J. Microbiol. Biotechnol. (2005), **15**(6), 1323–1329

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Predictive Modeling for the Growth of *Listeria monocytogenes* as a Function of Temperature, NaCl, and pH

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Received: March 31, 2005
Accepted: May 23, 2005

Abstract A mathematical model was developed for predicting the growth kinetics of *Listeria monocytogenes* in tryptic soy broth (TSB) as a function of combined effects of temperature, pH, and NaCl. The TSB containing four different concentrations of NaCl (2, 4, 5, and 10%) was initially adjusted to six different pH levels (pH 5, 6, 7, 8, 9, and 10) and incubated at 4, 10, 25, or 37°C. In all experimental variables, the primary growth curves were well fitted (r^2=0.982 to 0.998) to a Gompertz equation to obtain the lag time (LT) and specific growth rate (SGR). Surface response models were identified as appropriate secondary models for LT and SGR on the basis of coefficient determination (r^2=0.907 for LT, 0.964 for SGR), mean square error (MSE=3.389 for LT, 0.018 for SGR), bias factor (B_f=0.706 for LT, 0.836 for SGR), and accuracy factor (A_f=1.567 for LT, 1.213 for SGR). Therefore, the developed secondary model proved reliable predictions of the combined effect of temperature, NaCl, and pH on both LT and SGR for *L. monocytogenes* in TSB.

Key words: *Listeria monocytogenes*, surface response models temperature, NaCl, pH

Listeria monocytogenes is a facultative anaerobic foodborne Gram-positive coccobacillus pathogen that is widely distributed in nature [15, 20] and common in foods of both plant and animal origins [8]. This pathogen is of particular importance in the food industry and the public because of its ability to persist and grow under a wide range of unfavorable conditions such as low pH [5, 14, 15, 23–25, 31], high pH [14, 27], low water activity [26, 29], high osmolarity [14], and at refrigeration temperatures [14, 37].

Mathematical quantitative models that predict the growth of *L. monocytogenes* as a function of main environmental controlling factors will improve the shelf life and safety of foods [2, 6–8, 10, 16, 21, 22, 27]. However, earlier studies of predictive models describing the effects of environmental factors such as temperature, pH, water activity, CO_2, and preservative agents on the growth, survival, or inactivation of *L. monocytogenes* are still limited.

Therefore, the purpose of this study was to investigate the combined effects of temperature, NaCl, and pH on the growth kinetics of *L. monocytogenes* in a broth system with the goal of developing a model that could be used to predict the growth of the organisms in any combination of the variables.

Materials and Methods

Bacterial Culture

A cocktail of *Listeria monocytogenes* (ATCC 19112, 19113, and 19115) was used to determine growth characteristics

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and develop predictive models for growth. Each strain was maintained at −70°C in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI, U.S.A.) containing 50% glycerol stock. Each culture of *L. monocytogenes* was thawed at room temperature, and 10 μl of a mixed resuspended stock culture was then added in 9 ml of sterile TSB containing 0.6% yeast extract (Difco Laboratories). The culture was incubated at 37°C for 24 h. One milliliter of the culture was added into 100 ml of TSB and the culture was incubated at 37°C for 24 h. One milliliter of the starter culture was serially diluted in 9 ml of sterile 0.1% peptone water for inoculation into TSB.

Experimental Design

The effect of 120 combinations of factors was tested. These included:

- Temperature: 4, 10, 25, 37°C
- NaCl: 0, 2, 4, 5, 10% [w/v]
- pH: 5, 6, 7, 8, 9, 10

Preparation and Inoculation of Culture Media

TSB containing different concentrations of sodium chloride (NaCl) [0, 2, 4, 5 and 10% (w/v)] was autoclaved at 121°C for 15 min and allowed to cool. The pH of the media was then adjusted to an initial of either 5, 6, 7, 8, 9, or 10 using 1 N NaOH or 1 N HCl solution. Each pH adjusted medium was inoculated with 30 μl of the *L. monocytogenes* cocktail to obtain a starting population of approximately 10^2 CFU/ml.

Growth Temperature and Measurement

To enumerate growth during the storage period at either 4, 10, 25, or 37°C, 100 μl samples were removed from inoculated broth at appropriate intervals, serially diluted in 0.9 ml of 0.1% peptone water, spiral plated in duplicate on *Listeria* modified oxford agar (Difco Laboratories) plates, and incubated at 37°C for 24 h. Colonies were counted by standard plates count (SPC) and expressed as colony-forming unit (CFU)/g. The data were converted to values of $\log_{10}$CFU/ml.

Primary Modeling

Growth curves of viable cell count versus sampling time were iteratively generated from the experimental data using the Gompertz equation and fit to a nonlinear regression model (Prism, version 4.0, GraphPad Software, San Diego, CA, U.S.A.) to determine lag time (LT, in hours) and specific growth rate (SGR, in $\log_{10}$CFU/mL per hour) at each incubation temperature. The Gompertz parameter values were described by Gibson *et al.* [18].

$$Y=N0+C*\exp(\exp((2.718*SGR/C)*(LT-X)+1))$$

Y= Log cell number
X= Incubation time
N0= Log initial number of cells
C= Difference between initial and final cell numbers
LT= Lag time before growth, same units as X
SGR=Maximum specific growth rate

Secondary Modeling

A surface response model in terms of temperature, sodium chloride concentration, and pH was calculated on the LT and SGR. These two Gompertz parameters for *L. monocytogenes* growth data were determined by the least squares analysis of PROC GLM of the SAS version 8.1 [33]. The surface response model was described by Gibson *et al.* [17].

$$\ln LT \text{ or } \ln SGR=b_0+b_1A+b_2B+b_3C+b_4A^2+b_5B^2+b_6C^2+b_7AB+b_8AC+b_9BC+\varepsilon$$

A= Incubation temperature
B= Initial pH
C= Sodium chloride concentration
b_0–b_9= Regression coefficients
ε= Random error

Evaluation of Model Performance

The coefficient of determination (r^2), which is provided by GraphPad (GraphPad Software, San Diego, CA, U.S.A.), is often used as an overall measure of the prediction attained. It measures the fraction of the variation about the mean that is explained by a model.

The mean square error (MSE), the residual sum of squares divided by the number of degrees of freedom, is a measure of variability remaining that is not accounted for by deliberate changes in factors such as temperature, pH, and a_w.

$$MSE=(\sum\log(LT_{predicted}/LT_{observed})^2)/n$$
$$=(\sum\log(SGR_{predicted}/SGR_{observed})^2)/n$$

$LT_{predicted}$= the predicted lag time
$LT_{observed}$= the observed lag time
$SGR_{predicted}$= the predicted specific growth rate
$SGR_{observed}$= the observed specific growth rate
n= the number of observations

The bias factors (B_f) answers the question of whether, on average, the observed values lie above or below the line equivalence and, if so, by how much. It gives the structural deviations of a model.

$$B_f=10(\sum\log(LT_{predicted}/LT_{observed})/n)$$
$$=10(\sum\log(SGR_{predicted}/SGR_{observed})/n)$$

The accuracy factor (A_f) averages the distance between each point and the line of equivalence as a measure of how close, on average, predictions are to observe.

$$A_f=10(\sum\log|(LT_{predicted}/LT_{observed})|/n)$$
$$=10(\sum\log|(SGR_{predicted}/SGR_{observed})|/n)$$

RESULTS AND DISCUSSION

Primary Modeling

At the storage temperature of 4°C, no growth of *L. monocytogenes* was observed in the presence of all experimental variables for 3 weeks (data not shown). In addition, no growth of *L. monocytogenes* was observed in the combination of all experimental variables of either 10% NaCl or pH 5 at 4, 10, 25, and 37°C and in the presence of 5% NaCl adjusted pH 9 at 10°C for 3 weeks (data not shown). Therefore, the model in the current study involved 47 growth curves conducted under 120 combinations of temperature, NaCl, and pH in TSB media.

Traditionally, the Gompertz equation is used to fit bacterial growth curves for estimating LT and SGR in the U. S. Department of Agriculture (USDA) [3, 4, 6, 29]. Therefore, the current study used the Gompertz equation to fit growth curves for *L. monocytogenes*. Table 1 shows best-fit values for LT and SGR and goodness of fit in the primary model. In general, the data of LT and SGR for the TSB medium

Table 1. Best-fit LT and SGR and goodness of fit of the primary modeling.

Temp (°C)	NaCl (%)	pH	LT (h)	SGR (log CFU/h)	r^2 [a]	S_{yx} [b]	Temp (°C)	NaCl (%)	pH	LT (h)	SGR (log CFU/h)	r^2 [a]	S_{yx} [b]
10	0	5	NG[c]	NG	NA[d]	NA	25	4	5	NG	NG	NA	NA
10	0	6	21.790	0.050	0.996	0.157	25	4	7	7.520	0.294	0.982	0.337
10	0	7	17.683	0.050	0.992	0.244	25	4	8	13.590	0.635	0.991	0.301
10	0	8	20.290	0.054	0.993	0.214	25	4	9	16.295	0.220	0.992	0.292
10	0	9	28.220	0.056	0.991	0.254	25	4	10	NG	NG	NA	NA
10	0	10	NG	NG	NA	NA	25	5	5	NG	NG	NA	NA
10	2	5	NG	NG	NA	NA	25	5	6	10.050	0.221	0.995	0.209
10	2	6	26.507	0.048	0.996	0.143	25	5	7	8.739	0.216	0.995	0.191
10	2	7	25.509	0.056	0.995	0.180	25	5	8	13.5025	0.223	0.992	0.242
10	2	8	36.705	0.054	0.997	0.138	25	5	9	48.525	0.166	0.990	0.184
10	2	9	50.760	0.065	0.986	0.294	25	5	10	NG	NG	NA	NA
10	2	10	NG	NG	NA	NA	37	0	5	NG	NG	NA	NA
10	4	5	NG	NG	NA	NA	37	0	6	2.353	0.922	0.988	0.302
10	4	6	53.505	0.033	0.986	0.256	37	0	7	1.150	0.975	0.988	0.316
10	4	7	50.745	0.036	0.991	0.236	37	0	8	2.350	0.953	0.984	0.371
10	4	8	67.950	0.034	0.988	0.226	37	0	9	2.844	0.838	0.988	0.305
10	4	9	151.200	0.066	0.984	0.110	37	0	10	NG	NG	NA	NA
10	4	10	NG	NG	NA	NA	37	2	5	NG	NG	NA	NA
10	5	5	NG	NG	NA	NA	37	2	6	2.611	0.798	0.992	0.244
10	5	6	101.160	0.031	0.982	0.169	37	2	7	2.531	0.818	0.991	0.272
10	5	7	112.690	0.031	0.988	0.166	37	2	8	2.552	0.856	0.991	0.256
10	5	8	249.270	0.024	0.992	0.161	37	2	9	4.676	0.881	0.989	0.295
10	5	9	NG	NG	NA	NA	37	2	10	NG	NG	NA	NA
10	5	10	NG	NG	NA	NA	37	4	5	NG	NG	NA	NA
25	0	5	NG	NG	NA	NA	37	4	6	3.140	0.744	0.989	0.313
25	0	6	4.223	0.397	0.995	0.211	37	4	7	3.386	0.762	0.989	0.348
25	0	7	4.045	0.476	0.996	0.186	37	4	8	3.632	0.712	0.992	0.277
25	0	8	3.915	0.414	0.993	0.264	37	4	9	12.480	0.470	0.992	0.353
25	0	9	3.603	0.392	0.994	0.221	37	4	10	NG	NG	NA	NA
25	0	10	NG	NG	NA	NA	37	5	5	NG	NG	NA	NA
25	2	5	NG	NG	NA	NA	37	5	6	5.400	0.632	0.991	0.313
25	2	6	4.620	0.371	0.996	0.198	37	5	7	7.628	0.209	0.987	0.393
25	2	7	4.625	0.367	0.996	0.186	37	5	8	5.144	0.501	0.994	0.269
25	2	8	3.805	0.395	0.997	0.182	37	5	9	13.110	0.359	0.993	0.304
25	2	9	10.583	0.266	0.990	0.329	37	5	10	NG	NG	NA	NA
25	2	10	NG	NG	NA	NA							

[a] r^2, coefficient of determination.
[b] S_{yx}, standard error of the residuals.
[c] NG, no growth.
[d] NA, no application.

fitted a Gompertz equation model well, with a high degree of goodness of fit (r^2=0.982 to 0.998) and low S_{yx} values at all treatment factors (Table 1).

Secondary Modeling

While the growth kinetics of *L. monocytogenes* in response to temperature alone [12], temperature in combination with pH and NaCl or a_w [10, 38], and pH, NaCl, nitrite, and gaseous atmosphere [6, 7] have been modeled extensively, no predictive models have been constructed describing the effect of temperature in combination with NaCl and acidic to basic pH. Therefore, the model development phase of this involved 47 growth curves conducted under 120 combinations of temperature, NaCl, and acidic to basic pH in TSB medium. LT and SGR from these 47 growth curve fits were transformed to their natural logarithm to stabilize model variance [17] and regressed against model variables to obtain a surface response model.

Secondary models were developed for LT (Fig. 1) and SGR (Fig. 2) as a function of temperature, NaCl, and acidic to basic pH. When the overall main effects of NaCl concentrations or pH levels in TSB media stored at 10, 25, or 37°C were compared, the LT of the *L. monocytogenes* was increased by a basic [8, 9] or acidic pH level [6] or increase (0–5%) of NaCl concentration. Eight to nine folds or more increase of the LT of the *L. monocytogenes* was predicted in the presence of 5% NaCl than in the presence of 4, 2 and 0% NaCl at all pH levels stored at 10, 25, or 37°C. However, the LT of the *L. monocytogenes* was not further increased by pH 6 than by pH 7 containing 5% NaCl in TSB media under 10, 25, or 37°C. Although the SGR of the *L. monocytogenes* was somewhat similar among the different pH levels, it was steadily decreased by increase (0–5%) of NaCl concentration in TSB media stored at 10°C. The SGR of the *L. monocytogenes* was also steadily decreased by basic [8, 9] or acidic pH level [6] or increase (0–5%) of NaCl concentration in TSB media stored at 25°C or 37°C. Although the main effects of temperature for LT and SGR were not compared, LT and SGR appeared to be generally more and less, respectively, in lower temperature than in higher temperature studies.

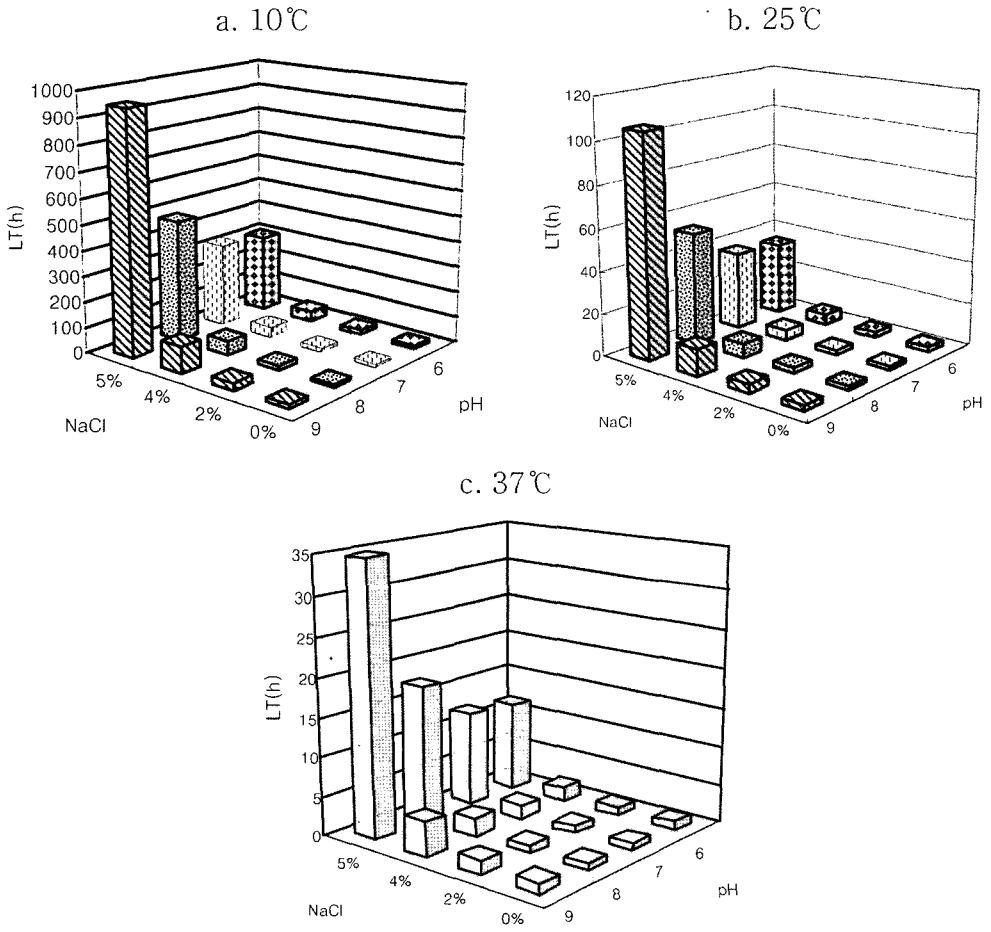

Fig. 1. Surface response models for the effects of temperature in combination with acidic to basic pH and NaCl on lag time (LT) of monocytogenes in TSB.

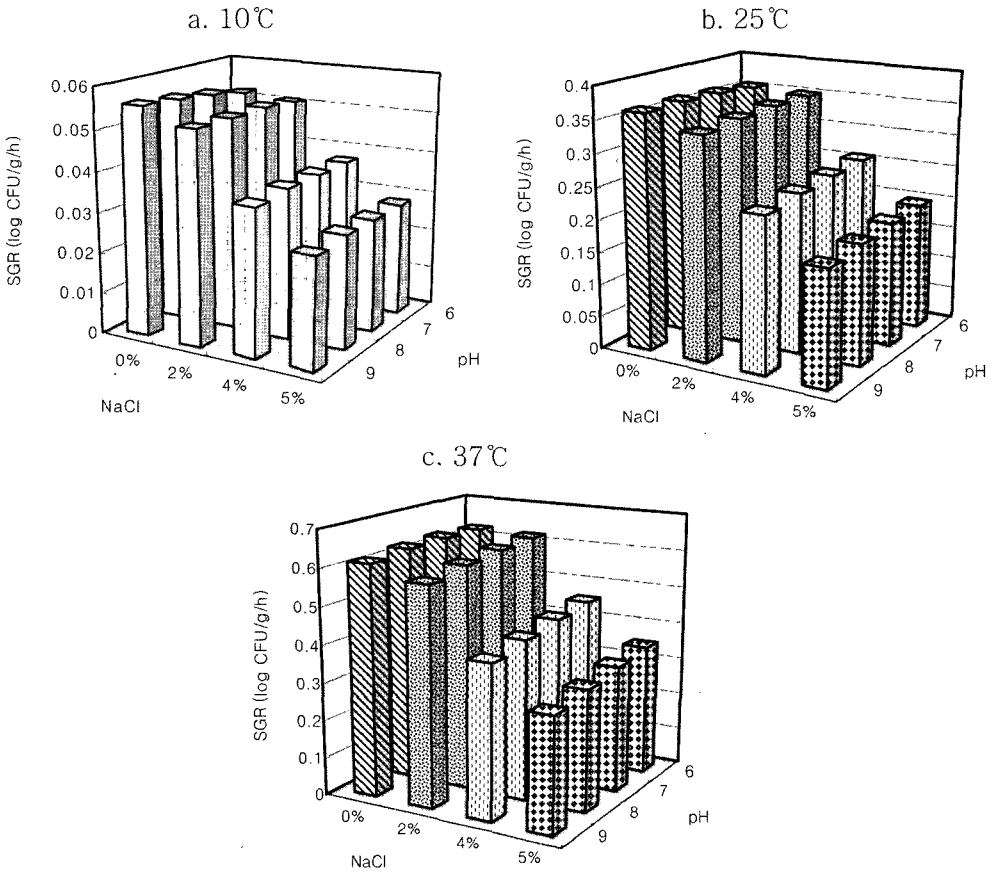

Fig. 2. Surface response models for the effects of temperature in combination with acidic to basic pH and NaCl on specific growth rate (SGR) of *L. monocytogenes* in TSB.

Evaluation of the Model Performance

Table 2 presents four different statistical indices of the secondary modeling step for LT and SDR in the TSB medium. The higher the value of r^2 ($0<r^2<1$), the better is the prediction by the model [13, 19, 34]. The lower the value of MSE, the better is the adequacy of the model to describe the data [1, 36]. $B_f<1$ indicates a "fail safe" model [30]. $B_f>1$ indicates a "fail dangerous" model [29]. Ross [33] also noted that for models describing pathogen growth rate, B_f in the range 0.9–1.05 could be considered good, in the range 0.7–0.9 or 1.06-1.15 considered acceptable, and <0.7 or >1.5 considered unacceptable. The larger the value of A_f, the less accurate is the average estimate. According to Ross [32], A_f of 2 indicates that the prediction is, on average, a factor of 2 different from the observed value; i.e., either half as large or twice as large. If there is no structural deviation (bias=1, both positive and negative deviations, on average the model is exact), inaccuracies can still be shown by the accuracy factor. Ross *et al.* [34] additionally noted that an acceptable model that predicts the effect of temperature, pH, and water activity on *Listeria* growth rate could be expected to have A_f in the range of 1.3–1.5.

Table 2. Statistical indices of the secondary modeling step for lag time (LT) and specific growth rate (SGR).

Model	r^2	MSE	B_f	A_f
LT	0.907	3.389	0.706	1.567
SGR	0.964	0.018	0.836	1.213

Based on the above statement about the four different statistical indices, our results indicated that developed surface response models for LT and SDR in the TSB medium were generally considered to be quite good or acceptable. On the other hand, the fits achieved for the secondary model for LT were not as good as the fits achieved for the secondary model for SDR, because two indices, A_f (1.567) and MSE (3.389), for LT were slightly high. Lag time duration has often been considered erratic, and evaluations of predictive models have shown that lag times are less reliably predicted than generation time [34] or growth rate [11, 12, 39].

Extrapolation of model predictions for the LT in the current study must be made with caution. However, the surface response model proved reliable predictions of the combined effect of environmental factors, temperature,

NaCl, and pH on both LT and SGR for *L. monocytogenes* in the TSB medium.

For risk management, further work is needed to confirm the predictive ability of the growth model for *L. monocytogenes* in food products. Furthermore, there is an urgent necessity in developing models of growth and death, and survival and transmission of *L. monocytogenes* occurred in diverse food matrices and food processing plants exposed to various environmental conditions. Therefore, each developed model will reduce the uncertainty over *L. monocytogenes* in food production, processing, and distribution, and thus will ensure food safety when microbial risk assessment (MRA) is executed.

Acknowledgment

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (03-PJ1-PG1-CH11-0003).

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