



## Synergistic effect of Pulsed Electric Fields and CocoanOX 12% on the inactivation kinetics of *Bacillus cereus* in a mixed beverage of liquid whole egg and skim milk

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### ABSTRACT

With a view to extending the shelf-life and enhancing the safety of liquid whole egg/skim milk (LWE–SM) mixed beverages, a study was conducted with *Bacillus cereus* vegetative cells inoculated in skim milk (SM) and LWE–SM beverages, with or without antimicrobial cocoa powder. The beverages were treated with Pulsed Electric Field (PEF) technology and then stored at 5 °C for 15 days. The kinetic results were modeled with the Bigelow model, Weibull distribution function, modified Gompertz equation, and Log-logistic models.

Maximum inactivation registered a reduction of around 3 log cycles at 40 kV/cm, 360 μs, 20 °C in both the SM and LWE–SM beverages. By contrast, in the beverages supplemented with the aforementioned antimicrobial compound, higher inactivation levels were obtained under the same treatment conditions, reaching a 3.30 log<sub>10</sub> cycle reduction.

The model affording the best fit for all four beverages was the four-parameter Log-logistic model.

After 15 days of storage, the antimicrobial compound lowered *Bacillus cereus* survival rates in the samples supplemented with CocoanOX 12% by a 4 log cycle reduction, as compared to the untreated samples without CocoanOX 12%. This could indicate that the PEF-antimicrobial combination has a synergistic effect on the bacterial cells under study, increasing their sensitivity to subsequent refrigerated storage.

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### 1. Introduction

*Bacillus cereus* is a Gram-positive, spore-forming, facultative anaerobic bacterium, which can grow over a wide range of temperatures (4 to 55 °C) pH values (4.9 to 9.3) and water activities (0.92 to 1.0) (Scientific Panel on Biological Hazards, 2005). *Bacillus* species can be found in many varieties of badly preserved food products, including milk, dairy, meat and bakery products, fermented soy beans, mashed potato products, vegetable purees, pasta products, coca herbs and spices (Chorin et al., 1997). Consumers may be unaware of the presence of *B. cereus* in a food product because it does not necessarily modify the organoleptic properties, therefore they consume it without suspicion (Christiansson et al., 1989).

There are numerous research reports on the prevalence of *B. cereus* in dairy products; furthermore, it has been found in many ingredients used in milk formulations (Larsen and Jorgensen, 1999). In milk products, in particular, the reported prevalence of *B. cereus* ranges from 10 to 100%, reaching levels of between 0.3 and 10<sup>3</sup> cells or spores g<sup>-1</sup> (Barkley and Delaney, 1980; Baker and Griffiths, 1993; Griffiths 1992). However, despite the high incidence of this bacterium in these

products, only a few outbreaks of diarrhea have been reported in which milk or milk-related products containing *B. cereus* were believed to have been the cause (Notermans et al., 1997).

Certain authors have concluded that *B. cereus* is unlikely to cause any problems at temperatures below 6 °C (Myllykangas, 1995; Rangasamy et al., 1993). However, at 7 °C, only a few degrees above the *T*<sub>min</sub>, psychrotrophic *B. cereus* can be problematic in pasteurized milk products (Larsen and Jorgensen, 1999).

Egg and egg derivatives have been linked to several enteric outbreaks compromising public health. Epidemiological studies by the Spanish National Epidemiological Centre indicate that diarrhea outbreaks caused by consumption of *B. cereus* in egg or egg derivatives represented 38.5% of all those occurring in the 2002–2004 period. Furthermore, the percentage of people affected as a result of incorrect handling and storage of products at home is over 59% (Instituto Estudios del Huevo, 2008). An increasing number of food industries are using pasteurized liquid whole-egg to formulate their products. Liquid whole egg enhances the nutritional value of the end product and, also, improves the functional properties required during the production process. Approximately 85% of egg production in Spain is sold as fresh eggs, while the remaining 15% is destined to industrial production of egg derivatives: omelettes, bakery products, ready-to-eat products and powdered milk formulations (Instituto Estudios del Huevo, 2008).

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Under current USA commercial pasteurization standards, processing whole-egg products with additives involves subjecting the product to a temperature of 61.1 °C for 3.5 min. Based on this critical time–temperature relationship, it follows that a lower-than-specified temperature will decrease pasteurization efficacy, while overheating may result in coagulation of the egg and formation of a film on the heat exchanger surface (Powrie et al., 1995). An alternative method to thermal pasteurization is the use of high intensity Pulsed Electric Fields (PEF). This emerging technology must also fulfill or surpass the main purpose of egg pasteurization, which is to provide a wholesome product by eliminating pathogenic bacteria. Both the quality attributes and long shelf-life extension achieved with PEF are very promising, because refrigerated liquid whole-egg products that have been thermally treated must be kept unopened at below 4 °C for 2–6 days maximum, depending on the microbial quality of the product ([www.institutohuevo.com](http://www.institutohuevo.com)). Nowadays, there is growing pressure on the food industry to reduce its reliance on synthetic chemical preservatives. Consequently, manufacturers are urged to develop alternative preservatives based on natural compounds. This demand could be met by the use of natural antimicrobial systems for preservation of foodstuffs. Herbs and spices have been known for their antimicrobial activity since ancient times. The safe use of herbs and spices and their components has led to their current status of “generally recognized as safe” (GRAS) food ingredients. Plants have an almost limitless capacity to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Delgado et al., 2004). In many cases these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Useful antimicrobial phytochemicals can be divided into several categories: phenolics and polyphenols; quinines; flavones, flavonoids, and flavonols; tannins; coumarins; alkaloids and lectins and polypeptides.

Recent studies have focused on the biological activity of various cocoa extracts or by-products from the chocolate industry, such as cocoa-bean husks, known to contain high concentrations of polyphenols.

Due to the *B. cereus* relevance in dairy and egg products outbreaks, a liquid whole egg and skim milk mixture beverage has been proposed in the present study to evaluate the *B. cereus* vegetative cell inactivation level using non-thermal PEF technology in combination with cocoa extract. This beverage may be an innovative product with high energetic value and possibly oriented to children, elderly or athletes.

## 2. Materials and methods

### 2.1. Microbiological

A pure culture of *B. cereus* (131) bacterium was provided freeze-dried by the Spanish Type Culture Collection (CECT).

The culture was rehydrated with 10 ml of brain heart infusion (BHI; Scharlab Chemie, Barcelona, Spain). After 20 min, the whole 10 ml was inoculated in 500 ml of BHI and incubated at 30 °C with continuous shaking at 200 rpm for 14 h to obtain cells in a stationary growth stage. Growth curves were obtained based on both optical density and plate counts.

The cells were centrifuged twice at 4000 ×g, 4 °C, for 15 min and then resuspended in BHI. After the second centrifugation, the cells were resuspended in 20 ml of BHI with 20% glycerol and then dispensed in 2-ml vials. The 2-ml samples were immediately frozen and stored at –80 °C until needed for the kinetic inactivation studies.

For the pulsed electric field treatments, stock cultures of *B. cereus* CECT 131 were grown in BHI at 30 °C for 14 h to stationary phase. After incubation, 0.1 ml of culture was transferred to 50 ml of BHI and incubated at 30 °C for 14 h. Cells were harvested by centrifugation at 2400 ×g for 15 min at room temperature (22 to 23 °C), washed with sodium phosphate buffer (0.1 M, pH 7), and resuspended in beverage to give approximately 10<sup>9</sup> cfu/ml. All samples were inoculated 20 min before treatment to allow cells to acclimatize to the new environment.

One milliliter of uninoculated beverage was transferred onto brain heart infusion agar (BHIA) plates and incubated at 30 °C for 6 days to make sure that the samples were sterile.

### 2.2. Antimicrobial substance

Cocoa (*Theobroma cacao*) has a number of uses in the food industry. It is most commonly used to make chocolate, but is also used for flavoring in drinks, cookies, ice-creams, and other products. Cocoa flavonoids are known to be beneficial to health due to their antioxidant properties. Most notably, they have been reported to lower cholesterol levels and blood pressure, promoting beneficial cardiac health (Dreosti, 2000). In the present study the cocoa powder CocomOX 12% (CCX) was used (CocomOX 12%<sup>®</sup> Natraceutical S.A., Valencia, Spain). It is primarily composed of highly bioavailable monomers and dimers, and is guaranteed to contain at least 12% cocoa polyphenols (Tomas-Barberán et al., 2007).

### 2.3. Treatment medium and inoculation

In the present research work, a mixed beverage of pasteurized skim milk (80% v/v) and pasteurized liquid whole egg (20% v/v) was formulated.

Therefore the study was carried out on four beverages: pasteurized skim milk (Beverage 1; B1–SM), pasteurized skim milk with CCX (2.5% w/v) (Beverage 2; B2–SM–CCX), a mixed beverage of pasteurized skim milk and liquid whole egg (Beverage 3; B3–LWE–SM), and a mixed beverage of pasteurized skim milk, liquid whole egg, and CCX (2.5% w/v) (Beverage 4; B4–LWE–SM–CCX).

All beverages were treated by PEF, thereby enabling us to test the effect of this technology on *B. cereus* inactivation in two media, B1 and B3, and the possible synergistic effect of CCX.

The effect of CCX on *B. cereus* growth was assessed at different temperatures, 4, 20, and 37 °C in both skim milk with (B3) and (B1) without antimicrobial cocoa powder; thus, the inhibitory/bacterostatic effect of the active principle was tested. Beverages 1 and 3 were inoculated with *B. cereus* reconstituted at a concentration of 10<sup>2</sup> cfu/ml. At 4 and 20 °C, samples were taken every 2 h and every hour, respectively. At 37 °C, samples were also taken every hour. All the samples were plated onto BHIA and incubated in duplicate at 37 °C for 24–48 h. Results were fitted to the Gompertz equation.

### 2.4. Measurement of physical properties

The initial electrical conductivity and pH of the four beverages were measured at room temperature (5, 25, 35 °C). The values of the electrical conductivity and pH are shown in Table 1. Electrical conductivity was measured with a conductivity meter (Crison 525 conductimeter, Crison Instruments, S.A., Alella, Barcelona, Spain). The pH was measured with a pH meter (Crison 2001 pH-meter, Crison Instruments).

**Table 1**  
Electrical conductivity and pH values for different beverages and temperatures

Beverage	5		25		35	
	<sup>a</sup> K	pH	<sup>a</sup> K	pH	<sup>a</sup> K	pH
B1	3.38±0.14	6.68±0.07	5.77±0.10	6.69±0.03	7.02±0.11	6.68±0.03
B2	3.58±0.10	6.50±0.11	5.97±0.08	6.55±0.04	7.22±0.03	6.53±0.02
B3	4.27±0.08	6.21±0.11	7.23±0.03	6.18±0.05	8.70±0.25	6.18±0.03
B4	4.25±0.11	6.20±0.08	7.16±0.04	6.21±0.02	8.45±0.12	6.19±0.02

B1: Pasteurized skim milk (SM).

B2: Pasteurized skim milk and CocomOX 12% (SM–CCX).

B3: Pasteurized skim milk and pasteurized liquid whole egg (LWE–SM).

B4: Pasteurized skim milk, pasteurized liquid whole egg, and CocomOX 12%. (LWE–SM–CCX).

<sup>a</sup> K: conductivity (mS/cm).

## 2.5. PEF treatment system

An OSU-4D bench-scale continuous PEF system, designed at Ohio State University (Columbus, OH 43210-1007, USA), was used to treat the samples. Eight co-field treatment chambers with a diameter of 0.23 cm and gap distance of 0.293 cm were connected in series. The pre- and post-treatment temperatures at the inlet and outlet of each pair of treatment chambers were monitored by type T thermocouples located at the entrance and exit chambers. All treatment chambers were kept at 20 °C for all treatment intensities, with thermo-regulation via a heater exchanger, which could be regulated to the desired working temperature. The pulse width was fixed at 2.5 μs. Pulse wave form, voltage, and current in the treatment chambers were recorded with a digital oscilloscope (Tektronic TDS 210, Tektronic Ins., OR, USA). At the beginning of each experiment, un-inoculated substrate was passed through the fluid handling system to remove air bubbles. Once the substrate had filled the system, the flow rate was adjusted to a velocity ( $v$ ) of 30 mL/min and the sample was collected in a graduated cylinder. The inoculated substrates were then pumped through the system for 2 min to displace un-inoculated medium. The initial temperature of the substrate was 5 °C. High-voltage pulsing was then turned on, and the pulse frequency and charge voltage were adjusted to the required levels. Treatment times ranged from 60 to 3895 μs, and the electric field strength was set at 15, 25, and 35 KV/cm. Final temperature was kept at below 20 °C for all treatments. A 5-ml sample was taken in a graduated tube from the inoculated medium, before and after each PEF treatment, for a count of viable cells. They were serially diluted in sterile 0.1% peptone water, plated in Brain Heart Infusion Agar (BHIA) and incubated for 24 h at 36 °C. A collection type strain (CECT 137) was used to ensure the validity of microorganism identity.

## 2.6. Statistical analysis

The experimental design used was: four substrates (B1, B2, B3, B4); 21 PEF treatment conditions, with three electric-field strengths and seven different treatment times for each level of electric-field strength. Each combination of treatment conditions (Table 2) and substrate was repeated in triplicate. The statistical analysis was performed using Microsoft Excel™ and Statgraphics® Centurion XV software.

## 2.7. Mathematical model

In the present study, three mathematical models were used to analyze the kinetic inactivation experimental data.

The Bigelow model (1921):

$$\text{Log}S = -\left(\frac{t}{D}\right) \quad (1)$$

where  $S$  is the survival fraction [ $N/N_0$ ] at a treatment time ( $t$ ),  $N$  is the final number of microorganisms at treatment time ( $t$ ) (cfu/mL),  $N_0$  is the initial number of microorganisms before treatment (cfu/mL), and  $D$  is the decimal reduction time, which is, mathematically, the negative inverse of the inactivation curve slope.

**Table 2**

Pulsed electric field treatment conditions by combination of electric field strength ( $E$ , kV/cm) and treatment time ( $t$ , μs) for the different substrates in *Bacillus cereus* inactivation

Treatment time (μs)	$E$ (kV/cm)		
t1	15	25	35
t2	60	60	60
t3	360	120	80
t4	420	220	100
t5	600	360	120
t6	850	500	150
t7	1000	600	180
t7	1900	750	200

The Weibull distribution function (1951):

$$\text{Log} \frac{N}{N_0} = -bt^n \quad (2)$$

where log is the decimal logarithm of the survival fraction [ $N/N_0$ ] at treatment time ( $t$ );  $b$  and  $n$  are scale and shape factors, respectively. The  $n$  factor interprets the shape of the survival curve, so that when  $n=1$  it draws a straight line on a natural log scale, when  $n<1$  the survival curve forms is concave (it forms tails), when  $n>1$  the survival curve is convex (it forms shoulders).

The log-logistic model was proposed by Cole et al. (1993):

$$\log N(t) = \alpha + \frac{\omega - \alpha}{1 + e^{4\sigma(\tau - \log(t))/(\omega - \alpha)}} \quad (3)$$

where  $\alpha$  = upper asymptote (log (cfu/mL));  $\omega$  = lower asymptote (log (cfu/mL));  $\sigma$  = the maximum rate of inactivation ((log (cfu/mL))/log min); and  $\tau$  = the log time to the maximum rate of inactivation (log min).

The modified Gompertz equation was originally proposed by Gibson et al. (1988) to model growth curves and was later used to model inactivation kinetics (Xiong et al., 1999):

$$\log \frac{N}{N_0} = Ce^{-e^{BM}} - Ce^{-e^{B(t-M)}} \quad (4)$$

where  $M$  is the time at which the absolute death rate is maximum (min);  $B$  (log (cfu/mL)/min) is the relative death rate at  $M$ ; and  $C$  is the difference in value of the upper and lower asymptotes.

The model used to analyze the growth behavior at different temperatures in SM and LWESM supplemented/not supplemented with CCX is the equation defined by Gompertz (Gibson et al., 1988):

$$N = N_0 + Ce^{-e^{(2.718*\mu)/(\lambda - t)} + 1} \quad (5)$$

where  $N$ , is the number of microorganisms (cfu/ml) at time  $t$ ;  $N_0$  is the initial number of microorganisms (cfu/ml);  $C$  is the difference between initial and final cell numbers;  $\lambda$  is the delay before growth (hours);  $\mu$  is the maximum specific growth rate.

The goodness of fit of the linear and nonlinear models was compared by computing the  $R^2$ , RMSE, and  $A_f$  values.  $R^2$  measures how well a linear or nonlinear model fits the data, and the higher the  $R^2$  value, the more suitable the model is for describing the data (Neter et al., 1996).

RMSE measures the average deviation between the observed and fitted values. A smaller RMSE value for a model indicates a better fit of data for that model:

$$\text{RMSE} = \sqrt{\frac{(\text{fitted} - \text{observed})^2}{(n - p)}} \quad (6)$$

where  $n$  is the number of observations and  $p$  is the number of parameters to be estimated.

The accuracy factor parameter ( $A_f$ ) described by Ross (1996) is defined by the following expression:

$$A_f = 10^{\sum \frac{|\log(\text{predicted}/\text{observed})|}{n}} \quad (7)$$

where  $n$  is the number of observations used to make the calculations. The predicted/observed ratio refers to the relationship between the survival fraction fitted by the model and the one obtained experimentally. The larger the  $A_f$ , the less accurate the average estimate, while a value of 1 indicates that the model produces a perfect fit for the data.

## 3. Results

The bacteriostatic effect of CCX was studied at three different incubation temperatures (5, 20, and 37 °C) in inoculated LWESM and SM beverages. Fig. 1 shows the  $\mu_{\text{max}}$  values in SM and SM-CCX and it is

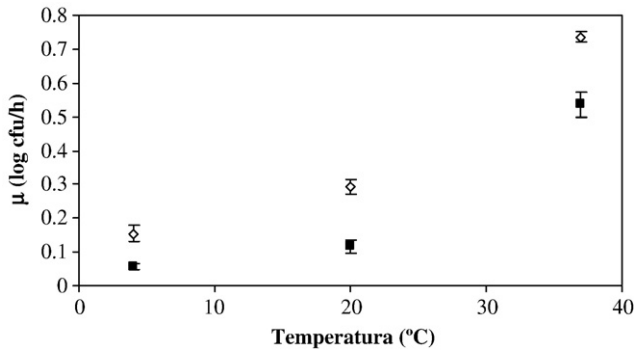


Fig. 1. Values of  $\mu_{\max}$  (log(cfu/ml)) parameter of the Gompertz model at three different temperatures of *Bacillus cereus* incubation.

evident that the values in the beverage with cocoa differ significantly ( $P \leq 0.05$ ) from those recorded for SM. The lower values for maximum specific growth rate ( $\mu_{\max}$ , log(cfu/h)) in beverages with CCX would indicate that the latter exerts a bacteriostatic effect on *B. cereus* growth.

*B. cereus* inactivation by Pulsed Electric Fields (PEF) was studied in two media: (i) a mixed beverage of pasteurized liquid whole egg (20% v/v) and pasteurized skim milk (80% v/v) (LWE–SM); and, (ii) pasteurized skim milk (SM).

Fig. 2 shows the curves of *B. cereus* inactivation for two substrates.

The effect of PEF on the *B. cereus* inactivation kinetics was studied first in the beverages without CocaoOX 12%. The maximum inactivation level, at an electric field strength intensity of 35 kV/cm and treatment time of 200  $\mu$ s (20 °C), was a  $3.03 \pm 0.02$  log cycle reduction in the LWE–SM beverage and a  $3.05 \pm 0.02$  log<sub>10</sub> cycle reduction in the SM beverage. For both beverages, LWE–SM and SM, the higher the electric field strength the higher the inactivation level

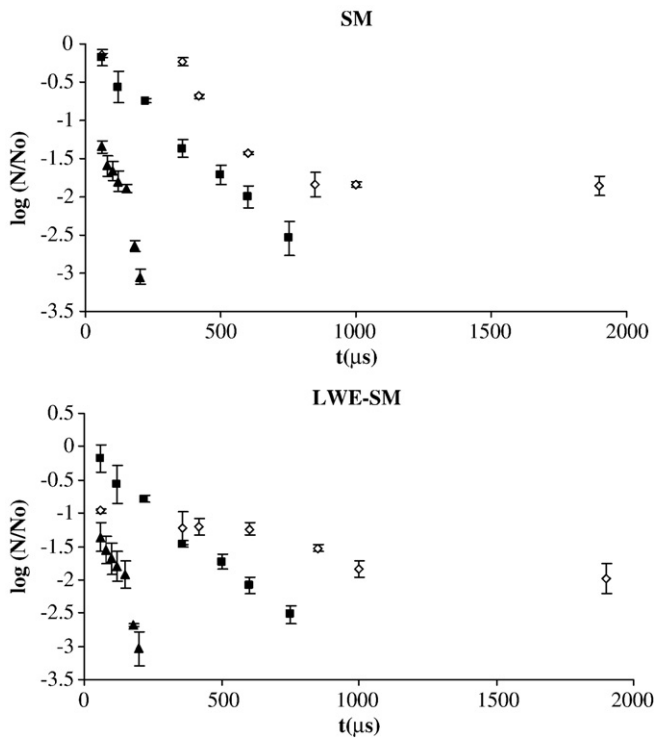


Fig. 2. Inactivation levels of *Bacillus cereus* at 15 ( $\diamond$ ), 25 ( $\blacksquare$ ), and 35 ( $\blacktriangle$ ) kV/cm in two substrates: Pasteurized skim milk (SM), and a mixture of pasteurized liquid whole egg (20% v/v) and pasteurized skim milk (80% v/v). Plot of the experimental points and error bars corresponding to three replicates.

obtained (35 > 25 > 15 kV/cm) and, for a given electric field intensity, the longer the treatment time, the higher the inactivation level.

Intensity treatments of 25 kV/cm–360  $\mu$ s and 35 kV/cm–60  $\mu$ s were found to be equivalent to an inactivation level of 1.356 log cycle reduction in the LWE–SM beverage. The same was observed between 15 kV/cm–1900  $\mu$ s and 35 kV/cm–150  $\mu$ s with an inactivation level of a 1.985 log cycle reduction.

The effect of the substrate was analyzed by an analysis of variance (ANOVA) but the results indicated that substrate did not significantly affect inactivation by PEF under different conditions ( $P$ -value > 0.05). Under the same treatment conditions *B. cereus* behaved similarly in both substrates after being subjected to PEF. One explanation for this could be the greater proportion of SM (80% v/v) in the LWE–SM beverage. The liquid whole egg (20% v/v) provides fats and proteins to the beverage and some authors believe these compounds afford protection against PEF treatments (Grahl and Märkl, 1996; Pina-Pérez et al., 2007), while other authors have not found any protective relationship to exist between complex food substrates and buffers (Dutreux et al., 2000).

### 3.1. Synergistic/antimicrobial effect of CocaoOX 12%

All PEF treatments were carried out with the LWE–SM and SM beverages supplemented with CocaoOX 12% (CCX) in a proportion of 2.5% (w/v), the minimum concentration at which a reduction in the viable population was observed by Busta and Speck (1968).

Data obtained from *B. cereus* exposure to PEF in LWE–SM and SM beverages, supplemented/not supplemented with CocaoOX 12%, were subjected to an analysis of variance (ANOVA). The inactivation levels achieved in supplemented/un-supplemented beverages were significantly different ( $P < 0.05$ ). According to results for bacteriostatic *B. cereus* growth, a synergistic effect was observed in the CocaoOX 12% supplemented LWE–SM beverage at all incubation temperatures. We use the term “synergistic effect” when the inhibitory action of the combination is higher than that of the hurdles applied separately.

The synergistic effect was significantly affected by electric-field strength ( $P$ -value < 0.05). Synergistic effect was higher at the lowest electric-field intensity (15 kV/cm), possibly due to the larger proportion of the reversible electroporation of the bacterial membrane that occurs at low treatment intensity levels (Ma et al., in press). Although the maximum level of inactivation in the LWE–SM beverage with CocaoOX 12% was reached at 35 kV/cm, 200  $\mu$ s (20 °C), the maximum antimicrobial synergistic effect was observed at 15 kV/cm, for treatment times of 850, 1000, and 1900  $\mu$ s. PEF treatment time influenced the synergistic effect between PEF and CCX in LWE–SM and SM beverages ( $P \leq 0.05$ ). At the lowest treatment times for each electric-field strength, from t1 to t3 (Table 1), the inactivation level did not differ substantially between the beverages with and without CocaoOX 12%. However, when the treatment time increased, from t4

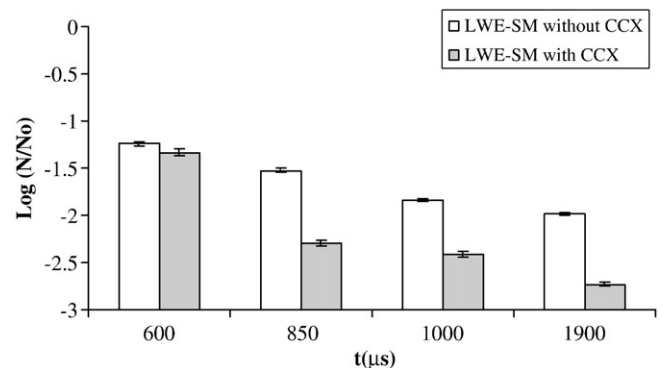


Fig. 3. Synergistic effect of 15 kV/cm PEF electric-field strength and CocaoOX 12% antimicrobial effect on *Bacillus cereus* inactivation in LWE–SM beverage.

to t7, the antimicrobial activity of CocaoOX had a synergistic effect on the inactivation results. The inactivation levels achieved under these treatment conditions in the LWE-SM beverage were 1.523, 1.834, and 1.986 log<sub>10</sub> cycle reductions, whereas the substrate supplemented with CocaoOX showed inactivation levels of 2.297, 2.427, and 2.730 log<sub>10</sub> cycle reductions, respectively. Fig. 3 shows the synergistic effect of CCX-PEF combination at treatment times from t4–t7 at 15 kV/cm.

Given the bacteriostatic effect of CCX, the results of the combined PEF-antimicrobial activity could be qualified as synergistic rather than additive, because the level of inactivation exceeds the additive effect on increasing treatment time, thereby becoming synergistic (Fig. 3). For example, the inactivation achieved in the LWE-SM beverage at 15 kV/cm–850 μs was a 1.60 log cycle, while that obtained for LWE-SM-CCX under the same conditions was a 2.30 log cycle. This result exceeds that obtained by the bacteriostatic and PEF net effects by a 1.20 log cycle.

This result is important given the main objective of this study is to reduce the treatment intensity required to inactivate *B. cereus* to safe levels. Accordingly, *B. cereus* can be reduced by a 2.297 log cycle at 15 kV/cm–850 μs in a CCX supplemented beverage, whereas a treatment intensity of 35 kV/cm–150 μs is required to achieve a similar inactivation level in LWE-SM beverages without CCX.

### 3.2. Modeling inactivation kinetics

Many hypotheses have been proposed to explain different types of deviations from linearity observed in the behavior of a wide variety of microorganisms inactivated by PEF technology (Baranyi and Roberts, 1994; Bhaduri et al., 1991; Cole et al., 1993; Xiong et al., 1999), such as the variability of sensitivity to lethal agents in the bacterial population, mixed bacterial populations in samples where each component has first-order inactivation kinetics, inactivation kinetics of a different order or a different kind of kinetics, or adaptation to stress that makes the remaining cells more resistant.

Interestingly, the survival curves obtained in our study could all be treated as linear ones, except at 15 kV/cm, where the shape of the curve showed a more or less pronounced sigmoidal curvature in different substrates. The tailing of the survival curves at 15 kV/cm started after approximately 2 log<sub>10</sub> cycle reductions. At higher electric field intensities (25 and 35 kV/cm), the higher the electric field intensity, the higher the slope of the linear curves (Fig. 2).

Table 3 shows the kinetic parameters for the four models applied to inactivate *B. cereus* in the two beverages with and without CocaoOX.

The biological significance of each parameter could be explained.

**Table 3**  
Goodness of fit of models to kinetic inactivation data for *Bacillus cereus* in four beverages (B1: pasteurized skim milk (SM); B2: pasteurized skim milk and CocaoOX 12% (SM-CCX); B3: pasteurized skim milk and pasteurized liquid whole egg (LWE-SM); B4: pasteurized skim milk, pasteurized liquid whole egg, and CocaoOX 12% (LWE-SM-CCX))

Beverage	<sup>1</sup> E	2 parameters Weibull model			4 parameters Log-logistic model			3 parameters Log-logistic model			3 parameters Gompertz equation			1 parameter Bigelow model		
		R <sup>2</sup>	RMSE	A <sub>f</sub>	R <sup>2</sup>	RMSE	A <sub>f</sub>	R <sup>2</sup>	RMSE	A <sub>f</sub>	R <sup>2</sup>	RMSE	A <sub>f</sub>	R <sup>2</sup>	RMSE	A <sub>f</sub>
B1	15	0.767	0.182	1.336	0.994	0.044	1.002	0.989	0.007	1.012	0.910	0.080	1.260	0.700	0.244	1.200
	25	0.868	0.114	1.246	0.991	0.074	1.002	0.975	0.021	1.023	0.993	0.008	1.020	0.993	0.006	1.019
	35	0.951	0.046	1.005	0.971	0.084	1.013	0.868	0.064	1.125	0.935	0.073	1.027	0.911	0.073	1.070
B2	15	0.967	0.012	1.001	0.967	0.057	1.022	0.884	0.019	1.111	0.903	0.043	1.300	0.712	0.223	1.214
	25	0.827	0.134	1.249	0.965	0.101	1.103	0.904	0.067	1.103	0.949	0.047	1.220	0.955	0.030	1.010
	35	0.967	0.031	1.003	0.972	0.079	1.110	0.887	0.044	1.201	0.940	0.066	1.030	0.890	0.091	1.086
B3	15	0.955	0.019	1.006	0.951	0.060	1.154	0.825	0.036	1.222	0.831	0.087	1.300	0.601	0.248	1.241
	25	0.908	0.090	1.264	0.991	0.022	1.001	0.982	0.015	1.015	0.993	0.008	1.004	0.989	0.009	1.021
	35	0.956	0.042	1.006	0.976	0.084	1.023	0.871	0.058	1.195	0.941	0.067	1.024	0.918	0.067	1.069
B4	15	0.763	0.375	1.054	0.997	0.006	1.007	0.920	0.125	1.036	0.902	0.186	1.300	0.801	0.236	1.064
	25	0.896	0.134	1.393	0.999	0.002	1.001	0.981	0.023	1.001	0.994	0.008	1.013	0.995	0.005	1.044
	35	0.872	0.154	1.011	0.979	0.035	1.020	0.952	0.035	1.014	0.971	0.042	1.060	0.979	0.021	1.200

<sup>1</sup>E → E: Electric field strength (kV/cm).

#### 3.2.1. Bigelow model

Decimal reduction time values (*D*) in the Bigelow model decreased with an increase in electric field intensity. In the LWE-SM beverage the *D* values decreased when the beverage was supplemented with CocaoOX 12% due to the lower resistance of cells to higher PEF treatment intensities.

#### 3.2.2. Weibull distribution function

*B. cereus* survival curves were fitted to the Weibull model because many authors have tested its efficacy in describing linear and non-linear inactivation curves. The fitted survival curves yielded shape factors that were *n* < 1, indicating that all the survival curves were concave upward. The scale factor (*b*) is considered as a measure of microorganism resistance to PEF treatment (kinetic parameter). The Weibull model was further analyzed to determine the possible effect of electric-field strength on the *b* and *n* values. The scale factor value (*b*) decreased when electric field intensity increased from 15 to 35 kV/cm in SM and LWE-SM. Concerning the *b* parameter in the LWE-SM beverage mixture, the *b* value obtained in LWE-SM-CCX at 15 kV/cm was considerably lower than the value obtained in the beverage without CocaoOX, indicating lower resistance of *B. cereus* cells in the beverage with CocaoOX.

The higher the electric field intensity, the higher the shape factor value (*n*), for each beverage. These results led us to conclude that these parameters, the scale and shape factors, were electric-field intensity dependent in all four beverages, in the range under study. The scale and shape factors were dependent on the substrate (*P* < 0.05) (Table 4).

#### 3.2.3. Modified Gompertz equation

Linton et al. (1995, 1996) used the modified Gompertz equation to fit non-linear survival curves for *Listeria monocytogenes* Scott A heated in infant formula and found that it was effective in modeling sigmoidal curves. We therefore fitted the data with the modified Gompertz equation and made a further study into the relationship between the parameters and the microbial death kinetics. The relative death rate (*B*) increased with electric field intensity from 15 to 35 kV/cm in the different beverages; however, no increase was observed between 15 and 25 kV/cm.

#### 3.2.4. Log-logistic model

The  $\sigma$  parameter corresponds to the maximum rate of inactivation and has been used to study the effect of electric-field intensity level on microbial inactivation. In our study, the  $\sigma$  value decreased for all the beverages in the range of electric-field intensities studied. This means

**Table 4**

Kinetic parameters of five studied models for *B. cereus* PEF inactivation in four beverages (B1: pasteurized skim milk; B2: pasteurized skim milk and CoccoanOX 12%; B3: pasteurized skim milk and pasteurized liquid whole egg; B4: pasteurized skim milk, pasteurized liquid whole egg, and CoccoanOX 12%)

Beverage	<sup>a</sup> E (kV/cm)	2 parameters		4 parameters	3 parameters	3 parameters	Bigelow (D)
		Weibull		Log-logistic	Log-logistic	Gompertz (B)	
		(b)	(n)	( $\sigma$ )	( $\sigma$ )		
B1	15	0.056	0.429	-2.683	-10.420	0.000584	1548.950
	25	0.046	0.524	-2.862	-5.490	0.000333	591.431
	35	0.037	0.716	-6.120	-8.170	0.001567	135.690
B2	15	0.167	0.286	-0.735	-1.390	0.000707	1612.830
	25	0.033	0.545	-2.964	-6.070	0.000383	701.321
	35	0.063	0.631	-5.886	-7.120	0.002576	130.124
B3	15	0.226	0.259	-0.642	-1.270	0.000505	1462.560
	25	0.048	0.519	-2.101	-5.400	0.000508	581.147
	35	0.036	0.723	-5.719	-8.320	0.001592	135.118
B4	15	0.032	0.531	-2.115	-13.900	0.000453	1194.440
	25	0.048	0.537	-3.515	-6.940	0.000339	513.639
	35	0.096	0.547	-6.079	-15.320	0.001679	135.078

<sup>a</sup> E (kV/cm): Electric field strength.

that the higher the electric-field intensity, the higher the level of inactivation reached.

The log-logistic model has been used by other authors to describe the microbial inactivation of vegetative cells (Ellison et al., 1994) and bacterial spores (Anderson et al., 1996) by heat. With vegetative cells, a linear relationship was found between  $\tau$  value (log of treatment time at which the maximum inactivation rate occurs) and treatment temperature. We also found a linear relationship between  $\tau$  value and electric-field strength level for both beverages, SM (B1) and LWESM (B3), with CoccoanOX 12% (B2 and B4) and without, as can be seen in the following equations:

B1 and B2

$$\tau = -0.030E + 7.361 \quad R^2 = 0.994$$

B3 and B4

$$\tau = -0.049E + 8.113 \quad R^2 = 0.901$$

The higher slope of the  $\tau$  curve in the beverages with pasteurized liquid whole egg (B3 and B4) is transformed into a higher effect of electric-field intensity enhancement, given the reduction in the time needed to reach the maximum level of inactivation ( $\tau$ , log(min)).

The correlating ability of the different models was tested by using the accuracy factor test (Af),  $R^2$ , and RMSE, using an independent databank (Table 3). The model that provided the best fit to the experimental data in all the beverages was the Log-logistic model:  $R^2$  0.979 $\pm$ 0.015; RMSE 0.053 $\pm$ 0.010; and Af values of 1.038 $\pm$ 0.030. The model that provided the second most accurate fit to the data in all the beverages was the modified Gompertz equation:  $R^2$  0.946 $\pm$ 0.035; RMSE 0.063 $\pm$ 0.028; and Af values of 1.129 $\pm$ 0.083. The Weibull model with two distribution parameters provides the third best fit to the data:  $R^2$  0.892 $\pm$ 0.070; RMSE 0.111 $\pm$ 0.082; and Af ranged from 1.001 to 1.391.

The four-parameter Log-logistic model could be reduced to a model with three parameters by simplifying the  $\tau$  parameter to an average value of 6.901 $\pm$ 0.301. The new model  $\sigma$  parameter and the accuracy of fit are shown in Table 4. The simplified three-parameter Log-logistic model provides a better fit ( $R^2$  0.918 $\pm$ 0.062; RMSE 0.042 $\pm$ 0.012; and Af 1.088 $\pm$ 0.070) than the two-parameter Weibull model. Consequently, we can conclude that the simplified three-parameter Log-logistic model is the best one for fitting the experimental data.

### 3.3. Storage

After treatments, cells were stored at 5 °C and evaluated for a period of 15 days in order to test the possible influence of CCX on the evolution of PEF-treated and untreated *B. cereus* cells under refrigeration. Figs. 4 and 5 show the evolution of refrigerated *B. cereus* cells in the LWESM beverage with and without CoccoanOX 12% during the storage period. As can be seen in Fig. 4, no death or growth of treated *B. cereus* cells was observed in LWESM beverage during the refrigerated storage period. However, treated cells in the LWESM-CCX beverage showed a decrease in the number of survivors during storage. This reduction in the number of survivor cell counts was greater at electric-field intensities of 15 and 25 kV/cm than at 35 kV/cm. At lower electric-field intensities the decrease observed was higher for treatment times ranging from 60 to 1900  $\mu$ s. At 15 kV/cm the reduction in counts of treated cells ranged from 7 to 15% at 5 days and from 25 to 35% at 15 days of storage. At 25 kV/cm the highest decrease was observed at 15 days of refrigerated storage, when it reached 75% (4 log cycle reduction) at 500  $\mu$ s.

Under conditions of low treatment intensity, Calderón-Miranda et al. (1999) found similar results to those obtained in our study. PEF application followed by nisin exposure caused additive inactivation of *Listeria innocua* in liquid egg. In the present study, the cell damage caused by PEF at lower treatment intensities could be the basis for the subsequent attack by the active compound, reducing ability of *B. cereus* cells to repair injury during the storage period. The increased permeability of the cell membrane not only allows the entrance of ions and other molecules but also sensitizes the cell to antimicrobials. LWESM contains inherent antimicrobial systems such as lysozyme. Lysozyme has a lytic action on the bacterial cell wall of Gram-positive and -negative bacteria (Li-Chan et al., 1995). Thus, the presence of two (lysozyme and CoccoanOX 12%) or more antimicrobials in LWESM can have a synergistic effect on *B. cereus* as a consequence of PEF.

According to Leistner (1992), when two hurdles have the same physiological target in a microbial cell, the effect of the combination of both is additive. An additive effect on the inactivation of *L. innocua* was observed by Calderon-Miranda et al. (1999) since both PEF and the antimicrobial act on the cell membrane. In the present study, the effect of the antimicrobial CoccoanOX 12% became apparent in the storage period, in combination with low temperatures (5 °C), leading to a decrease in the survivor population. Similar results were obtained by Ostovar (1973) with dark and milk chocolate inoculated with *Staphylococcus aureus* and stored at room temperature. The *S. aureus* concentration was reduced from 2 log<sub>10</sub> cycles to complete absence after two days in dark chocolate, whereas in milk chocolate 14 days were necessary for this to occur.

Busta and Speck (1968) observed that heat treatments enhanced the lethal effect of cocoa suspensions on *Salmonella typhimurium*, but heating was not essential since the lethal substance was present in the unheated suspension. At the concentration of CoccoanOX used in the present study (2.5% w/v), no reduction was observed during storage of untreated cells. However, an explicit synergistic effect of CCX and PEF treatments was observed in the beverages with CCX in comparison with the beverages without CCX.

### 4. Discussion

To facilitate the production of safe products it is essential to accurately predict the effectiveness of PEF processing against foodborne pathogens, which can be done using microbial inactivation kinetics (Chen and Hoover, 2003). PEF antibacterial ability is dependent on its electric parameters, the features of the microorganism in question, and the physicochemical properties of the suspending medium. The present results indicate that cell-death rate differed for each electric-field intensity, and that higher cell lethality was obtained by increasing electric-field intensity.

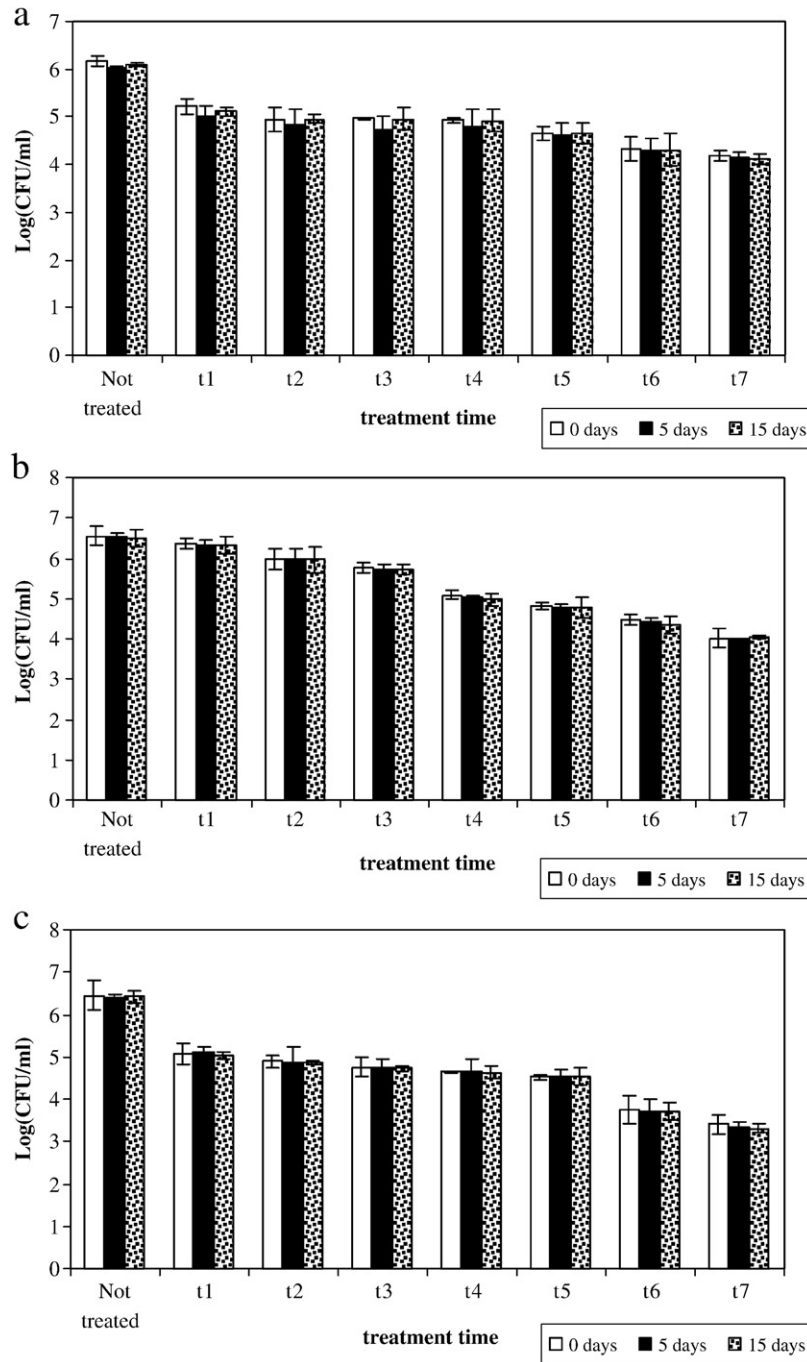


Fig. 4. Evolution of treated *Bacillus cereus* cells at a) 15kV/cm, b) 25 kV/cm and c) 35 kV/cm in LWE-SM beverage stored at 5 °C for 0 days, 5 days, and 15 days.

Although PEF treatment induces a loss of microorganism viability in general, the protective effect of milk and egg composition could imply a drop in PEF effectiveness (Martin et al., 1997). Nevertheless, various studies have indicated a synergistic effect when combining PEF and other non-thermal treatments, particularly antimicrobial compounds. In the present study, this synergistic effect was only observed at treatment time intensities of 120–200, 350–750, and 600–1900, depending on the electric field strength, 35, 25, and 15 kV/cm respectively.

Busta and Speck (1968) used the antimicrobial effect of cocoa to inactivate *Salmonella* spp. Garbis and Langlois (1967) reported that the growth of several test organisms in milk was retarded by the

presence of cocoa powder. A review of several reports on anthocyanin inhibition of microorganisms and the knowledge that anthocyanin compounds are present in cocoa suggest that these components may be the lethal agents. However, the cocoa anthocyanin fractions that are active against each microorganism have to be assayed in each case.

PEF applicability to industrial treatment of LWE-SM and SM has good prospects in view of the results obtained in our study. An initial concentration of  $10^4$  cfu/mL of vegetative *B. cereus* cells could be reduced by around 3.30 log cycles in beverages supplemented with CCX.

Recognition of the health potential of chocolate is a recent development, and most attention so far has focused on the possible

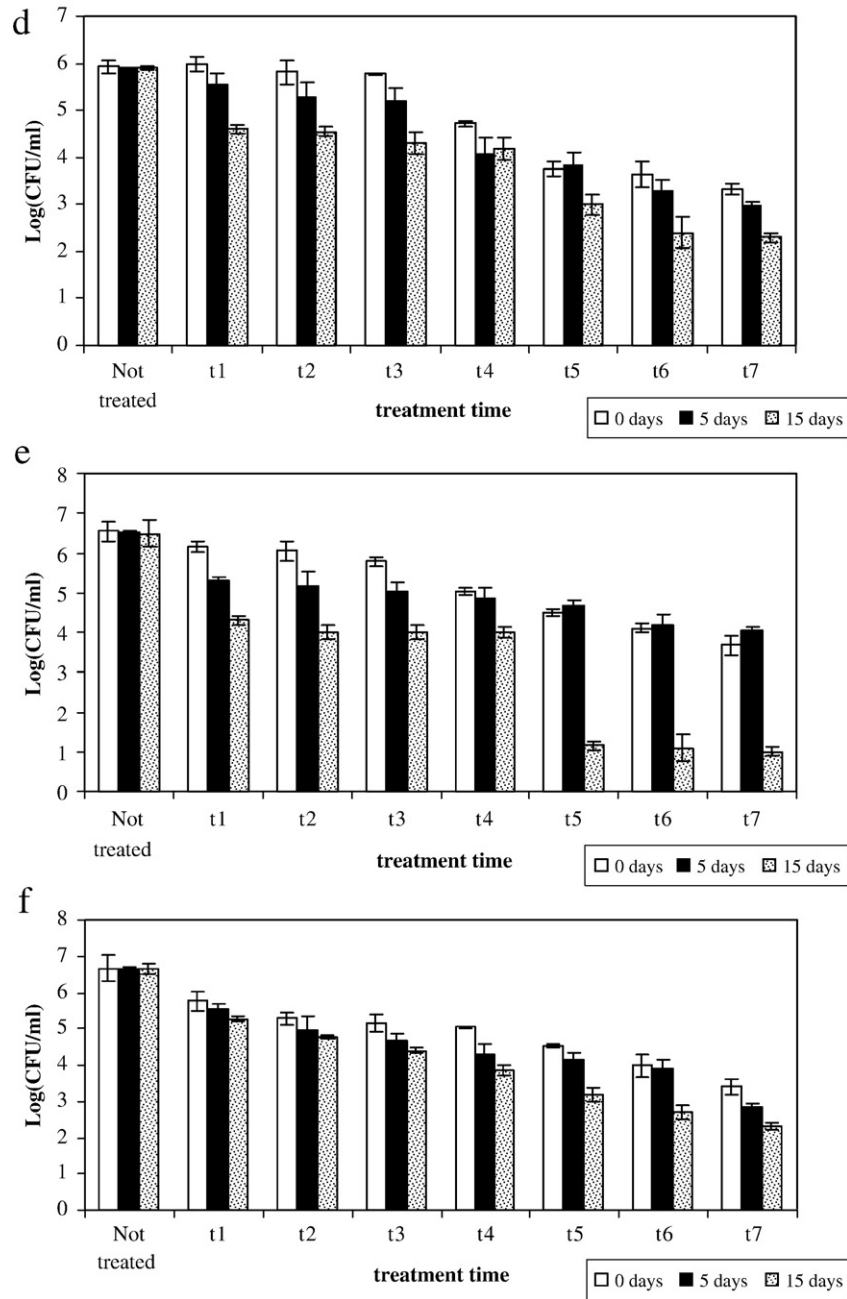


Fig. 5. Evolution of treated *Bacillus cereus* cells at d) 15 kV/cm, e) 25 kV/cm and f) 35 kV/cm in LWE-SM-CCX beverage stored at 5 °C for 0 days, 5 days, and 15 days.

benefits that may arise from the antioxidant activity of cocoa polyphenols. Most studies have reported on the protection afforded by extracts of cocoa powder and chocolate against LDL peroxidation, calculating that its potency, based on phenol content, slightly exceeds that of red wine. This in turn has led to suggestions that cocoa polyphenols may help to protect against the development of coronary heart and cardiovascular disease (Dreosti, 2000). A review of several reports on anthocyanin inhibition of microorganisms and the knowledge that anthocyanin compounds are present in cocoa suggest that these components may be the lethal agents.

## 5. Conclusions

The present study shows that CocoonOX 12% powder added to beverages containing pasteurized skim milk and liquid whole egg is

effective in reducing *B. cereus* counts after PEF treatments. A synergistic effect was observed between PEF and CocoonOX, but only at intensive treatment times. The death of *B. cereus* cells during the refrigerated storage period of the beverages shows that CocoonOX activity influences subsequent development of treated *B. cereus* cells.

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