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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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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 $\log_{10}$ 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.

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 10^3 cells or spores g^{-1} (Barkley and Delaney, 1980; Baker and Griffits, 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_{min}, 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 derivates 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). 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 10^9 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 CocoanOX 12% (CCX) was used (CocoanOX 12% ®Natraceutical S.A., Valencia, Spain). It is primarily composed of highly bioavailable monomers and dimmers, 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 10^2 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

Temperature (°C)						
	5		25		35	
Beverage	[a]*K*	pH	[a]*K*	pH	[a]*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 ± 003	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 CocoanOX 12% (SM–CCX).
B3: Pasteurized skim milk and pasteurized liquid whole egg (LWE–SM).
B4: Pasteurized skim milk, pasteurized liquid whole egg, and CocoanOX 12%. (LWE–SM–CCX).
[a] *K*: conductivity (mS/cm).