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Modelling the exposure to *Cronobacter sakazakii* by consumption of a cocoa-milk-based beverage processed by pulsed electric fields

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Infants' exposure (N*f*) to *Cronobacter sakazakii* via the consumption of infant-rich-in-polyphenols cocoa-milk-based beverages (CCX-M) treated with high-intensity pulsed electric fields (PEF) was evaluated. Monte Carlo simulation enabled the prediction of the variability in *C. sakazakii* load in beverages at the time of consumption to be estimated. Different scenarios (initial contamination levels; PEF treatment conditions; and time-temperature combinations of CCX-M beverages storage after treatment) were simulated. Cocoa addition and PEF treatment resulted in the most influential input factors to control bacterial final load. *Cronobacter* spp. exposure risk was reduced by a maximum of 100 times at 95% of iterations due to addition of cocoa at 5 g/100 mL, corresponding to scenario 3 (PEF: 15 kV/cm–3,000 µs; storage 120 h at 8 °C). Moreover, the probability of illness for a healthy population was reduced from 2.15 × 10^{-8}, in the baseline scenario, to 4.78 × 10^{-10} due to cocoa addition and application of 15 kV/cm–3,000 µs PEF treatment.

Keywords: Cronobacter sakazakii; exposure assessment; infant beverages rich-in-polyphenols cocoa; pulsed electric field (PEF)

Introduction

Infant liquid milk-based beverages frequently come from powdered infant formula milk (PIFM) which can be considered as risk products due to potential contamination by *Cronobacter sakazakii* (Lai 2001; Bowen and Braden 2006; FAO/WHO 2008). According to the preliminary

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assessment of the risks associated with *C. sakazakii* in PIFM conducted by FAO/WHO (2006), additional control measures after reconstitution of PIFM, such as the addition of an inhibitor or biopreservative agent to reconstituted PIFM (RPIFM), or the use of bactericidal/pasteurisation additional processes are required to obtain microbiologically safe RPIFM. Alternative treatments to those that use heat aim to ensure not only the microbiological safety of RPIFM but also the nutritional quality of this product, which could be affected by elevated temperatures (Barbosa-Cánovas and Bermudez-Aguirre 2010). Recent studies have been conducted on the possible impact of new non-thermal technologies on *C. sakazakii* inactivation. These include high hydrostatic pressure (HHP) (González *et al*. 2006; Pina-Pérez *et al*. 2007a; Arroyo *et al*. 2011a), pulsed electric field technology (PEF) (Pina-Pérez *et al*. 2007b; Arroyo *et al.* 2010a,b), ultrasound (Adekunte *et al*. 2010; Arroyo *et al*. 2011b) and natural antimicrobials (Nair, Joy and Venkitanarayanan 2004; Jang and Rhee 2008; Amalaradjou, Hoagland and Venkitanarayanan 2009). PEF technology is suggested as a possible non-thermal alternative process to control this microorganism in industrial and hospital settings (Pina-Pérez *et al*. 2007b, 2009a; Arroyo *et al*. 2010a,b). In recent years, the use of substances of a functional nature that act as ingredients in food formulation and, in turn, may also prevent the development of hazardous microorganisms (Marco *et al*. 2011) is gaining relevance. The hurdle technology concept, based on the successive or simultaneous application of barriers to microbial growth, is being used, increasing the intensity of physical preservation processes. In this respect, the availability and recent interest in certain minimally processed cocoa derivatives/concentrates and flavonoid-rich extracts, are encouraging the development of a variety of products with functional and antimicrobial properties. Polyphenols have been described to have significant antimicrobial effects against Gram positive and Gram negative bacteria (Busta and Peck 1968; Pina-Pérez *et al*. 2009b; Pina-Pérez, Rodrigo and Martinez 2011). Recently, Kim *et al*. (2009) obtained a reduction in *C. sakazakii* to undetectable levels due to muscadine seed extracts cell exposure (30–60 min). Pina-Pérez, Martínez López and Rodrigo (2013) in a previous study determined that 5 g/100 mL of cocoa added to milk formula, at a specified step in the refrigeration period (post-treatment), reduced *C. sakazakii,* achieving the maximum inactivation level obtained up to date due to PEF application.

To produce novel tasty and safe products for children, exposure risk assessment studies should be carried out at various stages from production to consumption, with a view to reducing the number of microorganisms at particular steps in the production chain. For quantitative risk assessment, a stochastic approach using frequency or probability distributions for model inputs is recommended, to take account of uncertainties and variabilities (Lammerding and Fazil 2000; Pouillot and Delignette-Muller 2010).

The overall objective of this study was to assess the exposure of children consuming infant rich-in-polyphenols-cocoa milk based (CCX-M) beverages to *C. sakazakii.* Inactivation results from PEF application under different conditions followed by the storage of treated beverages were mathematically simulated to estimate the final number of microorganisms at the time of consumption. The specific objectives of the present study were to estimate the exposure values at the time of consumption by healthy and immunodepressed children to generate data that could be used in future *C. sakazakii* risk assessments associated with novel products processed by hurdle technologies. By means of the stochastic methodology of analysis, the present work considered the research needs in the field of the validation of the effectiveness of novel technologies.

Materials and Methods

Bacterial strain and growth medium

The Spanish Type Culture Collection (CECT 858) provided the pure culture of *C. sakazakii* (equivalent to strain 29544 ATCC) in a lyophilised state. Obtaining stock culture was carried out according to Pina-Pérez *et al*. (2007a, 2009a). Prior to use, cells in the stationary phase were stocked and stored at −80 °C. The final concentration in the stocked vials was 10^9 Colony Forming Units (cfu) per mL.

Pulsed Electric Field equipment

An OSU-4D bench-scale continuous PEF system (Columbus, OH 43210-1007, USA) was used to inactivate *Cronobacter* spp. suspended in different CCX-M beverages. The bath temperature was set at 4 °C. The pulse, voltage and intensity of treatment were recorded by a digital oscilloscope (Tektronic TDS 210, Tektronic, OR). The flow was set at 30 mL/min using a gear pump (Cole-Parmer 75210-25, Cole – Instruments Parmer, IL). The square-wave bipolar pulse duration was 2.5 μs. The treatment times ranged between 60 and 3,000 μs, and the electric field intensity was 15, 25, and 35 kV/cm (see Table 1). The final temperature remained below 25±3 °C for all treatment conditions.

Beverage preparation and inoculation

Beverages were prepared as a mixture of CCX (12% minimum polyphenols concentration) added to RPIFM (10 g PIFM/100 mL) (Nutriben Natal®, Alter Farmacia, SA) at 1 g/100 mL (CCX-M-1%) at 2.5 g/100 mL (CCX-M-2.5%), and 5 g/100 mL (CCX-M-5%). A control beverage (10 g PIFM/100 mL) was processed without cocoa addition (CCX-M-0%) and was prepared according to the manufacturer' instructions. Beverages were inoculated to a final concentration of 10^8 cfu per mL.

The beverages were treated under the same PEF conditions, electric field-strength (E, kV/cm) – treatment time (t, μs) combinations. After PEF treatment, CCX-M beverages were kept under different storage conditions: 8 °C and 25 °C (stirring conditions at 250 rpm) with a total storage time from 24 h to 120 h.

Table 1. Pulsed electric field treatment conditions [E, electric field strength (kV/cm); t, treatment time, (μs)] applied to *Cronobacter sakazakii* control in cocoa-milk based beverages (CCX-M beverages)

E (kV/cm)	t (μs)
15	60
	240
	500
	700
	1,660
	3,000
25	60
	180
	240
	500
	860
	1,660
35	60
	240
	360
	420
	500
	700

Viable counts

Control and PEF treated samples were collected and serially diluted in 0.1% sterile peptone water, plated on TSA agar, and incubated at 37 °C 24 h. The experiments were performed by triplicate.

Exposure assessment model

The potential exposure to *C. sakazakii* by consumption of a single serving (100 mL) of CCX-M infant beverages was estimated. For this, an exposure assessment model was built to describe the distribution of the final *C. sakazakii* load probabilities after different PEF inactivation treatments and storage conditions, covering the process pathway from PEF processing of CCX-M infant beverage to consumption (Figure 1).

A summary of the input variable distributions, models, and data sources used to simulate the *C. sakazakii* final load in different CCX-M beverages is presented in Table 2. Inputs and assumptions of the global exposure model are defined below:

(1) It is assumed a product serving size was 10 g PIFM per 100 mL of novel CCX-M infant beverage.

(2) The initial concentration (N_0) of *Cronobacter sakazakii* in beverages was based on data of contamination levels in PIFM reported by FAO/WHO (2008) and estimated from a thousand samples. It was defined by a cumulative distribution function as follows: cumulative (–5.24;-2.79; {–3.30;–3.70;–4.20; –4.70;–5.24}; {0.21;0.43;0.65;0.91; 0.99}) log(cfu/g).

(3) PEF inactivation and growth during storage were fitted to mathematical models.

(4) To predict the microbial reduction level due to each PEF treatment, the Weibull model was fitted to the experimental data according to the following equation:

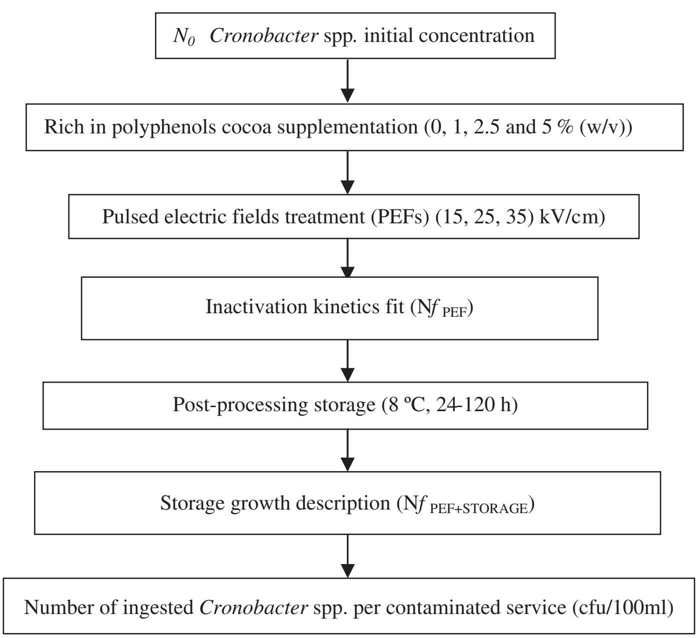

Figure 1. Framework of the Cronobacter sakazakii *exposure assessment process by consumption of PEF treated and stored rich-in-polyphenols-cocoa milk based infant beverages (CCX-M).*

Table 2. Overview of the *Cronobacter sakazakii* variables and distributions used in the exposure assessment model for PEF treated and stored infant rich-in-polyphenols-cocoa (0, 1, 2.5 and 5% [w/v]) milk based beverages (CCX-M) consumption

Variable	Description	Treatment conditions		Distribution	Source of hypothesis
N_0	Initial contamination level, log (cfu/g)	–		Cumulative (–5.24;–2.79; {–3.30;–3.70;–4.20;–4.70;–5.24}; {0.21;0.43;0.65;0.91;0.99})	FAO/WHO 2008
N_{PEF}	Final load after PEFs, cfu per serving before storage	–		Log N_{PEF}=(-) t·b^n	Pina *et al*. 2007b; Arroyo *et al*. 2010a
b	Scale parameter	CCX-M 0%	15 kV/cm	BetaGeneral(0.22;0.21;0.09;0.012)	Experimental data
			35 kV/cm	BetaGeneral(0.22;0.22;0.025;0.029)	
		CCX-M 1%	15 kV/cm	BetaGeneral(0.22;0.22;0.012;0.014)	Experimental data
			35 kV/cm	BetaGeneral(0.22;0.23;0.038;0.04)	
		CCX-M 2.5%	15 kV/cm	BetaGeneral(0.22;0.20;0.014;0.016)	Experimental data
			35 kV/cm	BetaGeneral(0.22;0.21;0.04;0.043)	
		CCX-M 5%	15 kV/cm	BetaGeneral(0.21;0.22;0.018;0.021)	Experimental data
			35 kV/cm	BetaGeneral(0.22;0.21;0.048;0.053)	
n	Shape parameter	–		Fixed value 0.561	Experimental data
μ_{max}	Maximum growth rate, log [(cfu/mL)/h]	CCX-M 0%	8 °C	Logistic(0.025;0.001)	Experimental data
			25 °C	BetaGeneral(0.19;0.22;0.38;0.39)	
		CCX-M 1%	8 °C	Logistic(0.025;0.0009)	Experimental data
			25 °C	BetaGeneral(0.21;0.21;0.37;0.37)	
		CCX-M 2.5%	8 °C	Logistic(0.021;0.0008)	Experimental data
			25 °C	BetaGeneral(0.22;0.21;0.37;0.37)	
		CCX-M 5%	8 °C	Logistic(0.013;0.001)	Experimental data
			25 °C	BetaGeneral(0.18;0.14;0.34;0.35)	
$t_{storage}$	Time of refrigerated storage	–		Triangular	HHS-FDA 2014
$Nf_{PEF+STORAGE}$	Final load after $_{PEF+STORAGE}$, cfu per serving	–		Log Nf_{PEF} + μ_{max} $t_{storage}$	Carrasco *et al*. 2010
S	Serving size per feeding (g)			Fixed 10 g/100 mL	Manufacturer instructions
V	Volume of CCX-M beverage (mL)			Fixed 100 mL	INE 2011

(Table 2. Continued)

Variable	Description	Treatment conditions	Distribution	Source of hypothesis
D	Number of bacteria ingested per contaminated serving (cfu/serving/infant)		FinalConc*serving size (Nf*S)	Experimental data
r_1	Dose-response parameter non-immunosuppressed		Fixed value (7×10^{-4})	Havelaar and Zwietering 2004
r_2	Dose-response parameter immunosuppressed		Fixed value (1.2×10^{-10})	Reij *et al.* 2009
P_i (i =1, 2)	Probability of illness by eating contaminated product		$P_{inf}(D;r)=1\text{-}e^{-rD}$	Teunis and Havelaar 2000

$$Log\left[\frac{Nf_{PEF}}{N_0}\right] = -\left(\frac{t}{b}\right)^n, \quad \text{(Equation 1)}$$

where log $[Nf_{PEF}/N_0]$ versus time t is the decimal logarithm of S; where S is the fraction of survivors $[Nf_{PEF}/N_0]$ at treatment time (t), *b* and *n* are the scale and shape factors, respectively. To ascertain the goodness of fit provided by the model, the following coefficients were used: corrected regression coefficient (R^2-corrected) and the root mean square error (RMSE) (Statgraphics Centurion XV, version 15.1.03, Statpoint Inc., 2005, USA).

(5) After treatment, cells were stored at different temperatures (8 and 25 °C) and the microbial increase during the storage period was measured. According to Carrasco *et al*. (2010), the equation of exponential growth (equation 2) was used to calculate the concentration of cells during the storage period. The final load after PEF application was used as the dynamic initial load in the storage stage mathematical simulation.

$$LogNf_{PEF+STORAGE} = LogNf_{PEF} + \mu_{\max} \cdot t, \quad \text{(Equation 2)}$$

where $Nf_{PEF+STORAGE}$ is the concentration of cells (cfu/mL) at the end of the stage considered, Nf_{PEF} is the concentration of cells (cfu/mL) at the beginning of the storage stage, μ_{max} is the maximum growth rate [log_{10} (cfu/mL)/h] and, t is the duration of the storage stage (h). Maximum specific growth rate [μ_{max}, log_{10} (cfu/mL)/h] distribution functions of obtained values at 8 and 25 °C are presented in Table 2.

(6) The time storage period (t_i) studied ranged from 24 h to 120 h (up to 5 days).

The number of *C. sakazakii* counts after PEF (Nf_{PEF}, cfu/mL) and after the complete process ($Nf_{PEF+STORAGE}$, cfu/mL) were the outputs of the exposure model.

(7) *Risk characterisation*. The probability of illness by consumption of contaminated CCX-M infant beverages was also obtained as an output of the exposure dose-response model proposed by Teunis and Havelaar (2000). Two populations were considered: immunodepressed and non-immunodepressed populations.

Figure 2 shows the flow diagram of the stochastic model. The model was constructed using the software Risk Analysis add-in for Microsoft Excel, @ Risk 4.5 (Palisade Corporation, Newfield, NY, USA). Monte Carlo simulation was run (100,000 iterations) by random sampling in distributions describing the variability of the input variables, using the Latin Hypercube sampling method.

"What if" scenarios

In the present study, the baseline model for each beverage was considered as the refrigerated storage of contaminated CCX-M beverages, at conditions of 8 °C – 24 h (FAO/WHO 2006), without previous PEF treatment. Different models called "What if" scenarios were run based on the modification of different initial conditions (initial load [N_0], PEF treatment, storage temperature, storage time). These scenarios represent the individual effect of each factor.

Five scenarios were considered as follows, for each beverage, in order to cover different possibilities regarding CCX-M consumption at home, with the aim of quantifying control measures that impact on CCX-M food safety:

a) Scenario 1: What if "CCX-M beverages were treated by the most effective PEF treatment according to Pina-Pérez *et al*. (2009a), 15 kV/cm–3,000 µs, before refrigerated storage 8 °C, 24 h?"

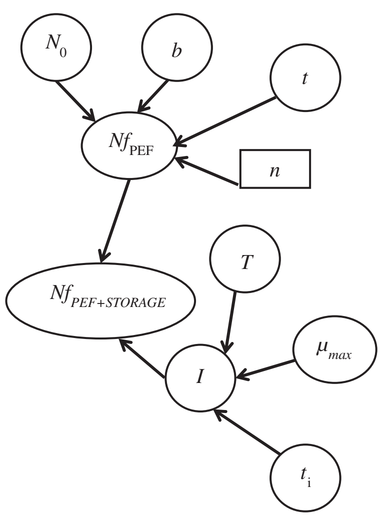

Figure 2. Flow diagram of the stochastic model used to evaluate the Cronobacter sakazakii *contamination of infant milk beverages not supplemented (CCX-M-0%)/supplemented with rich-in-polyphenols-cocoa at different concentration (1, 2.5, and 5% [w/v]) after PEF and storage. Circles are used for random variables and squares indicate fixed parameters.*

b) Scenario 2: What if "treatment intensity was increased from 15 kV/cm–3,000 µs to 35 kV/cm–700 µs?" Taking into account that the effectiveness of PEF depends on electric field strength and treatment time (Pina-Pérez *et al*. 2007b, 2009a; Arroyo *et al*. 2010a,b), the distribution function that defines the level of *C. sakazakii* reduction due to PEF treatment was estimated by Monte Carlo simulation based on an inactivation model.

c) Scenario 3: What if "the storage time of PEF treated (15 kV/cm–3,000 µs) beverages was prolonged from 24 h to 120 h at 8 °C?"

d) Scenario 4: What if "storage temperature of PEF treated (15 kV/cm–3,000 µs) CCX-M beverages was increased from 8 °C (maximum limit specified by CAC 2008) to 25 °C?" It was assumed that temperature follows a Normal distribution (25±2.5 °C).

e) Scenario 5: What if "the initial contamination level (N_0) exceeded considerably the likely FAO/WHO (2008 levels)? from 10^{-3} cfu/g to 10 cfu/g defined by a lognormal distribution [1 log cfu/g; 0.1].

Results and Discussion

Inactivation kinetics results

The levels of inactivation were influenced both by the PEF treatment (electric field strength [E] and treatment time [t]) and CCX addition. The effect of process parameters on *C. sakazakii* inactivation has been described previously by Pina-Pérez *et al*. (2007b, 2009a) and Arroyo *et al*. (2010a,b). The higher the cocoa concentration added to infant beverage, the higher the PEF inactivation effect, according to Pina-Pérez *et al.* (2013). The maximum inactivation level achieved in CCX-M-0% was 1.30 $\log_{10}$ cycles corresponding to 35 kV/cm–700 µs PEF treatment. At the same conditions, the maximum reduction level achieved in CCX supplemented beverages (5, 2.5 or 1 g/100 mL) was up to 2.10±0.12, 1.70±0.08, or 1.55±0.03 $\log_{10}$ cycles, respectively (Figure 3). The PEF survival curves were fitted to the Weibull model and kinetic parameters (scale and shape) were obtained. No significant differences ($P>0.05$) were observed between the shape parameter values in different beverages and at the PEF treatment conditions studied, so a simplified model substituting the n parameter by an average value ($n=0.561$) was obtained. Scale parameter values and the goodness of the simplified fit are presented in Table 3.

After PEF treatments, different beverages were stored at 8 °C from 24 to 120 h and *C. sakazakii* propagation was evaluated during the storage time. Cocoa addition at 5 g/100 mL (w/v) increased post-treatment inactivation rates ($P<0.05$) (to undetectable limits) after all PEF treatment conditions studied (15 kV/cm–3,000 µs; 8 °C, 120 h). According to Pina-Pérez *et al.* (2009a), 15 kV/cm–3,000 µs was the most effective treatment to control propagation of *C. sakazakii* cells at refrigeration temperatures. In this sense, and according to the present results, cocoa addition with refrigerated storage represents an additional barrier to proliferation of *C. sakazakii* post-PEF treatment.

Exposure assessment results

The outputs within the baseline model and "what if" scenarios, for control CCX-M and supplemented CCX-M milk beverages, are presented in Table 4. Regarding the PEF effect on CCX-M-0%, a significant contribution to the reduction in the population of *C. sakazakii* can be attributed to

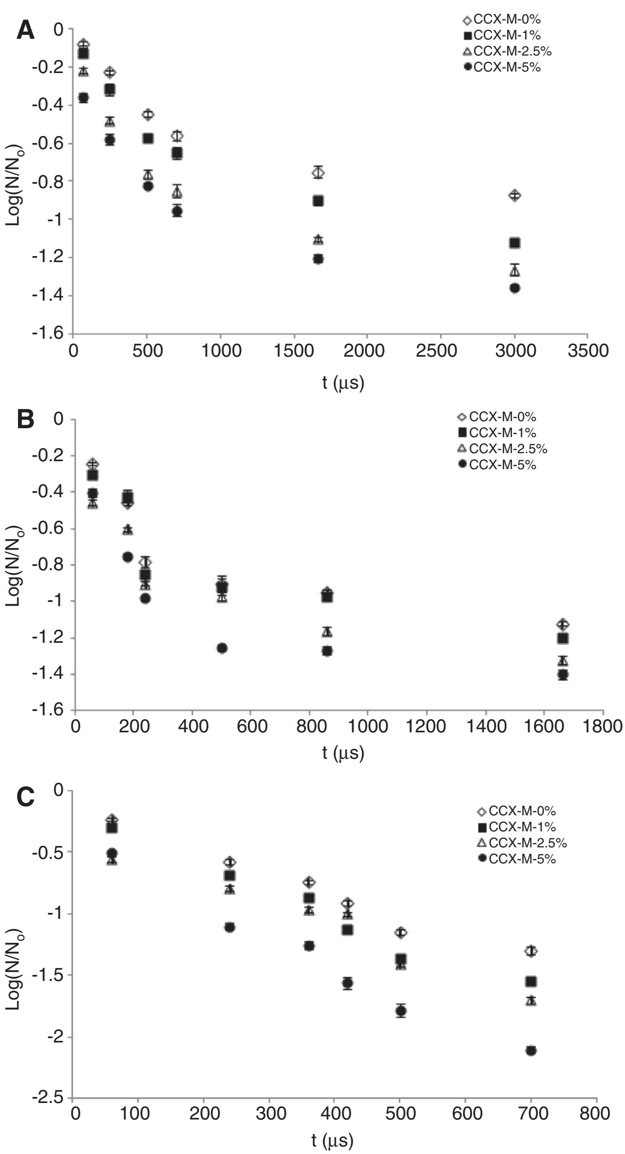

Figure 3. Pulsed electric field (PEF) survival curves (a) 15 kV/cm–3,000 μs, (b) 25 kV/cm–1,660 μs, (c) 35 kV/cm–700 μs, for Cronobacter sakazakii *in infant milk beverages not supplemented (CCX-M-0%)/ supplemented with rich-in-polyphenols-cocoa at different concentrations (1, 2.5 and 5% [w/v]).*

15 kV/cm–3,000 μs PEF application (baseline vs. scenario 1). However, no significant decrease in final load values (cfu per serving) was observed due to PEF intensity increase from 15 kV/cm–3,000 μs to 35 kV/cm–700 μs (scenario 1 vs. scenario 2), when these treatments were followed by a 24 h, 8 °C storage period. On the other hand, cocoa addition enhanced PEF effectiveness against *C. sakazakii* (scenarios 1, 2, and

Table 3. Simplified Weibull model kinetic parameter (a) values and goodness of fit (R^2-corrected and RMSE) for *Cronobacter sakazakii* inactivation in infant milk control beverage (CCX-M-0%) and infant rich-in-polyphenols cocoa-milk based beverages (CCX-M-1%; CCX-M-2.5%; CCX-M-5%), with a constant shape parameter ($n_{average}$ = 0.561)

E[1]	Beverage	a[2]	R^2 corrected	RMSE
15 kV/cm	CCX-M-0%	0.012	0.945	0.038
	CCX-M-1%	0.013	0.919	0.107
	CCX-M-2.5%	0.015	0.903	0.107
	CCX-M-5%	0.018	0.919	0.100
25 kV/cm	CCX-M-0%	0.011	0.965	0.040
	CCX-M-1%	0.011	0.969	0.043
	CCX-M-2.5%	0.013	0.963	0.042
	CCX-M-5%	0.014	0.972	0.036
35 kV/cm	CCX-M-0%	0.027	0.866	0.121
	CCX-M-1%	0.039	0.895	0.102
	CCX-M-2.5%	0.042	0.974	0.123
	CCX-M-5%	0.051	0.905	0.123

[1]E = electric field intensity (kV/cm).
[2]a = scale parameter of Weibull model.

3) and therefore, represents a significant control measure for reducing the risk of exposure to this pathogen (e.g. scenario 3; probability of illness in healthy population equals 7.35×10^{-6} in CCX-M-0% which is reduced to 1.56×10^{-8} in CCX-M-5%). Considering the assumptions related to scenario 1, cocoa addition at 5 g/100 mL controlled *C. sakazakii* cells by increasing PEF effectiveness by ~1 $\log_{10}$ cycle at the 95% of iterations. The probability of illness for non-immunodepressed populations, was reduced by 10–100 times in beverage supplemented with cocoa (e.g. scenario 2–3, respectively), and also significantly reduced for immunodepressed populations due to cocoa addition (e.g. scenario 4).

The most important cocoa intervention for *C. sakazakii* control occurred when storage temperature remained at 8 °C and storage time increased from 24 to 120 h (scenario 3). Under the conditions corresponding to scenario 3, the final load increased from baseline 1.17×10^{-6} up to 3.0×10^{-3} cfu per serving (95% of iterations) in infant milk beverage not supplemented with cocoa. In this case, the cocoa addition seems to act synergistically with refrigerated storage after PEF (Pina-Pérez *et al*. 2013), reducing the *C. sakazakii* cell counts to 8.87×10^{-5} cfu per serving at 95% of the iterations. The increase in sensitisation of treated cells due to the exposure to additional stress conditions post-treatment is well known (Pol *et al*. 2001; Rodrigo *et al*. 2007; Pina-Pérez *et al*. 2009b, 2012). In this sense, the addition of this natural antimicrobial ingredient in the formulation of beverages could reduce the exposure risk even more during shelf life.

In spite of PEF treatment, storage at room temperature (25 °C) (scenario 4) increased *C. sakazakii* cfu per serving up to 10^4 at 95% of iterations, with respect to the baseline (1.17×10^{-6} cfu per serving). This temperature could occur if a beverage remains outside the fridge prior to or during feeding at home (Joosten and Lardeau 2004). Consequently, the increase in storage temperature resulted in an enhanced exposure risk, increasing the probability of illness for healthy populations from 2.45×10^{-8} (scenario 1) to ≈1

Table 4. *Cronobacter sakazakii* outputs of the Monte Carlo simulation analysis of different "what if" scenarios combining PEF treatment and storage of control beverage (CCX-M 0%) and 5% (w/v) rich-in-polyphenols-cocoa milk based (CCX-M-5%) beverage

Scenarios	Beverages	CCX-M-0 %				CCX-M-5 %			
		Mean	SD	Percentiles		Mean	SD	Percentiles	
				5th	95th			5th	95th
Baseline									
	Final log cycles	–3.9	0.62	–4.81	–2.87	–4.09	0.62	–5.02	–3.14
without PEF treatment	Final load, cfu per serving	1.05×10^{-6}	9.40×10^{-8}	9.54×10^{-7}	1.17×10^{-6}	7.81×10^{-7}	6.54×10^{-8}	7.17×10^{-7}	8.52×10^{-7}
8 °C–24 h	Prob[1]	2.15×10^{-8}	2.75×10^{-8}	8.20×10^{-10}	7.53×10^{-8}	1.42×10^{-8}	1.89×10^{-8}	5.96×10^{-10}	5.42×10^{-8}
	Prob[2]	–	–	–	–	–	–	–	–
Scenario 1									
15 kV/cm–3,000 µs	Final log cycles	–4.50	0.15	–5.46	–3.44	–5.16	0.64	–6.15	–4.14
8 °C–24 h	Final load, cfu per serving	2.75×10^{-7}	2.52×10^{-8}	2.55×10^{-7}	3.19×10^{-7}	6.83×10^{-7}	2.31×10^{-8}	9×10^{-8}	6.66×10^{-6}
	Prob[1]	5.77×10^{-9}	7.93×10^{-9}	2.34×10^{-10}	2.45×10^{-8}	4.78×10^{-10}	5.93×10^{-11}	5.14×10^{-11}	4.91×10^{-9}
	Prob[2]	–	–	–	–	–	–	–	–
Scenario 2									
	Final log cycles	–4.62	0.67	–5.52	–3.58	–5.41	0.64	–6.40	–4.47
35 kV/cm–700 µs	Final load, cfu per serving	6.56×10^{-6}	8.61×10^{-6}	3×10^{-7}	2.63×10^{-6}	9.62×10^{-7}	1.21×10^{-6}	4.16×10^{-8}	3.10×10^{-6}
8 °C–24 h	Prob[1]	4.59×10^{-9}	6.08×10^{-9}	2.10×10^{-10}	1.84×10^{-8}	6.73×10^{-10}	8.47×10^{-10}	2.92×10^{-11}	2.18×10^{-8}
	Prob[2]	–	–	–	–	–	–	–	–
Scenario 3									
15 kV/cm–3,000 µs	Final log cycles	–2.88	0.92	–4.45	–1.44	–4.28	0.76	–5.41	–3.05
8 °C–120 h	Final load, cfu per serving	9×10^{-4}	0.0023	0	0.003	2.23×10^{-5}	5.50×10^{-5}	0	8.87×10^{-5}
	Prob[1]	7.35×10^{-6}	8.48×10^{-7}	6.22×10^{-6}	8.42×10^{-6}	1.56×10^{-8}	3.55×10^{-8}	2.69×10^{-10}	6.08×10^{-8}
	Prob[2]	1.26×10^{-13}	2.82×10^{-13}	0	6.63×10^{-13}	–	–	–	–
Scenario 4									
15 kV/cm–3,000 µs	Final log cycles	1.32	2.32	–2.62	4.39	0.14	2.08	–3.99	–3.31
25 °C–24 h	Final load, cfu per serving	637.32	1949.47	0	12,900	21.06	52.66	0	285
	Prob[1]	0.13	0.88	0	0.965	0.01	0.03	0	0.13
	Prob[2]	1.62×10^{-11}	3.45×10^{-11}	0	1.15×10^{-10}	1.66×10^{-12}	4.54×10^{-12}	0	1.59×10^{-11}
Scenario 5									
15 kV/cm–3,000 µs	Final log cycles	0.14	0.05	0.06	0.20	–0.06	0.12	–0.29	0.14
8 °C–24 h	Final load, cfu per serving	0.72	0.34	0.28	1.46	0.15	0.06	0.07	0.24
Log N_0=N(1,0.1)	Prob[1]	1.78×10^{-4}	1.44×10^{-5}	1.58×10^{-4}	1.98×10^{-4}	1.07×10^{-4}	4.29×10^{-5}	5×10^{-5}	1.78×10^{-4}
	Prob[2]	6.09×10^{-14}	2.89×10^{-14}	0.23×10^{-13}	1.23×10^{-13}	1.28×10^{-14}	5.81×10^{-15}	0.61×10^{-14}	2.09×10^{-14}

Prob[1] = Non-immunosuppressed population Probability of illness by consumption of contaminated product per day.
Prob[2] = Immunosuppressed population Probability of illness by consumption of contaminated product per day.

(scenario 4) at 95% of iterations, after consumption of un-supplemented beverage. Cocoa intervention at 5 g/100 mL significantly reduced the probability of illness for a healthy population ($Prob^1$=0.13, 95% of iterations) (scenario 4). The increase in storage temperature after PEF of CCX-M-0%, led to a final ingested dose of 12,900 cfu (per contaminated serving) at 95% of iterations. This ingested dose is considerably above the value of 1000 cfu considered as infective for this microorganism (Iversen and Forsythe 2003). However, cocoa supplementation at 5 g/100 mL provided a final *C. sakazakii* count per serving around 10 times below the concentration of concern.

The initial load variation had a significant effect on the final *C. sakazakii* numbers and probability of illness values, according to simulation results obtained for scenario 5. In this sense, different authors have pointed out the effectiveness of controlling this parameter in risk prevention (Ferrer *et al*. 2007; Pina-Pérez *et al*. 2010). The addition of cocoa at 5% (w/v) to infant beverage treated by PEF controls *C. sakazakii* levels below ~1 cfu per serving (95% of iterations), even with a high initial contamination level defined by Normal distribution *N*(1; 0.1), and under limit storage conditions (8 °C, 24 h) by FAO/OMS (2008).

For all scenarios studied, cocoa addition reduced the final *C. sakazakii* load from 0.6 to 1.70 $\log_{10}$ cycles, at 95% of iterations, depending on the scenario considered, and reduced the probability of illness for the two populations considered.

The simulation of the different scenarios indicates that the highest potential probability of illness could occur by the combination of different parameters under inappropriate conditions: (i) high initial load, (ii) high temperatures of infant beverage storage, and (iii) long storage times. In order to allocate the influence of each input on the output variation value (Pouillot and Delignette-Muller 2010), a sensitivity analysis was carried out. Table 5 shows the correlation coefficient (r) resulting from the analysis for each simulated scenario. Taking into account these results, N_0 ($\log_{10}$ (cfu/mL)) and storage temperature are the variables that exert the greatest influence on the final N*f* values, when no treatment has been applied (baseline scenario), according to previous studies (Kandhai *et al.* 2006; Beuchat *et al*. 2009). However, it can be shown that in scenarios with PEF application, PEF intensity (e.g. scenario 1 [r=–0.85] and 2 [r=–0.70]), cocoa addition and storage temperature (e.g. scenario 4 [r=0.70]), became significant determinants of the final load. Particularly, for scenario 3 (PEF=15 kV/cm–3,000 µs; storage= 8 °C, 120 h), when reconstituted PIFM was processed by PEF with storage at 8 °C for 120 h, cocoa addition represented

Table 5. Correlation coefficients from the sensitivity analysis performed at different *Cronobacter* spp. exposure assessment "what if" scenarios

Scenario	Correlation coefficients				
	Cocoa addition	N_0	PEF treatment	Storage temperature	Storage time
Baseline	0.05	0.97	–	0.68	0.52
Scenario 1	–0.75	0.40	–0.85	–0.45	–0.10
Scenario 2	–0.85	0.41	–0.70	0.11	0.05
Scenario 3	–0.98	0.50	–0.65	0.13	0.18
Scenario 4	–0.75	0.70	–0.18	0.70	0.58
Scenario 5	–0.10	0.97	–0.60	0.12	0.06

the most influential parameter controlling bacterial load (r=–0.98), which points to the potential of cocoa to control *C. sakazakii* proliferation specifically during storage of minimally processed products, even after extensive storage periods. On the other hand, for scenario 4 (PEF= 15 kV/cm–3,000 µs; storage=25 °C, 24 h) temperature (r=0.70) became one of the most influential parameters on the output value, due to its considerable increase with respect to the baseline model (from 8 to 25 °C). In this scenario that corresponds to storage temperature abuse, cocoa addition became a significant parameter contributing to *C. sakazakii* control. This result points to the relevance of temperature control and adopting CAC (2008) measures and recommendations in terms of handling, reconstitution and storage (Rosset, Noel and Morelli 2007) of products from infant powdered milk. When the initial load increased considerably (scenario 5) (N_0=1 $\log_{10}$ cfu/g; PEF=15 kV/cm–3,000 µs; storage=8 °C, 24 h), N_0 and PEF treatment were the two most influential parameters (r=0.97 and –0.80, respectively) defining the *C. sakazakii* final load at the time of consumption. These results could be explained by the influence of N_0 on final *C. sakazakii* exposure levels, and that at the most likely *C. sakazakii* contamination levels (EFSA 2007) contamination of PIFM could be reduced effectively by PEF technology.

Conclusion

The present study provides a preliminary approach to the estimation of the probability of *C. sakazakii* exposure after non-thermal processing of infant milk beverage supplemented/not supplemented with rich-in-polyphenols-cocoa. Knowledge concerning *C. sakazakii* risk exposure by consumption of novel PEF treated milk is required to determine the applicability of this technology, mainly at industrial level to manufacture beverages that add powder formula as an ingredient in formulation. In this regard, the present research is the first step in estimating the effectiveness of a hurdle combination (PEF and cocoa ingredient) technology and assessing the effect of inadequate handling conditions (high N_0 level, prolonged storage time and high storage temperatures [>8 °C]) which could occur, and therefore provides useful information from a risk prevention viewpoint.

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