The larger the *F* value within a regression model, the greater the impact of the model parameter on the dependent variable (i.e., ln λ or ln μ).

[b] The *P* value corresponds to the probability that the corresponding model parameter is not equal to zero as determined by the *F* test.

dratic effect). Thus, it is possible that expansion of the current data set to include growth kinetics over a broader range of previous growth pH (i.e., 4.5 to 9.5) may produce a model in which the effect of previous growth pH on λ of *Salmonella* Typhimurium is significant.

Validating the ability of response surface models to interpolate is a critical step in their development, but one that is rarely done in modeling experiments. Validation of response surface model predictions should be performed against new data obtained at intermediate levels of model variables, using the same strain and protocol as used during model development, to avoid confounding the validation results. Furthermore, an analysis method should be used that quantifies the accuracy and bias of the model predictions *(9, 17)* so that the reliability of the model predictions can be objectively assessed and compared between studies.

In the current study, the ability of our models to predict growth of *Salmonella* Typhimurium was validated using the aforementioned criteria. Lag time and μ from 30 growth curves conducted at intermediate levels of model variables were obtained and used to calculate MARE (model accuracy) and MRE of predictions (model bias) for each response surface model and data set combination (Table 2). As shown in Table 2, MAREs of the λ and μ models were low and similar for new data and data used in model development. In addition, MRE for λ and μ were very close to (Table 2) and randomly distributed around zero (Fig. 2), indicating that the model predictions were not biased.

Delignette-Muller et al. *(9)* pooled 468 prediction cases from seven response surface modeling papers and calculated MARE for λ and generation time (τ); MARE was 40.3% for λ and 36.2% for τ. The MARE reported by Delignette-Muller et al. *(9)* are higher than those reported in the current study (Table 2). When Delignette-Muller et al. *(9)* separated prediction cases for time to a 1,000-fold increase into data used in model development and new data, they found that MARE was lower (20.7 versus 46.2%) for data used in model development. In contrast, in the current study MARE for λ and μ were similar for data used in model development and new data (Table 2).

To compare further the current validation results to published data, MARE was calculated for data reported in nine modeling papers that used laboratory medium (Table 3). A total of 16 data set and model combinations representing 823 prediction cases was examined. Fifteen data sets were for data used in model development and one data set was for new data. In all cases, MARE of the current models was lower than MARE of published models. Thus, the low MARE of the current models indicates that they accurately predict λ and μ of *Salmonella* Typhimurium in BHIB within the range of factors modeled.

TABLE 2. *Validation results for response surface models that predict lag time (λ) and specific growth rate (μ) of* Salmonella *Typhimurium in brain heart infusion broth as a function of temperature, pH, and previous growth pH*

Model	Data	Cases	Relative error, %			
			Mean absolute ± SEM	Median	Minimum	Maximum
λ	Model development	75	10.3 ± 1.3	−0.4	−23.8	80.9
	Model validation	30	7.8 ± 1.1	−1.8	−20.9	19.8
μ	Model development	75	5.9 ± 1.0	−0.1	−20.4	70.5
	Model validation	30	6.6 ± 1.0	−2.8	−15.6	18.4

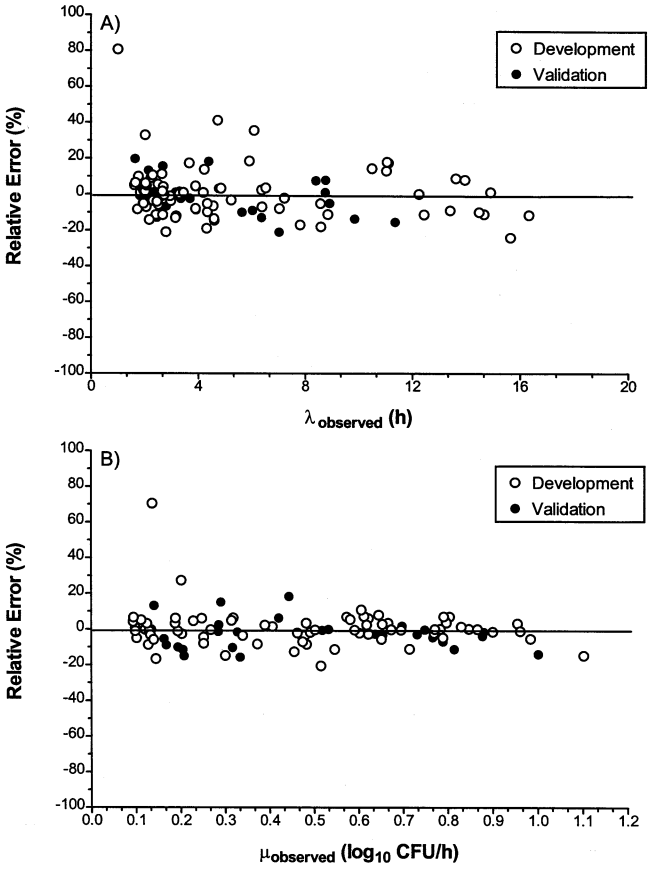

FIGURE 2. *Relative error of (A) lag time (λ) and (B) specific growth rate (μ) predictions from response surface models for* Salmonella *Typhimurium growth in brain heart infusion broth for data used in model development (○) and data used in model validation (●).*

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TABLE 3. *Mean absolute relative error (MARE) of published response surface models for microbial growth in laboratory medium*

Genus	Model[a]	Data[b]	Cases[c]	MARE ± SEM	Reference	Table(s)
Aeromonas	λ	New	27	62.3 ± 10.0	*(14)*	1, 4
Brochotrix	μ	Model	82	19.9 ± 1.9	*(11)*	3
Salmonella	τ	Model	20	18.5 ± 3.4	*(10)*	4
Escherichia	τ	Model	58	25.7 ± 3.3	*(5)*	3
Staphylococcus	τ	Model	74	30.4 ± 5.0	*(7)*	2
Aeromonas	τ	Model	61	72.0 ± 35.2	*(16)*	3, 4
Shigella	τ	Model	31	20.8 ± 2.9	*(21)*	4
Aeromonas	τ	Model	54	52.3 ± 8.0	*(15)*	3, 4
Yersinia	τ	Model	30	27.4 ± 6.2	*(1)*	2
Brochotrix	λ	Model	82	33.1 ± 3.6	*(11)*	3
Escherichia	λ	Model	58	41.5 ± 6.6	*(5)*	3
Staphylococcus	λ	Model	74	74.8 ± 14.2	*(7)*	2
Aeromonas	λ	Model	61	37.4 ± 4.2	*(16)*	3, 4
Aeromonas	λ	Model	54	54.0 ± 12.9	*(15)*	3, 4
Yersinia	λ	Model	30	56.9 ± 29.0	*(1)*	2
Aeromonas	λ	Model	27	28.1 ± 2.6	*(14)*	1, 4

[a] Growth characteristics: generation time (τ), lag time (λ), and specific growth rate (μ).
[b] Data used in model development (Model) or data not used in model development (New).
[c] Number of prediction cases obtained from the indicated table in the reference.