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Modelling bacterial growth of *Listeria monocytogenes* as a function of water activity, pH and temperature

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Temperature, pH and water activity are important factors controlling the microbiological safety of foods. To describe the growth rate of *Listeria monocytogenes* in relation to these factors, two equations have been developed. Both equations are based upon the Ratkowsky equation for temperature and growth rate. The first equation predicts growth rate at sub-optimal pH values, sub-optimal temperatures and sub-optimal water activities, the second model predicts growth throughout the entire pH range. The first model may be used to predict growth rates between pH 4.6–6.7, temperature range 5–35°C and a water activity range of 0.95–0.997. The second model is valid throughout the pH range of 4.6–7.4 and the same temperature and water activity range as the first model.

Key words: Growth model; pH; Water activity; Temperature; *Listeria monocytogenes*; Bělehrádek (square root)

Introduction

Microbial activity often influences the quality or safety of foods. To control microbial survival and outgrowth of microorganisms in foods, food preservation procedures are used. The most common preservation procedure is storing food at low temperatures. Other common ways to control microbial spoilage and growth of pathogens in foods are reduced pH in combination with low temperature and the adjustment of the water activity.

In recent years the interest in developing mathematical models that describe growth of microorganisms has increased. These models can predict changes in numbers of microorganisms in a product with time, dependent upon the physical and chemical condition of the product (Gould, 1989; Roberts, 1990).

Gompertz (1825) developed a model that describes human mortality as a function of age. For microbiological use this model was reparameterized by

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Zwietering et al. (1990) to include microbiologically significant parameters such as maximum specific growth rate (μ_m), lag time (λ) and asymptotic level of microorganisms (A) [$e = \exp(1)$].

$$Ln\left(\frac{N}{N_0}\right) = A \exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\right\} \quad (1)$$

where N is the number of microorganisms at time t and N_0 is the number of microorganisms at time $t = 0$.

A model that describes the relationship between maximum specific growth rate (μ_m) of a microorganism and temperature at sub-optimal conditions for growth is the square root model of Ratkowsky et al. (1982).

$$\sqrt{\mu_m} = b_1(T - T_{min}) \quad (2)$$

where b_1 is a regression coefficient; T is the actual temperature (°C) and T_{min} is the extrapolated minimum growth temperature (°C) for the microorganism.

This equation has been modified by McMeekin et al. (1987) to incorporate water activity (a_w).

$$\sqrt{\mu_m} = b_2\sqrt{(a_w - a_{w,min})}\,(T - T_{min}) \quad (3)$$

where b_2 is a new regression coefficient; a_w is the current water activity and $a_{w,min}$ is the extrapolated minimum water activity for growth of the microorganism.

Recently the square root model of Ratkowsky was modified by Adams et al. (1991) to describe the growth rate of *Yersinia enterocolitica* in response to pH and temperature (Eqn. 4):

$$\sqrt{\mu_m} = b_3\sqrt{(pH - pH_{min})}\,(T - T_{min}) \quad (4)$$

pH_{min} indicates the extrapolated minimum pH at which microorganisms will grow, pH indicates the actual acidity and b_3 is a regression coefficient. Eqn. 4 was validated for different acidulants; the value of the regression coefficient was different for each different acidulant, whereas the value for pH_{min} and the value for T_{min} were not affected by the type of acidulant.

Listeria monocytogenes is a pathogenic microorganism that is unique in its tolerance of factors that would normally prevent, or severely limit, growth. Walker et al. (1990) found growth of *L. monocytogenes* in chicken broth at a temperature as low as −0.4°C. The lowest water activity for growth was reported by Petran and Zottola (1989) to be 0.92. The minimum pH for growth is found to be 4.39 (George et al., 1988), the maximum pH for growth is reported to be 9.2 and the optimum pH for growth of *L. monocytogenes* is 7.0 (Petran and Zottola, 1989).

The objective of this work is to describe growth of *L. monocytogenes* as a function of pH, water activity and temperature.

Materials and Methods

Organism

Listeria monocytogenes (NCTC 9863).

Media

NaCl was added to Tryptone Soya Broth (TSB, Oxoid CM 129; 30 g/l) during preparation to give final concentrations of 5–80 g/l. Media at each NaCl concentration were made in 1500-ml volumes, adjusted to the appropriate pH (4.6, 4.9, 5.3, 6.0, 6.3, 6.7, and 7.4) using 5 M HCl and dispensed in 300-ml volumes.

Tryptone Soya Agar (TSA) for plate counts was prepared using TSB (30 g/l) with 1% agar (Lab M, no.2). The pH was adjusted to 7.2. Maintenance medium (1 g/l bacteriological peptone (Lab M), 8.5 g/l NaCl, pH adjusted to 6.4) was used for all serial dilutions for plate counts and for dilution of the inoculum. The stock culture was maintained on Nutrient Agar (NA, Difco) slopes at 4°C. All media were autoclaved at 121°C for 15 min.

Inoculum

L. monocytogenes was grown in Heart Infusion Broth (HIB) (Difco) for 24 h at 30°C (circa 10^8 cells/ml), diluted, and 1 ml used to inoculate each 300-ml volume of TSB, using a 1-ml repeating syringe (Eppendorf Multipette 4780, Baird and Tatlock Ltd, London) to give a final concentration of approximately 10^3 cells/ml.

Experimental procedure

TSB in 300-ml volumes was maintained overnight at the intended storage temperature prior to inoculation. Immediately after inoculation with *L. monocytogenes* and thoroughly mixing, TSB was aseptically dispensed in 10-ml volumes, using a peristaltic pump (Accuramatic-5, Jencons), into sterile 1 oz. (28.4 ml) universal bottles with plastic screw caps. Viable numbers in each treatment combination were determined by direct plating of 20 μl on TSA immediately after dispensing and the time was noted. The remaining bottles were incubated at the appropriate temperature.

At appropriate time intervals during storage, decimal dilutions were made from separate bottles of TSB and 20 μl of each plated onto TSA. The plates were incubated for 24 h at 30°C, colonies counted and viable numbers calculated according to the method of Farmiloe et al. (1954) and expressed as ln(cfu) per ml broth.

Determination of pH and NaCl

Measurements of pH were made using a Whatman PHA 230 pH meter. NaCl concentrations of TSB were determined using the Official Method of the Society for Analytical Chemistry (Hansen, 1973). The water activity was calculated from the NaCl concentration (Pitzer and Mayorga, 1973).

Experimental design

The limits of the experimental plan were chosen after a preliminary screening experiment to determine the growth limits of the strain used. Eighty-two combinations of the following conditions were used:

Storage temperature (°C): 5, 8, 10, 15, 20, 25, 30, 35;
pH: 4.6, 4.9, 5.3, 6.0, 6.3, 6.7, 7.4;
NaCl concentration (g/l) (a_w): 5 (0.9971), 20 (0.9885), 40 (0.9765), 60 (0.9637), 70 (0.9570), 80 (0.95).

The combinations were chosen (with some replicates) between the experimental limits (Table I).

TABLE I

Experimental plan

a_w	pH	Temperature (°C)							
		5	8	10	15	20	25	30	35
0.9971	4.6		*	*	*		*		*
0.9765	4.6			*		*			*
0.9765	4.9		*						
0.9570	4.9				*		*		*
0.9971	5.3	*		*2					
0.9885	5.3			*3			*2		
0.9765	5.3			*		*			
0.9637	5.3			*3			*2		
0.95	5.3			*					
0.9971	6.0	*3				*			*
0.9885	6.0	*2				*2			
0.9765	6.0	*3		*		*2	*	*	
0.9637	6.0	*2				*2			
0.95	6.0			*		*			*
0.9570	6.3	*							
0.9971	6.7	*		*					
0.9885	6.7			*2			*2		
0.9765	6.7					*			
0.9637	6.7			*2			*2		
0.95	6.7			*					
0.9971	7.4	*2		*	*		*		*
0.9885	7.4	*							
0.9765	7.4	*				*		*	
0.9637	7.4	*							
0.95	7.4	*	*	*	*		*		*

* One measurement.
2 and 3 indicate number of replicate measurements.

Statistical modelling

Modelling was carried out in two stages. At each combination of NaCl (water activity), temperature and pH the bacterial counts were fitted to the Gompertz equation (Eqn. 1) by a non-linear regression package using a Marquardt algorithm (Zwietering et al., 1990). The package estimates the maximum growth rate (μ_m), the final level of microorganisms ($A = \ln(N_\infty/N_0)$), the lag time (λ) and their 95% confidence intervals. For the second stage a statistical package for performing multi-variate non-linear regression was used to fit the growth rate to the controlling factors temperature, water activity and pH.

Results

All growth curves gave good fits with the Gompertz model ($r \geq 0.97$).

In the second stage of the modelling, two different relations between the maximum growth rate, pH, temperature and water activity were used to fit the data. The first relationship is based upon the combination of Eqns. 3 and 4. This model will only be valid in the sub-optimal pH, sub-optimal temperature and sub-optimal water activity range.

$$\sqrt{\mu_m} = b_4\sqrt{(a_w - a_{w,min})}\sqrt{(pH - pH_{min})}(T - T_{min}) \tag{5}$$

where b_4 is a regression coefficient and the other parameters are as above.

Ratkowsky et al. (1983) described a non-linear regression model for fitting growth rate and temperature throughout the entire biokinetic temperature range. This relationship is adapted for pH instead of temperature in Eqn. 6, where pH_{min} is the extrapolated minimum pH for growth, pH_{max} the extrapolated maximum pH for growth and b_5 and c are regression coefficients. This equation can be used to describe growth throughout the entire pH range.

$$\sqrt{\mu_m} = b_5(pH - pH_{min})\{1 - \exp[c(pH - pH_{max})]\} \tag{6}$$

The second relationship between growth rate, pH, sub-optimal temperature and sub-optimal water activity to apply in this case is derived from the combination of Eqns. 3 and 6. Here b_6 and c_{pH} are regression coefficients.

$$\sqrt{\mu_m} = b_6\sqrt{(a_w - a_{w,min})} \times (pH - pH_{min})\{1 - \exp[c_{pH}(pH - pH_{max})]\} \times (T - T_{min}) \tag{7}$$

In Table II the results of the regressions are shown. The first two rows of the table show models that do not use the entire data set. The first uses only that part of the data set where the pH is less than or equal to 6.3, from now onwards to be called Eqn. 5a. The second row shows a model that uses a larger pH range, here the pH

TABLE II

Regressed values for $a_{w,min}$, pH_{min}, pH_{max} and T_{min} for growth of *L. monocytogenes*

Equation	$a_{w,min}$	pH_{min}	pH_{max}	T_{min}(°C)
5a [a]	0.912	4.15	- [c]	−2.05
5b [b]	0.913	4.03	- [c]	−1.75
7 [d]	0.916	3.84	9.82	−2.55
Refs. [e]	0.92 (1)	4.39 (2)	9.2 (1)	−0.4 (3)

[a] Not the entire data set; pH ≤ 6.3 (53 datum points).
[b] Not the entire data set; pH ≤ 6.7 (65 datum points).
[c] Not present in model.
[d] Entire data set (82 datum points).
[e] (1) = Petran and Zotolla (1989), (2) = George et al. (1988), (3) = Walker et al. (1990).

is less than or equal to 6.7, from now onwards to be called Eqn. 5b. Both pH ranges are sub-optimal (Petran and Zotolla, 1989). The last row in the table shows the values derived from literature.

The predictions from both models were compared to the original data sets by comparing predicted growth rates at different constant conditions with the data set values. For this a 'general model' is introduced that uses the mean values of the measured data where all the controlling factors are constant (Eqn. 8). It describes the data sets as "... at constant conditions with respect to one temperature, one pH value and one water activity value the growth rate is found to be ...". Therefore it cannot be used as a predictive model.

$$\overline{\mu_m}(i) = \sum_{j=1}^{n} \frac{\mu_m(i,j)}{n} \tag{8}$$

where $\mu_m(i,j)$ is the jth growth rate at constant conditions i and $\overline{\mu}_m(i)$ is the mean growth rate at these constant conditions; n is number of replicates at constant conditions.

The statistical comparison was carried out using an F-ratio test. Although this testing method is not valid for non-linear equations it can give a good indication of the suitability of the model and how well it describes the data set. All models are tested against the general model that applies in the region of controlling factors. As stated by various workers this method must be seen as an approximation, not as a rigorous statistical analysis (Schnute, 1981; Zwietering et al., 1991).

The measuring error for the 'general model' is estimated by determining the deviation of the measured values from the mean value at a set of constant conditions. The residual sum of squares (RSS_1) is calculated from the deviation. The degrees of freedom (df_1) of this model are calculated as the total number of datum points minus the number of different constant conditions.

TABLE III

Statistical evaluation of the models

Equation	RSS_1	df_1	RSS_2	df_2	f	F
5a [a]	0.0495	16	0.2647	49	2.11	2.17
5b [b]	0.0923	20	0.3933	61	1.59	1.99
7 [c]	0.0924	21	0.4204	76	1.36	1.91

[a] Not the entire data set; pH $\leq$ 6.3 (53 datum points).
[b] Not the entire data set; pH $\leq$ 6.7 (65 datum points).
[c] Entire data set (82 datum points).

The error of prediction of a model is estimated by determining the deviation between the predictions from the model and the measured values. From these values the residual sum of squares (RSS_2) can be derived. The degrees of freedom (df_2) are calculated as the number of datum points minus the number of parameters in the model.

The lack of fit of the models can be estimated by taking the difference between RSS_2 and RSS_1. When this value is much smaller than the measuring error (RSS_1), the model is adequate. This comparison between the lack of fit and the measuring error can be quantified statistically by the f testing value:

$$f = \frac{(RSS_2 - RSS_1)/(df_2 - df_1)}{RSS_1/df_1} \tag{9}$$

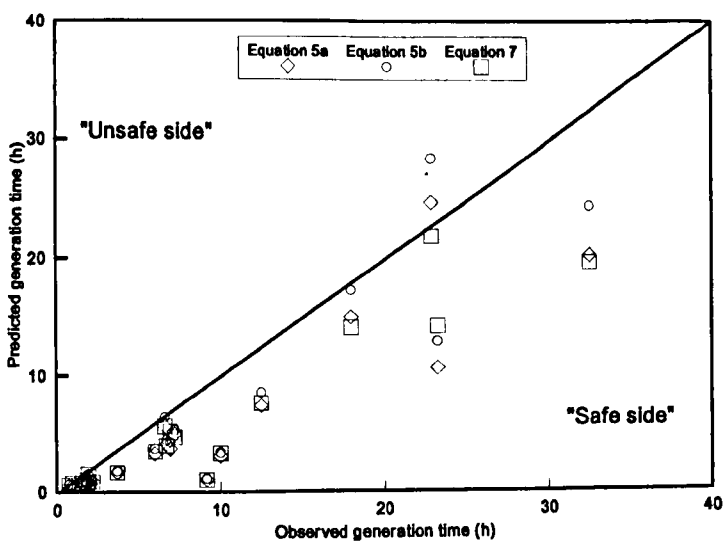

Fig. 1. Predicted generation time (h) versus observed generation time (h).

The value for f is tested against a 95% confidence interval $F_{df}^{df_2-df_1}$-value; if the value for f is smaller than the value for F the model is statistically acceptable.

In Table III the residual sum of squares of the general model (RSS_1), the degrees of freedom of the general model (df_1), the values for the residual sum of squares of the different models (RSS_2), the degrees of freedom of the models (df_2), the f and the F value are given. For all equations the value for f is smaller than the value for F, which indicates that all models are statistically acceptable. In Table IV the predictions of the generation time of *L. monocytogenes* from the different models are compared with generation times from literature for various strains of *L. monocytogenes* in various growth media.

Figure 1, which is derived from Table IV, shows the predicted generation time versus the observed generation time. Whenever the predicted generation time and the observed generation time are equal, the result can be found on the diagonal in Fig. 1 which leads to an exact prediction. The 'unsafe', left-hand side of the diagonal, represents an area in which the predicted generation time is longer than the observed generation time (the organisms grow faster than predicted). When the predicted values are smaller than the observed values the shelf-life of a food is estimated to be shorter than the real shelf-life. This area can be found on the right-hand side of the diagonal indicated by 'safe side'.

Discussion

Mathematical models to predict growth of microorganisms are promising tools to safeguard foods from spoilage or pathogenic microorganisms, especially when the model consists of easily determinable parameters. We tried to develop models that use growth parameters of microorganisms such as minimum pH, maximum pH, minimum temperature and minimum water activity and environmental variables pH, temperature and water activity.

The first two models based on Eqn. 5 can only be used below the optimal pH whereas the pH range for the third model (Eqn. 7) goes beyond the optimal pH. It should be noted that the models are valid in a temperature range from 5 to 35°C and a water activity range from 0.95 (80 g/l NaCl) to 0.997 (5 g/l NaCl), whereas the first model (5a) applies in a pH range from 4.6 to 6.3, the second model (5b) from 4.6 to 6.7 and the third model (7) a pH range from 4.6 to 7.4.

The first two models (sub-optimal pH and temperature) estimate the minimum water activity to support growth, very close to the reported minimum water activity for growth as can be seen in Table II. According to Petran and Zotolla (1989) the minimum value is 0.92 whereas the extrapolated value derived from Eqn. 5a is 0.912 and the value derived from Eqn. 5b equals 0.913.

The first model (Eqn. 5a) estimates the minimal temperature 1.65°C lower than the reported value in literature by Walker et al. (1990), and the estimated minimum pH is found to be 0.24 pH units lower than the reported minimum pH (George et al., 1988). Although this model cannot be used to describe growth

TABLE IV

Comparison of predicted and observed values for generation time of *L. monocytogenes*, with observed values from literature

a_w	pH	T (°C)	Substrate	Strain	Generation time [a] (h)				Ref [b]
					Obs.	Eqn. 5a	Eqn. 5b	Eqn. 7	
1.00	5.6	30	Unclarified cabbage juice	LCDC 81-861	9.16	1.06	1.13	1.04	1
1.00	6.2	30	Clarified cabbage juice	LCDC 81-861	1.52	0.75	0.82	0.79	1
0.997	6.2	30	Clarified cabbage juice	LCDC 81-861	1.81	0.78	0.85	0.82	1
0.994	6.2	30	Clarified cabbage juice	LCDC 81-861	1.80	0.81	0.88	0.85	1
0.991	6.2	30	Clarified cabbage juice	LCDC 81-861	1.92	0.84	0.91	0.88	1
0.989	6.2	30	Clarified cabbage juice	LCDC 81-861	2.18	0.88	0.95	0.91	1
0.986	6.1	6	Camembert cheese	OH	18.0	15.0	17.3	14.1	2
0.997	7.2	5	Growth medium	109	23.25	10.7	13.0	14.3	3
0.997	7.2	10	Growth medium	109	6.93	3.69	4.27	5.18	3
0.997	7.2	25	Growth medium	109	1.58	0.73	0.82	1.07	3
0.995	6.2	30	Growth medium	109	0.91	0.52	0.58	0.77	3
0.995	6.4	10	Whole milk	Scott A	6.6	5.65	6.39	5.64	4
0.995	6.4	4	Skimmed milk	Scott A	32.5	20.3	24.5	19.7	5
0.995	6.4	8	Skimmed milk	Scott A	12.5	7.38	8.51	7.60	5
0.995	6.4	13	Skimmed milk	Scott A	6.0	3.30	3.71	3.49	5
0.995	6.4	21	Skimmed milk	V 7	1.9	1.41	1.56	1.53	5
0.995	6.4	35	Skimmed milk	V 7	0.7	0.55	0.60	0.60	5
0.98	5.9	15	Asparagus	Scott A	6.7	4.14	4.47	3.97	6
0.98	6.5	15	Broccoli	Scott A	10.0	3.08	3.39	3.32	6
0.98	5.6	15	Cauliflower	Scott A	7.2	4.99	5.32	4.70	6
0.99	5.6	5	Lean meat	Scott A	22.9	24.86	28.57	21.98	7
0.99	5.6	25	Lean meat	Scott A	3.7	1.70	1.81	1.65	7

[a] Obs., observed generation time. Eqn. 5a = predicted generation time by Eqn. 5a; Eqn. 5b = predicted generation time by Eqn. 5b; Eqn. 7 = predicted generation time by Eqn. 7.

[b] 1, Conner et al. (1986); 2, Ryser and Marth (1987); 3, Wilkins et al. (1972); 4, Marshal and Schmidt (1988); 5, Rosenow and Marth (1987); 6, Berrang et al. (1989); 7, Chung et al. (1989).

throughout the entire pH and temperature range it estimates the growth minima for pH, temperature and water activity close to the reported values.

The second model (Eqn. 5b) estimates the minimum temperature a little better than the first model (1.4°C lower) (Walker et al., 1990). The minimum pH for growth is estimated 0.36 pH units lower than the value reported by George et al. (1988).

The third model (entire pH range, sub-optimal temperatures) also approximates the literature value for the minimum water activity very well, here the minimum water activity is 0.916. The estimated minimum growth temperature is 2.2°C lower than the literature value (Walker et al., 1990). The estimated minimum and maximum pH values are respectively 0.55 below (George et al., 1988) and 0.62 higher (Petran and Zotolla, 1989) than the reported values in the literature.

The reason for the discrepancies between the reported values in the literature and the regressed values from the model may be found in the problem of detecting growth itself, close to the growth boundaries (e.g. minimum pH, minimum temperature etc.). Here, two factors play a very important role. First of all, lag time increases near the growth extremes; secondly, the growth rate decreases near the extremes; at optimal values for pH and water activity the growth rate near the minimum for temperature approaches 0 div/h.

Considering Fig. 1 models 5a and 5b give one 'unsafe' prediction when used for the prediction of the generation time in lean meat at low temperature (Table IV). This temperature, however, is on the lowest temperature allowed for the usage of these models. Equation 7 gives a good estimate of the generation time in this case.

The predictions for the generation time of the models developed in this study are very good considering the facts that the models are developed for *L. monocytogenes* (NCTC 9863) whereas the observed generation times are for other species of *L. monocytogenes* (e.g. Scott A). Furthermore, most of the observed generation times are taken from foods whereas this study was carried out in broth. Most of the predictions are on the 'safe side' of the diagonal in Fig. 1. The predictions of Eqn. 7 are always on the 'safe side' which is a good reason to choose this model instead of the other two.

All models described are statistically accepted using the *F*-ratio test. This means that another criterion for the selection of the best model has to be formulated. The model that describes the full data set is the model that can be used in the largest range of controlling factors. The model that describes the full pH range (Eqn. 7) therefore can be considered the best model.

In some cases the product is microbiologically far more stable than the models might predict. This problem might be related to the presence of other inhibiting factors not taken into account in these models.

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