Journal of Food Safety and Food Quality 63, Heft 6 (2012), Seiten 165–196

Arch Lebensmittelhyg 63,
168–174 (2012)
DOI 10.2376/0003-925X-63-168

ISSN 0003-925X

Korrespondenzadresse:
amartinez@iata.csic.es

Department of food quality and preservation. IATA-CSIC, Avda Agustin Escardino, 7, 46980 Paterna, Valencia, Spain

Cronobacter sakazakii growth prediction in Reconstituted Powdered Infant Formula Milk

Modellierung des Wachstums von Cronobacter sakazakii in zubereiteter Säuglingsanfangsnahrung

Maria C. Pina-Pérez, Dolores Rodrigo, Antonio Martínez

Summary

In the present study, a detailed knowledge of the growth behaviour of *Cronobacter sakazakii* (858 CECT), at different temperatures, in Tryptic Soy Broth (TSB) and Reconstituted Powdered Infant Formula Milk (RPIFM), is condensed into mathematical models.

Growth kinetic data of *C. sakazakii* were obtained at four different temperatures: 4, 8, 25 and 37 ºC. The kinetic data were fitted to Baranyi and Gompertz primary models to determine initial density (N_o), lag time (λ), maximum growth rate (μ_{max}) and maximum population density (N_f). The Gompertz equation provided the best fit for experimental data and was selected as the most suitable primary model to describe the *C. sakazakii* growth behaviour. The results obtained revealed that the lag phase length was affected by temperature, decreasing with increasing temperature ranging from 101.89 h (8 ºC) to 0.834 h (37 ºC) in RPIFM. At the same time, the maximum growth rate (μ_{max}) was temperature-dependent, with values of 0.027 ((cfu/ml)/h) at 8 ºC, 0.384 ((cfu/ml)/h) at 25 ºC, and 0.924 ((cfu/ml)/h) at 37 ºC for RPIFM.

The present study contributes to support the good handling practices recommended by FAO/WHO after reconstitution of PIFM, and the requirement of thermal/non-thermal treatments after reconstitution, previous to neonatal feeding, to ensure RPIFM microbiological safety.

Keywords: Infant Formula Milk (IFM), *Cronobacter sakazakii,* growth, predictive microbiology, temperature

Zusammenfassung

In der vorliegenden Studie werden detaillierte Erkenntnisse über das Wachstumsverhalten von *Cronobacter sakazakii* (858 CECT) bei unterschiedlichen Temperaturen in CASO-Bouillon (Caseinpepton-Sojamehlpepton-Bouillon) und in zubereiteter Säuglingsanfangsnahrung in mathematische Modelle zusammengefasst.

Die kinetischen Wachstumsdaten von *C. sakazakii* wurden bei vier verschiedenen Temperaturen: 4, 8, 25 und 37 ºC ermittelt. Die kinetischen Daten wurden am Baranyi-Modell und am Gompertz-Modell angepasst, um anfängliche Dichte (N_o), Verzögerungsphase (λ), maximale Wachstumsrate (μ_{max}) und maximale Populationsdichte (N_f) zu bestimmen. Die Gompertz-Gleichung stellte sich für experimentelle Daten als passend heraus und wurde als primär Modell ausgewählt, um das Wachstumsverhalten von *C. sakazakii* zu beschreiben. Die Ergebnisse zeigten, dass die Verzögerungsphase von der Temperatur beeinflusst wurde, abnehmend mit ansteigender Temperatur von 101.89 h (8 ºC) auf 0,834 h (37 ºC) in Säuglingsanfangsnahrung. Gleichzeitig war die maximale Wachstumsrate (μ_{max}) temperaturabhängig, mit Werten von 0,027 ((KBE / ml) / h) bei 8 °C, 0,384 ((KBE / ml) / h) bei 25 °C und 0,924 ((KBE / ml) / h) bei 37 °C in Säuglingsanfangsnahrung.

Die vorliegende Studie trägt dazu bei, die von der FAO/WHO empfohlenen guten Handhabungspraktiken für zubereitete Säuglingsanfangsnahrung, zu unterstützen.

Schlüsselwörter: Säuglingsanfangsnahrung, *Cronobacter sakazakii,* Wachstum, prädiktive Mikrobiologie, Temperatur

Introduction

Epidemiological studies have implicated *Cronobacter* spp. (previously *Enterobacter sakakzakii*) (Iversen et al., 2008) in rare but life-threatening cases of sepsis, neonatal meningitis, bacteraemia, necrotizing enterocolitis (NEC) and necrotizing meningoencephalitis after ingestion (Muytjens and Cole, 1990) with mortality rates around 50 % (FAO/WHO, 2006). *Cronobacter* spp. is considered as opportunistic pathogen affecting neonatal, premature (< 37 weeks), low-weight (< 2000 g), immunodeppressed and VIH infants. International evidence suggests that powdered infant formula milk (PIFM) has been the main vehicle of *Cronobacter* spp. infection. Although the advantages of cost and storage of powder over the liquid form (sterile ready-to-eat), the PIFM is not a sterile product. The main sources of contamination and re-contamination of PIFM have been extensively reported (FAO/WHO, 2006; Skovgaard et al., 2007). Technology of powdered milk production (e. g spray drying and ingredients addition) and handle practices (e. g. lapses in preparation hygiene and extended holding at non-refrigerated temperatures) during reconstitution at home and hospital settings have been identified as the main risk prevention objectives.

In spite of contamination level of *Cronobacter* spp. of powdered milk formula appear to be very low (Reij et al., 2009), this microorganisms have the ability to survive at low a_w levels (a_w = 0.3–0.2) (Beuchat et al., 2009); pH levels from 5–9 to 3.5 (Edelson-Mammel et al., 2006), and in a wide temperature range [5–39.4] (Kandhai et al. 2006), with generation time values of 5 h at 10 °C, 40 min at 23 °C, 20 min at optimum (Lambert and Bidlas, 2007). So, it is important to get knowledge about the microbial behavior under different environmental conditions in order to prevent risk after PIFM reconstitution taking into account that the infectious dose value remains below 3 cfu/100g (NZFSA, 2010)

Up to date, *Cronobacter* spp. growth behavior has been scarcely reported (Kandhai et al., 2006; Lambert and Bidlas, 2007; Rosset et al.2007; Lenati et al., 2008). Microbiological risk assessment, associated to binomial RPIFM-*C. sakazakii,* at different consumption levels, requires a detailed knowledge of the *Cronobacter* spp. growth (Kandhai et al., 2006) and inactivation behavior (Pina-Pérez et al., 2007a, b; Koseki et al., 2009; Arroyo et al., 2010), condensed into mathematical models. Mathematical modelling of microbial growth has been used to estimate parameters (maximum growth rate and lag time) required to build up prediction models for use in risk analysis procedures (Whiting and Buchanan, 1997).

The objectives of the present study were: a) to achieve detailed knowledge about the growth behaviour of *C. sakazakii* (CECT 858) at different temperatures, in Tryptic Soy Broth (TSB) and RPIFM, condensed into mathematical models; and b) to compare the suitability of different primary and secondary growth models in order to define the *C. sakazakii* growth curves.

Material and Methods

Cultivation of microorganism

A pure culture of *C. sakazakii* (CECT 858) equivalent to 29544 ATCC was provided freeze-dried by the Spanish Type Culture Collection.

The culture was rehydrated with 10 ml of Tryptic Soy Broth (TSB) (Scharlab Chemie, Barcelona, Spain). After 20 min, the 10 ml was inoculated in 500 ml of TSB and incubated at 36 ºC for 24 h with continuous agitation at 200 rpm to obtain cells in a stationary growth stage. A stock of culture vials was obtained and kept frozen (–80 ºC) in Tryptic Soy Broth (TSB) (Scharlab Chemie, Barcelona, Spain) supplemented with 20 % glycerol.

Powdered Infant Formula Milk

The PIFM was rehydrated in accordance with the manufacturer's instructions. Sterile water (1000 ml) was mixed with 160 g of PIFM and the mixture was shaken at 1000 rpm. The mixture was allowed to stand at room temperature before inoculation.

Inoculation

Stocked culture vials were regenerated by transferring 50 µl into 10 ml of TSB and incubating at 36 ºC for 24 h to reach the stationary phase. The culture was centrifuged (4000 x g for 15 min) using a Sorvall RC-5B refrigerated superspeed centrifuge. The pellet was washed with 10 ml of buffered peptone water (1 % w/v). Appropiately diluted washed culture was then used to inoculate fresh flasks containing 1 l of reconstituted PIFM or 1 l of TSB to obtain a target inoculum of approximately 10^2 cfu/ml. The flasks were then stored under controlled isothermal conditions at 4, 8, 25 and 37 ºC. Triplicate samples of each storage temperature were taken at appropiate time intervals to allow for efficient kinetic analysis of microbial growth. For enumeration of *C. sakazakii,* 0.1 ml volumes of serial dilutions of RPIFM and TSB were plate counted on TSA in duplicate and incubated at 36 ºC for 24 h. A fourth sample was obtained for validation of growth models.

Each culture was maintained at constant conditions over the experimental period (no time-varying conditions). The number of data points was between 20 and 30, with variable frequency of sampling. Growth curves were obtained and repeated in triplicate.

Mathematical modelling

C. sakazakii growth data (log cfu/ml) in RPIFM and TSB incubated under four different isothermal conditions (4, 8, 25, 37 ºC) were fitted into the following models: Baranyi and Roberts growth model and modified Gompertz equation. We assumed that temperature T was the only varying environmental parameter influencing *C. sakazakii* growth in this study.

Primary models. Sigmoidal functions have been the most popular ones used to fit microbial growth data because these functions consist of four phases, similar to the microbial growth curve. The one most commonly used is the Gompertz equation (GMP) introduced by Gibson, Bratchell and Roberts (1988):

$$\log_{10} N = A + Ce^{-e^{-B(t-M)}} \quad (1)$$

where N is the number of cells at time *t,* A is the value of the upper asymptote; B is the relative death rate at M; C is the difference in value of the upper and lower asymptote; M is the time at which the absolute death rate is maximal; minus sign before C means the inactivation of microorganisms. The following kinetic parameters can be derived from equation (1) (McMeekin et al. 1993):

Journal of Food Safety and Food Quality 63, Heft 6 (2012), Seiten 165–196

maximum growth rate μ_{max} ($\log_{10}$((cfu/ml)/h))

$$\mu_{max} = \frac{BC}{e} \quad (2)$$

Lag phase duration (hours)

$$\lambda = M - \frac{1}{B} + \frac{\log_{10} N_0 - A}{\frac{BC}{e}} \quad (3)$$

Minimum cell concentration (the value of the lower asymptote), N_{min} (cfu/ml)

$$\log_{10} N_{min} = A - C = \log_{10} N_0 + Ce^{-e(BM)} - C \quad (4)$$

Baranyi and co-workers introduced in predictive microbiology a mechanistic model for bacterial growth (BAR). Baranyi and Roberts (1994) described bacterial growth by a pair of differential equations, which can be used in cases in which temperature changes with time. For isothermal conditions, however, there is an explicit solution which can be expressed as:

$$y(t) = yo + \mu A(t) - \frac{1}{m}\ln\left(1 + \frac{e^{m\mu A(t)} - 1}{e^{m(y_{max} - yo)}}\right) \quad (5)$$

where

$$A(t) = t + \frac{1}{v}\ln(e^{-vt} + e^{-ho} - e^{-vt-ho}) \quad (6)$$

y_o is the natural logarithm of the initial concentration, y_{max} is the natural logarithm of the cell concentration reached in stationary phase, μ is the maximum specific growth rate, v is a curvature parameter to characterize the transition to the exponential phase, and ho is the product of μ and the lag. The parameter m characterizes the curvature before the stationary phase. When $m = 1$ the function reduces to a logistic curve, a simplification of the model that is often assumed. The primary growth model of Baranyi and Roberts (1994) was fitted to the raw growth data by means of DMFit excel Add-In (J. Baranyi, Institute of Food Research, Norwich, UK). In the present research work, it has been used maximum growth rate μ_{max} (expressed in $\log_{10}$ (cfu/h)) throughout the paper, which is different from the maximum specific growth rate (μ), expressed in ln cfu/h.

Secondary model. The maximum growth rates estimated in isothermal conditions were modelled as a function of storage temperature using the square root model (Ratkowsky et al., 1982):

$$\sqrt{\mu_{max}} = b(T - T_{min}) \quad (7)$$

where b is a constant, T (ºC) the incubation temperature and T_{min} is the theoretical minimum temperature for growth of the organism, estimated by extrapolation of the regression line to $\mu_{max} = 0$.

Data analysis and model evaluation

The statistical analysis was performed by using Statgraphics (Statgraphics® Centurion XV, Statpoint Incorporated, USA) software. This analysis included the average and standard deviation calculations for the three repetitions and a variance analysis (ANOVA) to detect significant differences between substrates. The analysis also included assessing the goodness of fit of the models by using regression coefficient (adjusted-R^2) and root mean square error (RMSE). A fourth experimental data for each temperature was obtained in order to validated the obtained growth models. Accuracy and bias factors (Ross, 1999) were used to provide an indication of the average deviation between model predictions and observed results, being the closeness to a value of 1 is an effective and practical measure of predictive model validity. Similarly, it is possible to apply accuracy and bias factors using the parameter values to make a comparative assessment of the models' applicability at the safe stage.

Results and discussion

Representative growth curves of *C. sakazakii* in RPIFM and TSB were obtained (4, 8, 25 and 37 ºC) (Tab. 1) and fitted to the primary models of Baranyi and Roberts and Gompertz equation in order to estimate the kinetic parameters: maximum growth rate (μ_{max}), lag phase (λ) and maximum population density (N_f) (Tab. 2). At 4 ºC the concentration of *C. sakazakii* remained at the initial inoculum levels (1.8 x 10^2 cfu/ml) or decreased with time (data not shown), according to Lambert and Bidlas (2007). According to Iversen and Forsythe (2003) these findings confirm the importance of proper refrigerated storage temperatures after reconstitution of infant formula powders to validate that this organism does not growth. However, the temperature of many home refrigerators ranges from 7 to 10 ºC (FDA, 1992). These commonly found but potentially abusive temperatures would allow *C. sakazakii* to grow, with generation time value of 47 min in RPIFM at 25 ºC (room temperature) according to present results.

The results of our study show that *C. sakazakii* (858 CECT) is able to grow well in both studied substrates within the range from 8 ºC to 37 ºC. No significant differen-

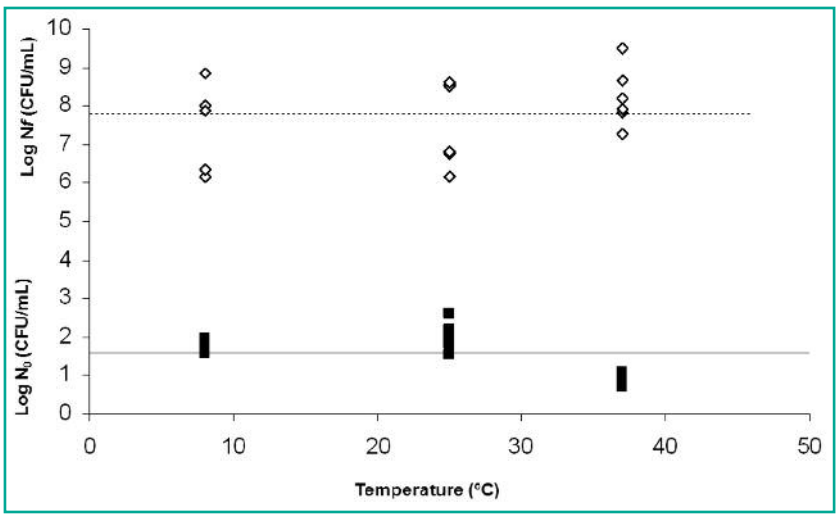

FIGURE 1: *Initial inoculum (■) and maximum population densities (◇) of strain CECT 858 incubated at three different temperatures: 8, 25 and 37 ºC. The dotted line represents the average value for the maximum population density data from 8 to 37 ºC. The solid line represents the average value for the initial inoculum data.*

ces ($p > 0.05$) were observed on the N_f, μ and λ values. The N_f parameter had an average value of 10^8 cfu/ml (Fig. 1). The initial inoculum level, which varied between ± 10^2 and 10^3 cfu/ml, did not seem to affect the maximum population density, N_f ($p > 0.05$). The temperature-independence of the maximum population density observed in the present study has also been reported previously (Álavi et a., 1999; Koutsoumanis, 2001).

The lag phase, defined by the λ parameter, is temperature-dependent in both substrates. The λ value decreases when the temperature increases because the time necessary to start growing exponentially is reduced when the temperature is increased by a supposed increase in the metabolic activity that simultaneously occurs. These results are supported by work done by Hector et al. (1988), who found a similar effect in fungi, with an increase in storage temperature producing a decrease in the length of the lag phase. In the present study the average value at 25 ºC was 3.242, similar to the *C. sakazakii* lag time of 2.760 in RPIFM reported by Nazarowec and Farber (1997) at 23 ºC.

To find the relationship between storage temperature and lag time a linear equation was initially applied. The correlation coefficients obtained ($R^2 < 0.844$) indicated that a linear relationship did not fit well. The relationship between experimental lag time and T (ºC) was described by using an exponential equation of λ. The results obtained for the two models were as follows:

GMP (IFM):

$$\lambda = 329.32e^{-0.1681T} \quad R^2 = 0.97 \qquad (8)$$

GMP (TSB):

$$\lambda = 611.76e^{-0.1974T} \quad R^2 = 0.99 \qquad (9)$$

BAR (IFM):

$$\lambda = 354.82e^{-0.187T} \quad R^2 = 0.97 \qquad (10)$$

BAR (TSB):

$$\lambda = 511.67e^{-0.197T} \quad R^2 = 0.97 \qquad (11)$$

As expected, maximum growth rate (μ_{max}) was temperature-dependent and increased when the temperature increased, in both substrates with 0.027 and 0.035 (h^{-1}) at 8 ºC, 0.384 and 0.473 (h^{-1}) at 25 ºC, and 0.924 and 0.909 (h^{-1}) at 37 ºC values, for RPIFM and TSB respectively (Gompertz model). Kandhai et al. (2006) reported μ_{max} values ranging from 0.115 h^{-1} at 10 ºC to 2.242 h^{-1} at 37 ºC for *C. sakazakii* ATCC 29544 in reconstituted powdered infant formula. These values are slightly higher than the

TABLE 1: *Cronobacter sakazakii experimental growth data (log_{10} and standard deviation) obtained at different incubation temperatures (8, 25, 37 ºC) in reconstituted powdered infant formula milk (RPIFM) and Tryptic Soy Broth (TSB).*

	RPIFM			TSB		
time	8 ºC	25 ºC	37 ºC	8 ºC	25 ºC	37 ºC
0	1.88±0.07	2.28±0.43	1.03±0.07	1.78±0.17	1.83±0.09	1.17±0.14
1			1.57±0.05			1.91±0.13
2		2.30±0.40	2.45±0.61		1.91±0.28	2.73±0.21
3			2.89±0.51			3.59±0.01
4		2.63±0.19	3.78±0.63		2.55±0.48	4.24±0.04
5			4.69±0.95			4.96±0.06
6		3.25±0.23	5.59±0.54		3.38±0.45	5.52±0.07
7			6.13±0.59			6.53±0.04
8		4.04±0.12	6.93±0.46	1.83±0.16	4.17±0.34	7.19±0.14
9			7.33±0.48			7.87±0.07
10		4.50±0.16	7.84±0.15		4.73±0.22	7.91±0.11
11			8.06±0.16			7.90±0.12
12	1.92±0.12	5.12±0.03	8.15±0.17		5.42±0.31	7.85±0.29
13			8.05±0.20			7.88±0.40
14		6.32±0.07	8.07±0.25		6.49±0.35	7.87±0.47
16		7.16±0.15			7.42±0.25	
18		7.34±0.16			7.62±0.31	
20		7.76±0.04			8.39±0.08	
22		7.86±0.02			8.68±0.03	
24	2.05±0.01	7.88±0.17		2.10±0.11	8.79±0.07	
26		7.95±0.19			8.75±0.12	
28		8.02±0.22			8.77±0.08	
30		8.10±0.24			8.75±0.12	
32		8.29±0.04		2.12±0.18	8.76±0.08	
34		8.33±0.01			8.82±0.10	
36	2.11±0.12	8.38±0.12			8.88±0.11	
38		8.44±0.01			8.80±0.20	
40		8.50±0.06			8.89±0.23	
42		8.68±0.10			8.82±0.15	
44		8.68±0.11			8.92±0.14	
46		8.70±0.12			8.80±0.22	
48	2.15±0.17	8.73±0.10		2.04±0.24		
50		8.75±0.09				
52		8.76±0.09				
54		8.77±0.14				
56	2.17±0.18	8.77±0.22		2.05±0.23		
58		8.77±0.22				
60						
72	2.22±0.67			1.94±0.52		
84	2.19±0.05			2.19±0.22		
96	2.39±0.07			2.39±0.15		
108	2.42±0.01			2.42±0.19		
120	2.43±0.48			2.43±0.32		
132	2.89±0.21			2.89±0.41		
144	3.05±0.15			2.25±0.23		
168	3.47±0.16			3.10±0.65		
216	4.56±0.86			3.52±0.94		
264	6.14±0.61			4.29±0.77		
360	7.61±0.03			7.45±0.14		
408	7.73±0.07			7.80±0.45		
456	7.93±0.20			7.94±0.42		
504	8.17±0.22			8.19±0.36		
552	8.13±0.20			7.81±0.20		
600	8.41±0.04			8.17±0.41		

Journal of Food Safety and Food Quality 63, Heft 6 (2012), Seiten 165–196

TABLE 2: *The Baranyi and Gompertz biological parameters for Cronobacter sakazakii growth curves at 8, 25 and 37 ºC.*

	Baranyi				Gompertz			
	[c]Log N_0	[d]Log N_f	[e]μ ((cfu/ml)/h)	[f]λ (h)	Log N_0	Log N_f	μ ((cfu/ml)/h)	λ (h)
[a]RPIFM								
8 ºC	2.007±0.06	8.352±0.58	0.025±0.01	101.971±8.72	2.061±0.21	8.355±0.94	0.027±0.01	101.89±4.81
25 ºC	1.740±0.31	8.550±0.03	0.303±0.04	1.929±0.23	2.112±0.41	8.662±0.37	0.384±0.03	3.242±0.61
37 ºC	1.027±0.11	8.111±0.37	0.799±0.01	0.478±0.78	0.957±0.15	8.570±1.16	0.924±0.05	0.834±0.56
[b]TSB								
8 ºC	2.017±0.10	8.180±0.487	0.029±0.01	132.054±20.15	2.055±0.08	8.079±0.50	0.035±0.01	139.600±16.54
25 ºC	1.700±0.08	8.822±0.010	0.400±0.05	2.181±0.08	1.844±0.06	8.944±0.12	0.473±0.07	3.435±1.76
37 ºC	1.144±0.04	8.017±0.152	0.780±0.007	0.479±0.05	1.113±0.13	8.490±1.32	0.909±0.04	0.475±0.14

[a]RPIFM: reconstituted powdered infant formula milk; [b]TSB: Tryptic soy broth; [c]log N_0: $\log_{10}$ of initial number of microorganisms; [d]log Nf: $\log_{10}$ of final number of microorganisms; [e]μ ((cfu/ml)/h): maximum growth rate; [f]λ (h): lag time

values obtained in the present study for *C. sakazakii* (858 CECT).

The maximum growth rate was further modelled as a function of temperature using the square root model. The parameters of the model and the correlation coefficients were as follows:

GMP (IFM):

$$\sqrt{\mu_{máx}} = 0.027 * T - 0.059 \quad R^2 = 0.999 \qquad (12)$$

GMP (TSB):

$$\sqrt{\mu_{máx}} = 0.026 * T - 0.011 \quad R^2 = 0.994 \qquad (13)$$

BAR (IFM):

$$\sqrt{\mu_{máx}} = 0.025 * T - 0.052 \quad R^2 = 0.996 \qquad (14)$$

BAR (TSB):

$$\sqrt{\mu_{máx}} = 0.024 * T - 0.013 \quad R^2 = 0.995 \qquad (15)$$

The model satisfactorily described the effect of temperature on growth of the pathogen with R^2 values in the range [0.994–999]. The experimental minimum temperature value obtained for *C. sakazakii* (858 CECT) was 4 ºC according to our results. However the prediction by square root model provides a minimum temperature value of 2.15 ºC for GMP model and RPIFM substrate. A similar value (2.19 ºC) was obtained by Kandhai et al (2006) using the expanded square root model of Ratkowsky.

The correlation coefficient (adjusted-R^2), RMSE, accuracy factor (A*f*) and bias factor (B*f*) were calculated for the primary models (Tab. 3). The high adjusted-R^2 correlation coefficients, low RMSE values and A*f* and B*f* values close to 1 show that both models, Baranyi and Gompertz, have a reasonably good fit for *C. sakazakii* growth at the temperatures studied. However, the Gompertz model fits the data obtained for *C. sakazakii* in RPIFM better than the Baranyi model, with adjusted-R^2 values ranging from 0.992 to 0.998, RMSE values from 0.018 to 0.072, A*f* values from 1.023 to 1.054 and B*f* values ranging from 1.000 to 1.009. Consequently, for *C. sakazakii* growth data, it might be more suitable to use the Gompertz equation for further study and future risk assessment processes according to goodness of fit. The results are in agreement with previous studies of growth and survival of other important microorganisms in milk, including *C. sakazakii* ATCC 29544 (Baranyi and Roberts, 1995; Delignette-Muller et al., 2005; Nazarowec-White and Farber, 1997).

A second means of comparing predicted and observed values is a graphical method that plots predicted μ_{max} values versus observed ones in RPIFM substrate (Ross and McMeekin, 1994; Sutherland, Aherne and Beaumont, 1996; Witjes et al., 1993). Ideally, the points should lie along the line of equivalence (LOE), μ_{max} predicted value should equal the μ_{max} observed value. Figure 2 illustrates the LOE plot. In the case of points above the line, the observed μ_{max} values are lower than the predicted and so the prediction can be regarded as "safe" (fail-safe). The observed versus predicted μ_{max} value for the Gompertz equation at 8 ºC fell on the LOE value. The same is illustrated for the Baranyi model at the same temperature. The data corresponding to 25 and 37 ºC for the Gompertz equation were above the LOE, indicating that these observed values were smaller than all the predicted ones; thus, the predicted GMP provided a higher margin of safety than the BAR model, in which the point at 25 ºC fell on the LOE value. From the safety viewpoint, the Gompertz model provides more suitable μ_{max} values to avoid hazardous practices. As indicated above, the Baranyi and Gompertz models differ slightly in the fit of the growth curves at the various temperatures studied.

The relationships obtained for μ_{max} (log (cfu/ml)/h (eq. 8) and λ (h) (eq. 12) versus Temperature (T (ºC)), were introduced in the corresponding equations (eq. 2 and 3) and integrated into the modified Gompertz model (eq. 1). An additional set of experimental data (log(N(t)) were fitted to the global model by means of a non-linear regression (Statgraphics

TABLE 3: *Goodness of fit of Baranyi and Gompertz models for the growth curves of C. sakazakii in TSB and RPIFM.*

	Baranyi				Gompertz			
	adjusted-R^2	[c]RMSE	[d]A*f*	[e]B*f*	adjusted-R^2	RMSE	A*f*	B*f*
[a]RPIFM								
8 ºC	0.997	0.095	1.023	1.012	0.998	0.026	1.027	1.008
25 ºC	0.979	0.056	1.049	0.998	0.992	0.018	1.023	1.000
37 ºC	0.998	0.106	1.054	1.005	0.997	0.072	1.054	1.009
[b]TSB								
8 ºC	0.991	0.068	1.038	0.999	0.995	0.038	1.034	1.008
25 ºC	0.998	0.080	1.126	0.875	0.996	0.023	1.030	1.004
37 ºC	0.996	0.050	1.035	1.007	0.988	0.074	1.039	1.004

[a]RPIFM: Reconstituted Powder Infant Formula Milk; [b]TSB: Tryptic Soy Broth; [c]RMSE: Root Mean Square Error; [d]Af: Accuracy factor; [e]Bf. Bias factor

Centurion XV). The experimental data were compared with those predicted by the model, and the goodness of fit was calculated by means of accuracy factor (A*f*) and bias factor (B*f*), indicating an error within 8.5 to 12 % in the predictions. Figure 3 shows the response surface for the global model of *C. sakazakii* growth in the range of temperature within 8 to 37 ºC.

The parameter *h*o, "adaptation work", related to the physiological state of the cells, was calculated as the product μ_{max} x λ based on the values of μ_{max} and λ observed under the isothermal conditions tested for the Gompertz equation. ho values ranged [4.90–3.04] at 8 ºC; [1.62–1.24] at 25 ºC; and [0.43–0.52] at 37 ºC, for TSB and RPIFM respectively. The physiological state parameter (α*0*) [α*0* = exp(–*h*o)] for the Gompertz model showed that at 25 ºC the cells were growing at 20 and 28 % of the potential maximum growth rate (Baranyi and Roberts, 1995), in TSB and RPIFM respectively, meanwhile at 37 ºC, α*0* was 60 % of the potential maximum growth rate of *C. sakazakii* for RPIFM, and 65 % for TSB. These results are in contrast with several studies that have suggested that the *h*o parameter and its transformation α*0* should be constant at different storage temperatures when the pre-inoculation history of the culture is identical (Pin et al., 2002). However, other studies have reported a significant effect of temperature on the *h*o parameter (Alavi et al., 1999; Delignette-Muller et al., 2005; Mellefont and Ross, 2003; and Xanthiakos et al., 2006).

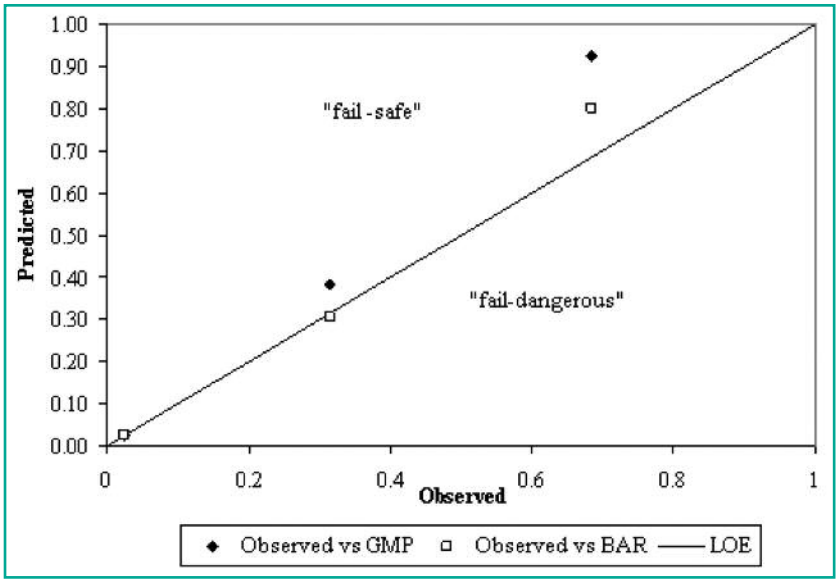

FIGURE 2: *Graphical comparison of observed versus predicted μ_{max} values for growth of C. sakazakii strain 858 CECT in RPIFM using the Line of Equivalence method (LOE).*

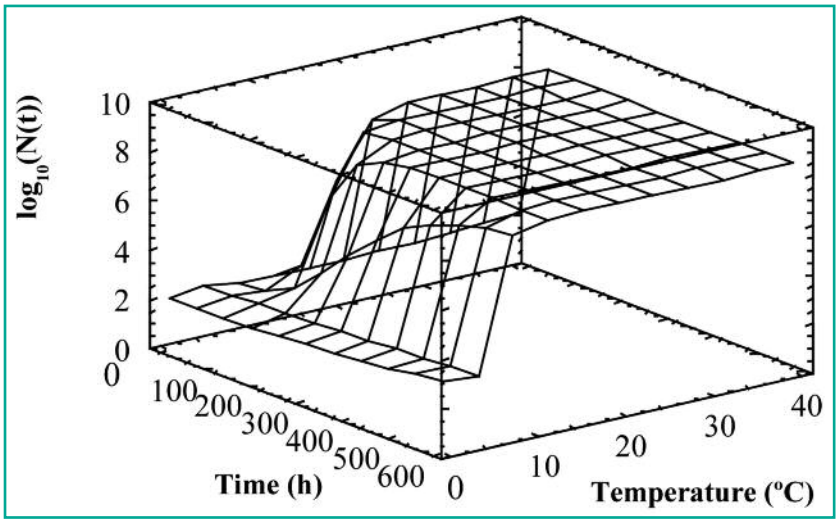

FIGURE 3: *Response surface plot of global Gompertz model to describe the C. sakazakii growth in RPIFM at temperature range within 8 to 37 ºC.*

Conclusions

Present research works points out the high temperature dependence of *Cronobacter sakazakii* μ_{max} and λ growth parameters, supporting the recommendations of FAO/WHO related to time-temperature of RPIFM storage, previous to consumption The study reveals the suitability of Gompertz fit to describe *C. sakazakii* kinetic growth behaviour. This study contributes to get knowledge about the mathematical quantification of this microorganism proliferation, a relevant aspect taking into account some households practices related to infant formula milk handling.

Acknowledgements

M. C. Pina is grateful to the Conselleria de Empresa, Universidad y Ciencia for providing I+D+I special action referred AE/2007/151. D. This study was carried out with funds from CICYT project no. AGL2010-22206-C02-01.

References

Alavi SH, Puri VM, Knabel SJ, Mohtar RH, Whiting RC (1999): Development and validation of a dynamic growth model for *Listeria monocytogenes* in fluid whole milk. J. Food Protect. 62(2), 170–176.

Arroyo C, Cebrián G, Pagán R, Condon S (2010): Resistance of *Enterobacter sakazakii* to pulsed electric fields. Innovat Food Sci Emerg Tech 11, 314–321.

Baranyi J, Roberts TA (1994): A dynamic approach to predicting bacterial growth in food. Int. J. Food Microbiol. 23, 277–294.

Baranyi J, Roberts TA (1995): Mathematics of predictive food microbiology. Int J. Food Microbiol, 26, 199–218.

Beuchat LR, Kim H, Gurtler JB, Lin L-C, Ryu J-H, Richards GM (2009): *Cronobacter skazakii* in foods and factors affecting its survival, growth, and inactivation. Int J. Food Microbiol, 136: 204–213.

Delignette-Muller ML, Baty F, Cornu M, Bergis H (2005): Modelling the effect of a temperature shift on the lag phase duration of *Listeria monocytogenes.* Int J. Food Microbiol, 100, 77–84.

Journal of Food Safety and Food Quality 63, Heft 6 (2012), Seiten 165–196

Edelson-Mammel S, Porteous MK, Buchanan RL (2006): Acid resistance of twelve strains of *Enterobacter sakazakii,* and the impact of habituating the cells to an acidic environment. J. Food Sci. 71: M201–M207.

FAO/WHO. (2006): *Enterobacter sakazakii* and *Salmonella* in powdered infant formula: meeting report, Microbiological Risk Assessment Series 10.

FDA (1992): L Bacteriological analytical manual, 7th ed AOAC International, Arlington, VA, USA.

Fernández A, Collado J, Cunha LM, Ocio MJ, Martínez A. (2002): Empirical model building base don Weibull distribution to describe the joint effect of pH and temperature on thermal resistance of *Bacillus cereus* in vegetable substrate. Int J. Food Microbiol, 77: 147–153.

Gibson AM, Bratchell N, Roberts TA (1988): Predicting microbial growth responses of salmonellae in laboratory medium as affected by pH, sodium chloride and storage temperature. Int J Food Microbiol. 6, 155–178.

Hector HL, Gonzalea SL, Vacmondo R (1988): Influence of temperature on growth rate and lag phase of fungi isolated from Argentina Corn. Int. J. Food Microbiol 6, 179–183.

Iversen C, Forsythe S (2003): Risk profile of *Enterobacter sakazakii,* an emergent pathogen associated with infant milk formula. Trends Food Sci. Technol. 14, 443–454.

Iversen C, Lehner A, Mullane N, Bidlas E, Cleenwerck I, Marugg J, Fanning S, Stephan R, Joosten H (2008): The taxonomy of *Enterobacter sakazakii:* proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. Nov. *Cronobacter sakazakii* subsp. *sakazakii,* comb nov. *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. Nov., *Cronobacter muytjensii* sp. Nov., *Cronobacter dublinensis* sp. Nov. and *Cronobacter genomospecies* I. BMC Evolutionary Biology, 7: 64.

Kandhai MC, Reij MW, Grognou C, van Shothorst M, Gorris LGM, Zwietering MH (2006): Effects of Preculturing conditions on Lag Time and Specific Growth Rate of *Enterobacter sakazakii* in Reconstituted Powdered Infant Formula. Applied Environ Microbiol 72(4), 2721–2729.

Koseki S, Matsubara M, Yamamoto K (2009): Prediction of a required Log reduction with probability for *Enterobacter sakazakii* during High-Pressure Processing using survival/death interface model. Applied Environ Microbiol 1885–1891.

Koutsoumanis KP (2001): Predictive modelling of the shelf life of fish under non-isothermal conditions. Applied Environ Microbiol 67, 1821–1829.

Lambert JW, Bidlas E (2007): A study of the gamma hypothesis: predictive modelling of the growth and inhibition of the *Enterobacter sakazakii.* Int Journal Food Microbiol, 115: 204–213.

Lenati RF, O'Connor DL, Hébert KC, Farber JM, Pagotto FJ (2008): Growth and survival of *Enterobacter sakazakii* in human breast milk with and without fortifiers as compared to powdered infant formula. Int J Food Microbiol 122: 171–179.

McMeekin TA, Olley JN, Ross T, Ratkowsky DA (1993): Predictive Microbiology: Theory and Application. Wiley, New York.

Mellefont L, Ross T (2003): The effect of abrupt shifts in temperature on the lag phase duration of *Escherichia coli* and *Klebsiella oxytoca.* International Journal of Food Microbiology 83, 295–305.

Muytjens HL, Cole LA (1990): *Enterobacter sakazakii* meningitis in neonates: causative role of formula? Pediatric Infectious Diseases J 9, 372–373.

Nazarowec-White M, Farber JM (1997): *Enterobacter sakazakii:* a review. Int J Food Microbiol 15, 207–214.

NZFSA. (2010): *Cronobacter* spp. (Formerly *Enterobacter sakazakii*). ESR Ltd.

Pin C, García de Fernando GD, Ordóñez JAA, Baranyi J (2002): Analysing the lag-growth rate relationship of *Yersinia enterocolitica.* Int J. Food Microbiol. 73, 197–201.

Pina-Pérez MC, Rodrigo D, Ferrer C, Rodrigo M, Martínez A (2007a): Inactivation of *Enterobacter sakazakii* by Pulsed Electric fields in buffered peptone wáter and infant formula milk. Int Dairy J 17, 1441–1449.

Pina-Pérez MC, Rodrigo D, Saucedo D, Martínez A (2007b): Pressure Inactivation kinetics of *Enterobacter sakazakii* in Infant Formula Milk. J Food Protect 70(10), 2281–2289.

Ratkowsky DA, Olley J, McMeekin TA, Ball A (1982): Relationship between temperature and growth rate of bacterial cultures. J Bacteriol 149(1), 1–5.

Reij MW, Jongenburger I, Gkogka E, Gorris LGM, Zwietering MH (2009): Perspective on the risk to infants in the Netherlands associated with *Cronobacter* spp. occurring in powdered infant formula. Int J Food Microbiol 136, 232–237.

Ross T, McMeekin TA (1994): Predictive Microbiology. Int J Food Microbiol, 23, 241–264.

Ross T (1999): Predictive Microbiology for the Meat Industry. Meat and Livestock Australia, North Sydney. 196 pp.

Rosset P, Noel V, Morelli E (2007): Time-temperature profiles of infant milk formula in hospitals and analysis of *Enterobacter sakazakii* growth. Food Control 18 (11) 1331–1476.

Skovgaard N. (2007): New trends in emerging pathogens. International Journal of Food Microbiology 120: 217–224.

Sutherland JP, Aherne A, Beaumont AL (1996): Preparation and validation of a growth model for *Bacillus cereus:* the effects of temperature, pH, sodium chloride and carbon dioxide. Int J Food Microbiol 30: 359–372.

Whiting RC, Buchanan RLB (1997): Predictive modelling. In: Doyle MP, Beuchat LR, Montville TJ (Eds.), Food Microbiology Fundamentals and Frontiers. American Society for Microbiology Press, Washington, DC, pp. 728–739.

Wijtzes T, McClure PJ, Zwietering MH, Roberts TA (1993): Modelling bacterial growth of *Listeria monocytogenes* as a function of water activity, pH and temperature. Int J Food Microbiol 18, 139–149.

Xanthiakos K, Simos D, Angelidis AS, Nychas GJE, Koutsoumanis K (2006): Dynamic modelling of *Listeria monocytogenes* growth in pasteurized milk. J Applied Microbiol 100, 1289–1298.

Address of corresponding author:
Dr. Antonio Martínez
Department of food quality and preservation
IATA-CSIC
Avda Agustin Escardino, 7
46980 Paterna, Valencia
Spain
amartinez@iata.csic.es