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Cocoa powder as a natural ingredient revealing an enhancing effect to inactivate *Cronobacter sakazakii* cells treated by Pulsed Electric Fields in infant milk formula

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ABSTRACT

This work was carried out to study the potential effect of combining polyphenol-rich cocoa powder (CocoanOX 12%, CCX) with Pulsed Electric Field (PEF) technology to inactivate *Cronobacter sakazakii* cells inoculated into infant milk formula (IMF).

This effect was studied for three different concentrations of cocoa powder, 1%, 2.5% and 5% (w/v), and for different addition times, 0, 2 and 4 h, before and after PEF treatment (15, 25 and 35 kV/cm), to determine the influence of both factors on inactivation and subsequent evolution of the treated cells under refrigerated conditions (8 °C, 12 h).

The results indicated that combined PEF and CCX application, and the moment of CCX addition, pre-treatment/post-treatment, significantly affected the level of *C. sakazakii* inactivation achieved and subsequent evolution of the treated cells over 12 h at 8 °C ($p \leq 0.05$). The maximum inactivation level, 4.41 $\log_{10}$ cycles, was achieved when CCX was added 4 h after PEF (15 kV/cm − 3000 μs) and the treated cells were kept under refrigerated (8 °C) storage for up to 12 h.

1. Introduction

Infant liquid milk-based beverages frequently come from a powdered infant formula with the potential risk of *Cronobacter sakazakii* contamination (Lai, 2001; Lehner, Fricker-Feer, & Stephan, 2011; Muytjens, Roelofs-Willemse, & Jasper, 1988; Reich, König, von Wiese, & Klein, 2010; Zeller-Péronnet et al., 2012). Related to these products, several studies up today, have identified documented cases of *Cronobacter* spp. infection in children under three years of age, worldwide, some of which ended in patient mortality (FAO/WHO, 2008; Norberg et al., 2012).

Having in mind further improvement of food safety and in order to preserve the nutritional factors included in powdered milk formula-based beverages for children, alternatives to heat treatment are being developed (Nakimbugwe, Masschalck, Anim, & Michiels, 2006; Rodríguez-González, Walkling-Ribeiro, Jayaram, & Griffiths, 2011; Zenker, Heinz, & Knorr, 2003). Hurdle technology advocates combinations of various preservation techniques in order to guarantee food safety by means of additional or synergistic effects. High-intensity Pulsed Electric Field (PEF) technology shows great potential as a non-thermal pasteurization method, leaving intact the most sensitive components of a wide variety of liquid foods (Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Belloso, 2007; Ross, Griffiths, Mittal, & Deeth, 2003). However, according to *C. sakazakii* inactivation levels reported by Pina-Pérez, Rodrigo Aliaga, Ferrer Bernat, Rodrigo Enguidanos, and Martínez López (2007), PEF may not be sufficient as a general infant formula industrial preservation method. In recent years, the addition of antimicrobially active natural compounds has been used, adding to the intensity of physical preservation processes (e.g. heat treatment, high hydrostatic pressure (HHP), Pulsed Electric Fields (PEF)) (Garcia-Graells, Masschalck, & Michiels, 1999; Periago, Conesa, Delgado, Fernández, & Palop, 2006; Sobrino-López & Martín-Belloso, 2008). Owing to legal issues affecting a number of antimicrobials, interest is arising in the use of substances of a functional nature that act as ingredients in food formulation and that, in turn, may also prevent the development of hazardous microorganisms (Marco et al., 2011). In this respect, the availability of 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. The antioxidant capacity of flavonoids (Bubonja-Sonje, Giacometti, & Abram, 2011; Cowan, 1999), and possible beneficial implications for human health (Cooper, Donovan, Waterhouse, & Williamson, 2008) have been demonstrated to exhibit a high bioavailability in humans when these cocoa derivatives/

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concentrates are administered in a milk drink (Tomas-Barberán et al., 2007). With regard to the most suitable moment at which to add these substances, before or after treatment, various studies have published the influence of the addition time of flavouring/texturizer/colouring/antimicrobial substances on the possible interaction effects occurring between them and the preservation treatment (Gallo, Pilosof, & Jagus, 2007; Gálvez, Abriouel, López, & Omar, 2007; Lee, Heinz, & Knorr, 2003; Terebiznik, Jagus, Cerrutti, De Huergo, & Pilosof, 2000).

This research work aims to study PEF inactivation of *C. sakazakii* in infant milk formula (IMF), supplemented with polyphenol-rich cocoa powder at various concentrations. The main objectives of this study are to: 1) determine the potential effect of cocoa powder on *C. sakazakii* inactivation by PEF; 2) determine the effect of cocoa powder addition time (0, 2, 4 h), before or after treatment, on the sensitivity of cells to PEF technology; 3) monitor the possible effect/influence of cocoa powder on the development of treated cells during refrigerated storage. These research aims contribute to a better understanding of PEF- and cocoa-based hurdle technologies.

2. Material and methods

2.1. Bacterial strain and growth medium

The pure culture of *C. sakazakii* (equivalent to strain 29544 ATCC) was supplied lyophilized by the Spanish Type Culture Collection (CECT 858).

The freeze-dried culture was rehydrated in 10 mL of tryptic-soy broth (Tryptic Soy Broth, TSB) (Scharlab Chemie, Barcelona, Spain) for 20 min. Then this cell suspension was inoculated in 500 mL of TSB and incubated at 37 °C under continuous stirring at 200 rpm for 24 h to obtain cells in their stationary growth phase.

Stationary-phase cells were collected after double centrifugation at 5000× *g*, 5 °C for 15 min. The pellet obtained in the second centrifugation was resuspended in 15 mL of TSB, and divided into 2 mL vials, with 1 mL of cell suspension and 1 mL of a solution prepared from glycerol (20%) and TSB. These vials were frozen at −80 °C. This primary stationary-phase cell culture, stored at −80 °C, had a concentration of 10^9 Colony Forming Units (CFU) per mL. These vials were used in the PEF assays.

2.2. PEF equipment

In order to treat samples OSU-4D equipment, designed by Ohio State University, was used at a laboratory scale setup. Eight treatment chambers with a diameter of 0.23 cm were connected in series. Two cooling coils were connected before and after each pair of chambers and submerged in a circulating refrigerated bath to maintain a treatment temperature below 25 ± 3 °C (inlet temperature, 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 (*t*, μs) ranged between 60 and 3000 μs, and the electric field intensity (*E*, kV/cm) was 15, 25, or 35 kV/cm (Table 1). Samples were collected previous (untreated controls) and after each treatment and serially diluted in sterile peptone water at 0.1%, and plated on Tryptic Soy Agar (TSA, Scharlaub, Barcelona, SPAIN) and incubated at 37 °C for 24 h. Colony Forming Units (CFU) were counted. Suspensions of untreated cells were used to determine the initial load as CFU/mL. The experiments were performed in triplicate.

Table 1
Pulsed Electric Field (PEF) treatment conditions (*E* (kV/cm) and *t* (μs)) applied to *C. sakazakii* inactivation.

E (kV/cm)	*t* (μs)
15	60
	240
	500
	700
	1660
	3000
25	60
	180
	240
	500
	860
	1660
35	60
	240
	360
	420
	500
	700

2.3. Cocoa powder rich in polyphenols (CCX)

In this study, we used a cocoa powder marketed as CocoanOX 12% (CCX), which resulted from a patented process to preserve the cocoa's natural content of cocoa polyphenols, more specifically flavonoids (Naturex S.L., Valencia, Spain).

Comparison with conventional cocoa powder revealed that CocoanOX 12% has a polyphenols content ≥12 g/100 g of product (dry weight). Moreover, it contains 8 times more epicatechin (31.49 mg/g) and procyanidin B_2 (17.14 mg/g) than conventional cocoa powder.

2.4. Formula composition and inoculation

In this study, infant milk formula (IMF) was used, that was reconstituted from powdered infant formula (IMF-0% CCX) and, to which cocoa powder was added at three different concentrations: 1% (w/v) (IMB-1% CCX); 2.5% (w/v) (IMB-2.5% CCX) and 5% (w/v) (IMB-5% CCX). The different formulas were all treated under the same electric field-strength (*E*, kV/cm) − treatment time (*t*, μs) conditions.

The combined effect of PEF and cocoa concentration on *C. sakazakii* inactivation was studied in depth, also considering the time of cocoa powder addition, before or after PEF treatment (0, 2 and 4 h). IMB, supplemented or not, before or after treatment, was kept refrigerated at 8 °C (with stirring at 250 rpm) for a total storage time of 12 h.

Electrical conductivity (Crison 525 conductivity meter, Crison Instruments, S.A., Alella, Barcelona, Spain) and pH (Crison GLP21 pH-meter, Crison Instruments, S.A., Alella, Barcelona, Spain), were measured at room temperature (25 °C): IMB conductivity value $k = 0.278$ S/m, and pH value 6.8; IMB-1% CCX (pH = 6.98 ± 0.10; $k = 0.320 \pm 0.10$ S/m); IMB-2.5% CCX (pH = 7.16 ± 0.04; $k = 0.328 \pm 0.05$ S/m) and IMB-5% CCX (pH = 7.24 ± 0.08; $k = 0.332 \pm 0.07$ S/m).

The different formulas were inoculated to a final concentration of $1–3 \times 10^7$ CFU/mL, with the 2-mL vials kept frozen at −80 °C.

2.5. Statistical analysis

Factorial design with at least three replications was used for all experimental groups. An analysis of variance (ANOVA) was