Predictive models for the growth of *Cronobacter sakazakii* in reconstituted powdered infant formula

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Received: 03 August 2018 / Accepted: 18 December 2018 / Published online: 21 February 2019
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**Abstract:** *Cronobacter sakazakii* has been implicated in foodborne illnesses in neonates and infants resulting from the consumption of contaminated infant formula. The objective of this research was to develop predictive models for the growth of *C. sakazakii* in infant milk formula (IMF) and infant soy formula (ISF). Growth kinetics for a five strain cocktail of *C. sakazakii* were obtained at several isothermal conditions at 8.5, 10, 15, 20, 25, 28, 32, 35, 37, 40, 45, and 47 °C in reconstituted IMF and ISF. Initial protocol resulted in clumping of colonies leading to difficulty in enumerating *C. sakazakii*. Protocol was then modified by addition of Tween-80 and stomaching the samples, which resulted in breaking up of colonies effectively. The growth data were fitted to three primary models (Baranyi, Gompertz, and Logistic) to describe the growth of *C. sakazakii* at each isothermal condition. For IMF, the pseudo-R² and the root mean square error (RMSE) ranged from 0.96-0.99 and 0.07-0.34 log CFU/mL, respectively. For ISF, the pseudo-R² and the RMSE ranged from 0.98-0.99 and 0.08-0.27 log CFU/mL, respectively. Two different secondary models were used to describe the effect of temperature on growth rate of *C. sakazakii* for each product. For the modified Ratkowsky’s equation, pseudo-R² and the RMSE values were 0.99 and 0.004-0.0169 (log CFU/mL)/h, respectively. For the Gamma model, psuedo-R² and the RMSE values were 0.99 and 0.004-0.006 (logCFU/mL)/h, respectively. *C. sakazakii* grew faster in IMF, when compared to ISF. Primary and secondary models were integrated and solved numerically to determine the growth of *C. sakazakii* at varying temperature profiles. Six dynamic models were validated with one sinusoidal and three ‘real-life’ temperature profiles. The dynamic models from Baranyi (RMSE ranging from 0.12-0.39 log CFU/mL) and logistic models (RMSE ranging from 0.25-0.79 log CFU/mL) predicted *C. sakazakii* growth better, compared to the Gompertz dynamic models (RMSE ranging from 0.46-0.67 log CFU/mL). These predictive models can help improve microbial risk assessment and develop appropriate risk management strategies.

**Keywords:** Cronobacter sakazakii, Infant formula, Predictive modeling

**Introduction**

Colonization of neonates with gram-negative bacteria is inevitable (Arseni et al., 1987) with the most common being *Klebsiella*, *Serratia*, and *Enterobacter*. It is estimated that the prevalence of neonatal sepsis and meningitis is roughly 1-5/1,000 live births, but increases to 1/230 in premature infants (Ferrieri 1990). Increased susceptibility stems from maternal and environmental factors. Cross contamination of newborn infants from personnel or from contaminated equipment is increased when combined with immunologic immaturity of preterm infants (Ferrieri 1990).

*Enterobacter sakazakii* is a newly recognized yellow-pigmented species of the family *Enterobacteriaceae* (Farmer et al., 1980). With new polyphasic taxonomic approaches, *C. sakazakii* was reclassified into a new genus, *Cronobacter* (Iversen et al., 2008). *Cronobacter* spp. is synonymous with *C. sakazakii*. Although the natural habitat and source of infection of *C. sakazakii* is questionable, foodborne illness outbreaks among infants in neonatal intensive care units (NICU) indicates strong correlation of *C. sakazakii* as a natural contaminant in powdered infant formula (PIF).

*C. sakazakii* has been isolated from a range of foods including UHT milk (Skladal et al., 1993) and reconstituted powdered infant...
Minimizing the risk of illnesses resulting from *C. sakazakii* infection in infants requires (i) minimizing the risk of contamination of PIF at the processing facility and (ii) the potential risk of pathogen growth during preparation and feeding of the neonates/infants in the NICU. The hospital reported in Noriega et al. (1990) changed their protocol from rinsing their blender between preparing the formula, which had heavy growth of *C. sakazakii*, to terminal pasteurization of reconstituted formula in the bottles. The hospital in Himelright et al. (2002) changed their policy by completely replacing the principle formula type for NICU infants from powdered formula to a commercially sterile, ready-to-feed liquid formula. Although these changes are simple, processing and production of commercial, sterilized liquid formulas are generally more expensive and require larger transport and storage facilities (Van Acker et al., 2001).

Regardless of the measures implemented at processing facilities, it would be prudent to develop and implement guidelines at the hospitals and other similar settings to minimize the risk of pathogen growth during preparation and feeding of the infants. But even with specific guidelines, temperature abuse and improper handling of infant formula could occur. Accurate prediction of potential *C. sakazakii* growth in infant formula can help in improving microbial risk assessment and developing appropriate risk management strategies.

**Material and methods**

**Organism**

Five *C. sakazakii* isolates were obtained from environmental sources of a processing facility and stored at -70 °C. Each strain was transferred three successive times in tryptic soy broth (TSB) (10 mL; Fisher) and incubated overnight at 35 °C. Each strain was then transferred to TSB and incubated at 35 °C for 16-17 h, separately to obtain cells in the exponential phase of growth. Five mL of TSB from each strain were then combined and centrifuged 2683 ×g for 15 min at 4 °C. The cell pellet was resuspended in 10 mL peptone water.

**Preparation, inoculation of infant formula, and enumeration**

Commercially available brands of milk and soy powdered infant formula were obtained from a grocery store. The formulations were prepared according to the manufacturer instructions in sterile containers using sterile, tap water. The infant formula was inoculated with the appropriate dilution of the mixed culture to provide an initial *C. sakazakii* population of 2.7 ± 0.1 log CFU/mL. Infant formula was manually mixed and 10 mL aliquots were transferred to sterile screw cap test tubes. The test tubes were then placed in water-baths set to temperatures of 8.5, 10, 15, 20, 25, 28, 32, 35, 37, 40, 42, 45, or 47 °C. Approximately 10 min was allowed for temperature stabilization. At least 12 samples were collected for each temperature (from the lag phase to the stationary phase) at various times to enumerate *C. sakazakii*. Samples from 8.5 – 20 °C water-baths were submerged in a hot water-bath to quickly warm the sample to ambient temperature to aid in sampling as slime formation (ropiness) was observed at different sampling points. A 1 mL aliquot of Tween-80 (1.0% solution in distilled deionized water) was added directly to the sample and further dilutions were prepared using 0.1% Tween-80 dilution blanks. Samples were then transferred to sterile stomacher bags (Nasco Whirl-Pak, 4 oz) and stomached for 2 min in a (Seward Model 400, Tekmar Company). Two independent replications for each temperature were performed using fresh culture and a new can of formula. Serial dilutions of the sample were prepared in sterile 0.1% Tween-80 and plated on violet red bile glucose agar (VRBGA) in duplicate and incubated for 16-24 h at 35 °C. Typical red colonies were enumerated as *C. sakazakii* and counts were expressed as log CFU/mL.

**Primary models**

Primary microbiological growth models describe the growth of microorganisms over time under constant environmental conditions such as temperature. Growth profiles were recorded for the 13 static temperatures. For each growth profile, parameters were calculated manually and specified into a preprogrammed non-linear regression program, PROC NLIN, in the SAS package (SAS, 2007) to fit the data using Marquardt iterative method for the estimation of the parameters.

**Baranyi’s Model**

Baranyi and Roberts (1994) published the solution for the natural logarithm of the cell concentration, $y = \ln(x(t))$, at constant temperature:

$$y(t) = y_0 + \mu_b F(t) - \log e \left( 1 + \frac{e^{\mu_b t F(t)} - 1}{e^{\mu_b t F(t) - y_0}} \right)$$

where, $F(t) = t + \frac{1}{\nu} \log e \left( e^{-\nu t} + e^{-\nu t - h_o} - e^{-\nu t - h_o} \right)$.

Baranyi’s model includes four parameters: $y_0$, initial microbial population (ln CFU/mL); $y_{max}$, maximum growth (ln CFU/mL); $\mu_b$, Baranyi maximum specific growth rate (h$^{-1}$); and $h_o$ which is the product of (h$^{-1}$) and the lag phase duration (h). is defined as the growth (ln CFU/mL) at time, (h). To determine the lag phase
periods (h), the converged values of was divided by the converged values.

To evaluate the goodness of fit, pseudo-R², a statistic closely corresponding to R² in the non-linear case, was used (Schabenberger, 2005):

\[ Pseudo - R^2 = 1 - \frac{SS(Residual)}{SS(Total_{corrected})}, \]  
(3)

where SS(Residual) is the sum of the squares of residuals and SS(Total_corrected) is the total sum of squares.

The root mean squared error (RMSE) is calculated by

\[ RMSE = \sqrt{\frac{(O - P)^2}{N - p}}, \]  
(4)

where \( O \) and \( P \) are observed and predicted microbial population in log CFU/mL and \( N \) is the number of observations, and \( p \) is the number of model parameters.

The model performance was evaluated using the Bias Factor (BF) and Accuracy Factor (AF) and were calculated using the following formulae:

\[ BF = \frac{1}{10} \sum \log(P/O)/N \]  
(5)

\[ AF = 10 \sum |\log(P/O)|/N \]  
(6)

A BF value greater than 1 indicates the model over predicted and vice versa. An AF value of 1.1 indicates the average deviation of the predicted values from the observed values is 10\% (Jeyamkondan et al., 2001).

**Modified Gompertz & Logistic Models**

The modified Gompertz equation (Gibson et al., 1987, 1988)

\[ L(t) = y_o + (y_{max} - y_o) \times \exp\left[-\exp\left(-\mu_g(t - m)\right)\right], \]  
(7)

and the logistic equation (Gibson et al., 1987, 1988),

\[ L(t) = y_o + \left(\frac{y_{max} - y_o}{1 + e^{(-\mu_1(t - m))}}\right), \]  
(8)

containing the following parameters: \( L(t) \) is the log(CFU/mL) of cell concentration; and \( y_o \), \( y_{max} \) are the initial and final cell concentrations; \( t \) the growth time; \( \mu_g \) Gompertz maximum specific growth rate (h\(^{-1}\)); \( \mu \) Gompertz theoretical minimum temperature (°C); \( T_{opt} \) optimum temperature (°C); \( T_{min} \) theoretical minimum temperature (°C); and \( T_{opt} \) theoretical maximum temperature (°C).

**Dynamic Model**

Dynamic models integrate the primary and secondary models to predict growth over time. Integrating the three primary models...
with the two secondary models resulted in six dynamic models. Huang (2003) differentiated Eq. (6) with respect to time and the resulting equation was

\[ \frac{dL}{dt} = \mu_y (y_{\text{max}} - y_o) \times \exp\{-\mu_y (t - M)\} \times \exp\{-\mu_y (t - M)\} \]

q. (5) can be rearranged as

\[ \frac{L - y_o}{y_{\text{max}} - y_o} = \exp\{-\mu_y (t - m)\} \]

\[ \ln \left( \frac{L - y_o}{y_{\text{max}} - y_o} \right) = -\mu_y (t - m) \]

By rearrangement of Eq. (6), and substitution of Eq. (13) and (14), Eq. (12) can be written as

\[ \frac{dL}{dt} = \mu_y (L - y_o) \ln \left( \frac{y_{\text{max}} - y_o}{L - y_o} \right) \]

Differentiating the Logistic equation (Eq. (7)) results in

\[ \frac{dL}{dt} = \frac{\mu_y (y_{\text{max}} - y_o) \left( e^{-\mu_y (t - m)} \right)}{ \left( 1 + e^{-\mu_y (t - m)} \right)^2 } \]

(16)

Eq. (7) can be rearranged as

\[ (y_{\text{max}} - y_o) = (L - y_o) \left[ 1 + e^{-\mu_y (t - m)} \right] \]

\[ [1 + e^{-\mu_y (t - m)}] = \frac{y_{\text{max}} - y_o}{L - y_o} \]

(18)

\[ e^{-\mu_y (t - m)} = \left( \frac{y_{\text{max}} - y_o}{L - y_o} \right) - 1. \]

(19)

By rearrangement of Eq. (7) and substitution of Eq. (17), (18), and (19), Eq. (16) can be written as

\[ \frac{dL}{dt} = \frac{\mu_y (L - y_o) \left( y_{\text{max}} - L \right)}{y_{\text{max}} - y_o} \]

(20)

In Eq. (15) and (20), the initial value of L must be given, but at L(0), L equals y_o, which makes the equation singular (Huang, 2003). To overcome this, Huang (2003) developed the pseudo-initial value of L by the equation L(0)=y_o+?L(0).

In the case of the Baranyi model, the following two differential equations must be solved:

\[ \frac{dQ}{dt} = \mu_b (T(t)) \] and (21)

\[ \frac{dy}{dx} = \frac{1}{1 + e^{-Q(t)}} \left[ \mu_b (T(t)) \right] (1 - e^{y-y_{\text{max}}}) \]

(22)

where y=ln(t)(x) and Q=ln(t)(q).

The differential forms the primary models, which allowed for prediction under non-isothermal conditions, were solved using numerical methods. Forth order Runge-Kutta method (Kam, 2006) was used to solve first order differential equations in the form of

\[ \frac{dy}{dx} = f(x, y), y(0) = y_o. \]

This algorithm discretizes the time domain as

\[ t_n = t_o + \Delta t \cdot n, \quad \text{for } n = 0, 1, 2, \ldots \]

and evaluates the function f(t,y(t)) at the beginning, end, and midpoint of each time interval (Amézquita, 2004). A popular solution to the Runge-Kutta forth order algorithm is (Amézquita, 2004, Kam, 2006)

\[ y_{n+1} = y_n + \frac{1}{6} \left( k_1 + 2k_2 + 2k_3 + k_4 \right) \]

\[ k_1 = \Delta t \cdot f(x_n, y_n) \]

\[ k_2 = \Delta t \cdot f \left( t_n + \frac{1}{2} \Delta t, y_n + \frac{1}{2} k_1 \right) \]

\[ k_3 = \Delta t \cdot f \left( t_n + \frac{1}{2} \Delta t, y_n + \frac{1}{2} k_2 \right) \]

\[ k_4 = \Delta t \cdot f \left( t_n + \Delta t, y_n + k_3 \right) \]

Parameters were entered in MATLAB 7.0.1 for estimations of growth over time during a specific temperature profile. Runge-Kutta forth-order methods were implemented in MATLAB 7.0.1 and the example programs are given in the appendix. The sinusoidal equation chosen was T=17.5×sin(2πx/4)+27.5. The temperature started at 27.5 °C then rose to 45 °C and fell to 10 °C, in 4 h for five cycles. Three real-life temperature profiles were made using scenarios (Table 1) from microbiological risk assessment model for C. sakazakii from Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA) (http://www.mramodels.org/ESAK/default.aspx). Worst case scenarios were chosen to obtain C. sakazakii growth to validate developed models. The temperature profiles were programmed into a commercial software package (NesCom 3.0 Software, Newington, NH) to replicate the desired temperature profile in the water baths (RITE 740, Thermo Neslab, Newington, NH).

**Results and discussion**

**Protocol**

Growth of C. sakazakii at 8.5 °C showed ropiness and clumping within the sample, resulting in significant differences in counts between dilutions and within the sample itself. Homogenization of the sample using a stomacher (Seward Model 400, Tekmar
Company) alleviated the issue and provided more precise enumeration of *C. sakazakii*.

Subsequently, predictive models for growth of *C. sakazakii* were validated using a sinusoidal profile. When sampling at lower temperatures, issues related to differences in populations between the dilutions were encountered. Addition of Tween-80 to the initial sample and the use of Tween-80-supplemented diluents in preparation of subsequent dilutions alleviated the issue and consistent counts were observed between duplicate plates. Tween-80 is a surfactant and emulsifier that can be used as a dispersing agent (Windholz et al., 1983) and allows easier plating and enumeration. Stomaching, warming the sample, and the use of Tween-80 alleviated the clumping issue, providing consistent results in enumeration of *C. sakazakii*. The growth data for *C. sakazakii* under isothermal temperature profiles were repeated following the new protocol (use of Tween-80 supplemented diluents, warming, and stomaching the sample). Fig. 1 shows the differences in observed growth of *C. sakazakii* in IMF at 10 °C using the previous and revised protocols.

No mention of difficulty in accuracy of making dilutions or enumeration has been reported in literature. In most of the literature on *C. sakazakii* growth, the microbial growth data was not fully disclosed. Richards et al. (2005) incorporated a technique that somewhat resembles stomaching: vigorously shaking the sample containers of formula before drawing 5 mL aliquots, which were then combined with 45 mL of *Enterobacteriaceae* enrichment broth supplemented with nalidixic acid and pyruvate and then subjected to vigorous agitation. This protocol may not be sufficient if *C. sakazakii* has produced the capsular exopolysaccharide, resulting in clumping of the cells, apparently reducing the microbial counts. Our protocol allows for the homogeneity of the formula, clumps of *C. sakazakii*, and any biofilm formation on the inner wall of the test tubes. Iversen et al. (2004) discussed the ability of *C. sakazakii* to adhere to silicon, latex, and polycarbonate. Neonatal infections associated with brushes, blenders, and spoons (Simmons et al., 1989; Bar-Oz et al., 2001) show the ability of *C. sakazakii* to adhere to surfaces and the enhanced attachment and adherence might be explained by the formation of exopolysaccharide capsular material (Scheepe-Leberkühne and Wagner, 1986). The authors reported that polysaccharide formation occurs within 4 h at 27 °C. They reported that *C. sakazakii* produced an exopolysaccharide which showed extremely high viscosity in solutions and gel formation with trivalent cations. In the present study, it was observed that with addition of Tween-80 to the sample and stomaching the infant formula, growth data from each temperature profile improved and was not as erratic in

### Table 1. Description of temperature abuse profiles created in JEMRA (temperature profiles were slightly altered to allow waterbath to keep up with the time-steps)

| Parameters                | JEMRA 1 | JEMRA 2 | JEMRA 3 |
|---------------------------|---------|---------|---------|
| Reconstitution (Water)    | Temperature (°C) | Time (h) | Temperature (°C) | Time (h) | Temperature (°C) | Time (h) |
| Preparatoin (Air)        | 30      | 25      | 35      |
| Holding (Air)            | 25      | 1       | 30      | 1       | 30      |
| Re-warming (Formula)     | 10      | 2       | 10      | 1       | 15      |
| Feeding (Air)            | 45      | 0.5     | 40      | 0.5     | 40      | 0.6     |
|                          | 30      | 4       | 27      | 4       | 30      | 4       |

### Table 2. Comparison of lag periods of *C. sakazakii* in reconstituted powdered infant milk formula calculated from each primary model

| Temperature (°C) | Lag Period (h) | Baranyi | Gompertz | Logistic |
|-----------------|----------------|---------|----------|---------|
| 8.5             | 155            | 165.38  | 176.06   | 238.59  |
| 10              | 75             | 88.07   | 98.56    | 134.76  |
| 15              | 8              | 9.43    | 12.16    | 23.04   |
| 20              | 2              | 2.79    | 3.58     | 8.55    |
| 25              | 1              | 1.76    | 1.84     | 4.95    |
| 28              | 1              | 0.65    | 0.90     | 2.69    |
| 32              | 1              | 0.54    | 0.86     | 2.29    |
| 35              | 0.5            | 0.51    | 0.63     | 1.95    |
| 37              | 0.5            | 0.51    | 0.72     | 1.83    |
| 40              | 0.5            | 0.26    | 0.42     | 1.31    |
| 42              | 0.5            | 0.48    | 0.85     | 1.85    |
| 45              | 1              | 0.64    | 0.79     | 1.87    |
| 47              | 3              | 0.53    | -0.04    | 0.60    |
Fig. 1. Comparison of growth results of *C. sakazakii* in reconstituted powdered infant milk formula at 10 °C (’ Old Protocol, ’ %’ New Protocol with Tween-80)

Fig. 2. Primary model results for the growth of *E. sakazakii* in reconstituted powdered infant milk formula (’ ◆’ Rep 1, ’ %’ Rep 2, ’ – ’ Baranyi, ’ – – ’ Gompertz, ’ – – – ’ Logistic)
enumeration compared to the growth data generated using the previous protocol.

Primary models

Figs. 2 - 3 show the predicted and observed C. sakazakii growth profiles in IMF at various temperatures. For each primary model, predicted growth followed the observed growth well for both infant milk and soy formula. Table 2 and 3 show the lag periods and generation times calculated from each primary model. Calculating the lag periods from Gompertz parameters provided several negative values, which were obviously incorrect. A disadvantage of the Gompertz model is that the slope cannot be zero and because of the lower asymptote of the sigmoid curve must be below the initial inoculum concentration, which can result in the duration of the lag phase to be negative (Baranyi et al., 1993). Lag periods calculated using the Baranyi equation matched the observed lag periods well except for values for ISF at 20 °C. At 47 °C, all models under-predicted the lag period for IMF. Overall, the lag period was slightly higher in IMF than in ISF.

Kandhai et al. (2006) reported lag times of 83.3 ± 18.7 and 1.73 ± 0.43 h at H” 10 and 37 °C, respectively. In the present study lag times of 88 and 74 h, at 10 °C was calculated in IMF and ISF, respectively. At 10 and 20 °C, the generation times from Baranyi parameters were 10.4 h and 1.5 h, respectively in IMF. Iversen and Forsythe (2003) reported at 10 and 21 °C, the generation times were 10 and 1.25 h, respectively. Iversen and Forsythe (2004) reported generation times of 14 and 0.75 h at 10 °C, and room temperature, respectively. Nazarowec-White and Farber (1997) reported generation times of 0.67 and 4.18 h at 23 and 10 °C in infant formula.

Secondary models

Fig. 4 and 5 show the predicted and observed C. sakazakii growth rates as a function of temperature. The Ratkowsky (secondary) model from Baranyi model parameters showed a slightly higher C. sakazakii growth rate in IMF compared to ISF. Ratkowsky models developed from Gompertz and Logistic primary models showed similar growth rates between IMF and ISF.

To limit over-prediction at optimum temperatures, the \( \mu_{\text{opt}} \) parameter in SAS was bound to be less than 2.1 h\(^{-1} \) for IMF and less than 1.8 h\(^{-1} \) for ISF in the Gamma-Baranyi model. Without bounds in SAS, Gamma models from Gompertz and Logistic followed the observed values very well and did not over-predict. Table 4 shows the measurements of bias and accuracy factors

Table 3. Comparison of generation periods of C. sakazakii in reconstituted powdered infant milk formula calculated from each primary model

| Temperature(°C) | Baranyi |  | Gompertz |  | Logistic |  |
|----------------|---------|---|-----------|---|----------|---|
| 8.5            | 17.33   |  | 15.34     |  | 10.34    |  |
| 10             | 10.38   |  | 8.72      |  | 6.07     |  |
| 15             | 3.39    |  | 2.94      |  | 2.05     |  |
| 20             | 1.55    |  | 1.35      |  | 0.94     |  |
| 25             | 0.88    |  | 0.80      |  | 0.54     |  |
| 28             | 0.70    |  | 0.60      |  | 0.42     |  |
| 32             | 0.51    |  | 0.43      |  | 0.30     |  |
| 35             | 0.43    |  | 0.38      |  | 0.26     |  |
| 37             | 0.39    |  | 0.34      |  | 0.24     |  |
| 40             | 0.36    |  | 0.31      |  | 0.22     |  |
| 42             | 0.34    |  | 0.29      |  | 0.20     |  |
| 45             | 0.38    |  | 0.34      |  | 0.23     |  |
| 47             | 1.15    |  | 1.00      |  | 0.72     |  |

Table 4. Measurements of bias and accuracy factors for \( \mu_{\text{opt}} \) in reconstituted powdered infant milk and soy formula

| Secondary Model | Milk B\(_f\) | A\(_f\) | Soy B\(_f\) | A\(_f\) |
|-----------------|-------------|--------|-------------|--------|
| Ratkowsky-Baranyi | 1.1239     | 1.1879 | 1.2390      | 1.3702 |
| Ratkowsky-Gompertz | 0.9186     | 1.1894 | 0.9701      | 1.1033 |
| Ratkowsky-Logistic | 0.8891     | 1.2573 | 0.9396      | 1.1577 |
| Gamma-Baranyi   | 1.0645     | 1.1555 | 1.2585      | 1.5213 |
| Gamma-Gompertz  | 0.9672     | 1.1046 | 0.8867      | 1.3532 |
| Gamma-Logistic  | 0.9958     | 1.0445 | 0.9448      | 1.1710 |
for $\mu_{\text{max}}$ in reconstituted powdered infant milk and soy formula. When there is a perfect agreement between the predicted and observed values, the bias and accuracy factors equal 1 (Tamplin et al. 2005).

Jo et al. (2010) developed models for *C. sakazakii* in IMF using four temperatures: 10, 20, 30, and 40 °C. Fig. 6 shows the differences between Jo et al. (2010) secondary model against our secondary models. By not modeling the entire temperature range at which the organism grows, Jo et al. (2010) secondary model does not curve downward after passing the optimum growth temperature.

Iversen et al. (2004) reported optimum temperatures of 37-43 °C for growth of *C. sakazakii*, which were consistent with the findings in this study for both infant milk and soy formula. Iversen et al. (2004) reported a mean specific growth rate of 2.88 h$^{-1}$ at 40 °C for IMF. In the present study, the observed maximum specific growth rates were 1.87 and 2.1 h$^{-1}$ (IMF) and 1.7 and 1.8 h$^{-1}$ (ISF) for 40 and 42 °C, respectively.

Fig. 3. Primary model results for the growth of *E. sakazakii* in reconstituted powdered infant milk formula (‘*’ Rep 1, ‘%’ Rep 2, ‘--’ Baranyi, ‘-- --’ Gompertz, ‘- - -’ Logistic)
Kandhai et al. (2006) concluded that neither the lag times nor the maximum specific growth rates were significantly different for strains of different origin. Also, the cell history had no significant effect on either the specific growth rate or the lag time during subsequent cultivation in reconstituted infant formula. They used the modified Gompertz equation as their primary model and the Bélehrádek-type (expanded square root model of Ratkowsky) and Rosso models as their secondary models. They reported a specific growth rate of $2.29 \pm 0.45 \text{ h}^{-1}$ at $37 \, ^\circ\text{C}$. In the present study, a maximum specific growth rate of $1.7 \, \text{h}^{-1}$ at $37 \, ^\circ\text{C}$ was found in IMF.

**Dynamic models**

Tables 5 and 6 show the RMSE values (log CFU/mL) for the dynamic models that were validated in the laboratory. Figs. 7 - 10 show the predicted and observed growth of *C. sakazakii* during two of the non-isothermal temperature profiles that were validated in the laboratory for IMF and ISF. The dynamic model allows the user to input isothermal temperature profiles, therefore going back to the primary model for static temperature predicted growth is not needed. For temperatures between 20 and $45 \, ^\circ\text{C}$, the dynamic models from Baranyi models predicted well when integrated with
Ratkowsky, but slightly under-predicted when integrated with Gamma. When predicting at lower temperatures and 47 °C, the dynamic models over-predicted *C. sakazakii* growth in infant milk and soy formula. The \( h_s \) values were fixed at the average which would explain the over-prediction at the lower temperatures. The value reflects the lag period; therefore a smaller indicates a shorter lag period. For the modified Gompertz model, the curvature in the exponential phase tends to over-estimate the maximum specific growth rate.

Table 5. RMSE values (log CFU/mL) for the dynamic models for non-isothermal temperature validations

| Formula | Temperature Profile | Gamma Baranyi | Gompertz | Logistic |
|---------|---------------------|---------------|----------|----------|
| Milk    | Sinusoidal          | 0.23          | 0.74     | 0.45     |
|         | JEMRA 1             | 0.25          | 0.83     | 0.34     |
|         | JEMRA 2             | 0.20          | 0.91     | 0.39     |
|         | JEMRA 3             | 0.25          | 0.69     | 0.12     |
| Soy     | Sinusoidal          | 0.40          | 0.96     | 0.71     |
|         | JEMRA 1             | 0.28          | 0.61     | 0.22     |
|         | JEMRA 2             | 0.16          | 0.66     | 0.19     |
|         | JEMRA 3             | 0.41          | 0.45     | 0.13     |

Table 6. RMSE values (log CFU/mL) for the dynamic models for non-isothermal temperature validations

| Formula | Temperature Profile | Ratkowsky Baranyi | Gompertz | Logistic |
|---------|---------------------|-------------------|----------|----------|
| Milk    | Sinusoidal          | 0.29              | 0.61     | 0.79     |
|         | JEMRA 1             | 0.49              | 0.59     | 0.61     |
|         | JEMRA 2             | 0.19              | 0.47     | 0.46     |
|         | JEMRA 3             | 0.26              | 0.56     | 0.43     |
| Soy     | Sinusoidal          | 0.29              | 0.67     | 0.71     |
|         | JEMRA 1             | 0.25              | 0.62     | 0.44     |
|         | JEMRA 2             | 0.14              | 0.46     | 0.25     |
|         | JEMRA 3             | 0.26              | 0.65     | 0.38     |
growth rate, (Baranyi et al., 1993b; McClure et al., 1993). This might account for the over-prediction by the modified Gompertz and Logistic dynamic models. The value was the mean of the values from the isothermal growth profiles rather than the maximum value to offset over-prediction in the stationary phase. Even though this value was set at the average, the maximum population was lower at temperatures below and above the optimum temperatures.

For the sinusoidal temperature profile, when using Baranyi’s parameters for either Ratkowsky or Gamma dynamic models, prediction of growth followed the observed growth very well. The dynamic models from Gompertz and Logistic models over-predicted growth when integrated with the Gamma model for the sinusoidal profiles. When integrated with the Ratkowsky model, the Logistic model still over-predicted but the Gompertz model initially under-predicted (during the first few hours), with subsequent tendency to over-predict for both IMF and ISF.

For the abusive temperature profiles created from JEMRA, dynamic models created from Baranyi or Logistic models for either Ratkowsky or Gamma models, prediction of growth during these temperature abuse profiles followed the observed growth very well. There were some slight under-predictions, though, for several points in these temperature profile validations with the Gamma-Baranyi dynamic model. Dynamic models from Gompertz
models over-predicts during these temperature-abuse profiles when integrated with Gamma but under-predicts when integrated with Ratkowsky. Dynamic models from Logistic models follow pretty well with some slight over-predictions towards the end of the temperature profile.

From the results of the validations, the dynamic models best suited for prediction of growth under temperature varying conditions would be the Baranyi and Logistic models integrated with the Ratkowsky model. By having both sets of results, a range of growth could be analyzed for the potential growth of _C. sakazakii_ for a particular temperature profile. Rosset et al. (2007) reported temperature profiles of the infant formula during preparation and feeding to the infants. These temperature profiles were used to evaluate growth using the dynamic models, with initial populations of 1 CFU/mL (Muytjens et al., 1988). For the first feeding and manufacturing profiles, carefully following preparation guidelines minimizes growth of _C. sakazakii_ (log
CFU/mL or g). The last feeding temperature profile resulted in the prediction of 0.5 and 1.5 log CFU/mL growth for the Ratkowsky model integrated with Baranyi and Logistic models, respectively. Even if the Ratkowsky-Logistic dynamic model over-predicts, by having both dynamic models, a range of potential growths can be predicted. If proper protocols are followed, inhibition of growth should be expected. On the other hand, if higher contamination levels already exist because of improper cleaning or handling, or if temperature abuse occurs, growth of C. sakazakii to unacceptable levels should be expected. Also, cross-contamination should be addressed. Even if contamination levels are as low as 1 CFU/100 g in the PIF, cross-contamination of the reconstituted PIF could easily result in outbreaks. If equipment is not cleaned properly like in past outbreaks, following proper preparation protocols would not prevent outbreaks.

Telang et al. (2005) concluded that C. sakazakii counts from infant formula for inoculated preterm formula or inoculated human milk samples, unfortified or fortifed, did not increase significantly over 6 h. Under ideal preparation conditions leaving out formula for 6 h might not result in significant growth, but as stated above, cross-contamination or temperature abuse could result in an illness. To address the potential risk of non-trained hospital workers, the risk of leaving reconstituted PIF at room temperature, using JEMRA and parameters from FAO/WHO (2006), a temperature profile was generated to depict a preparation scenario that includes a 6 h hang-time. With different preparation recommendations for tube feeding, the temperature profile could include a re-warming step that allows for 15 min heating in a water-bath set at 37 °C. Reconstitution temperature and ambient room temperature were set at 25 °C. FAO/WHO (2006) stated that a NICU could have a room temperature of up to 35 °C, which was inputted into JEMRA’s risk model parameters as the ambient room temperature during the 6 h hang-time. The Ratkowsky-Baranyi dynamic model resulted in prediction of 4 log CFU/mL C. sakazakii growth in IMF and ISF. The Ratkowsky-Logistic dynamic model predicted C. sakazakii growth to 7 log CFU/mL. The difference of these two predictions could be explained by the tendency of the Ratkowsky-Logistic dynamic model to over-predict.

Conclusions

All of the primary models predict C. sakazakii growth in infant milk and soy formula very well compared to the observed growth. Secondary models predict the maximum specific growth rates of C. sakazakii well with some slight over-prediction by the Ratkowsky model at higher temperatures. The Gompertz dynamic models resulted in considerable variation in predicted C. sakazakii growth. The models that proved to be accurate in predicting the growth of C. sakazakii in reconstituted PIF were Baranyi and logistic primary models integrated with the Ratkowsky secondary model. Although the Ratkowsky-logistic dynamic model predicts higher growth in some instances, this could be the upper limit of growth and the user could evaluate a range of growth on the risk to the infants.

With the infectious dose still uncertain and when dealing with already seriously ill infants, serious concern should be taken to develop recommendations for preparation and handling of PIF subsequent to preparation. There are so many variables from temperature abuse and different ambient room temperatures to extremely busy hospital personnel making seemingly simple mistakes that could result in significant growth of C. sakazakii in reconstituted PIF.

There seems to be a lot of controversy about the interpretation of ‘level of contamination’, ‘probability of ingestion’, ‘single cell’ ingestion, infectious dose, etc (Havelaar and Zwietering, 2004; Iversen and Forsythe, 2003; Iversen and Forsythe, 2004). Iversen and Forsythe (2004) stated: “To argue whether it takes 13 h or 17.9 h at 21 °C to reach a particular cell number is missing the point; rehydrated infant milk formula should not be left at 21 °C for a long period of time - that’s temperature abuse”. Biering et al. (1989) showed that C. sakazakii seemed to be present in the milk powder in low numbers, which would not result in these infections. The authors reported that the rules pertaining to the handling to the formula in the pediatric and NICU units were not always adhered to and that the formula was occasionally kept at 35-37 °C for extended periods of time in bottle heaters.

The focus should be equally on preparation and sanitation, if not more than the models, because these models cannot predict efficiently if preparation and sanitation rules are not followed. With these problems taken more seriously, these predictive models will be an important asset in risk analysis.

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