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Korrespondenzadresse:
conaml@iata.csic.es

[1]Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Burjassot, Valencia, Spain; [2]Natraceutical S. A., Quart de Poblet, Valencia, Spain

Effect of Flavanol-Rich Cocoa Powder on the Growth of *Bacillus cereus* Spores in Reference Media

Effekt von Flavonol-reichem Kakaopulver auf das Wachstum von Bacillus cereus-Sporen in Referenzmedien

Carmen Ferrer[1], Aurora Marco[1], Dolores Rodrigo[1], Begoña Muguerza[2], Antonio Martínez[1]

Summary

The effect of flavonol-rich cocoa powder on the growth of *Bacillus cereus* spores in reference media was evaluated considering different concentrations of flavonol-rich cocoa powder and temperature combinations. Results indicated that the ingredient had a bacteriostatic effect. Growth curves of *B. cereus* in the presence of flavonol-rich cocoa powder were fitted to the modified Gompertz equation to obtain kinetic parameters. A significant decrease in the specific growth rate ($p<0.001$) was observed when increasing the flavonol-rich cocoa powder concentration. Regarding the elongation of the lag phase, the effect of increasing the flavonol-rich cocoa powder concentration was significant at 32 °C. This study confirms the potential for flavonol-rich cocoa powder to prevent *B. cereus* outgrowths, which could be considered as an additional control measure in the case of cold chain break, as it is able to reduce the growth rate of *B. cereus* in beverages containing cocoa.

Keywords: antimicrobial activity, microbial kinetics, growth rate

Zusammenfassung

Der Effekt von Flavonol-reichem Kakaopulver auf das Wachstum von *Bacillus-cereus*-Sporen in Referenzmedien wurde mit verschiedenen Konzentrationen von Flavonol-reichem Kakaopulver und Temperaturkombinationen untersucht. Die Ergebnisse zeigten, dass der Inhaltsstoff einen bakteriostatischen Effekt hat. Die Wachstumskurven von *B. cereus* in Gegenwart von Flavonol-reichem Kakaopulver wurden an die Gompertz-Gleichung angepasst, um kinetische Parameter zu ermitteln. Eine bedeutende Senkung in der spezifischen Wachstumrate ($p<0,001$) wurde bei steigender Konzentration des Flavonol-reichen Kakaopulvers beobachtet. Bezüglich der Elongation der Verzögerungsphase, war der Effekt der Erhöhung der Konzentration von Flavonol-reichem Kakaopulver bedeutend bei 32 °C. Diese Studie bestätigt das Potential des Flavonol-reichem Kakaopulvers zur Vorbeugung des Auskeimens von *B. cereus*-Sporen. Dies könnte als zusätzliche Kontrollmaßnahme bei Unterbrechungen der Kühlkette betrachtet werden, da es die Reduzierung der *B. cereus*-Wachstumsrate in Kakao enthaltenden Getränken ermöglicht.

Schlüsselwörter: Antimikrobielle Aktivität, mikrobielle Kinetiken, Wachstumsrate

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Introduction

In the past two decades or so, Europe has seen a dramatic rise in the concern amongst citizens with respect to the quality, safety and long-term health effects of their food. Many consumers remain suspicious of the effect of industrialisation of food production on the health of the consumer and his family (European Technology Platform – Food for Life, 2007). For this reason, there is an increasing interest in "green" food products, that is, with fewer synthetic additives but increased safety, quality and shelf-life; which has led to an interest in the use of natural ingredients with antimicrobial activity as aids in food preservation.

Recent studies have documented the antibacterial effect of plant essential oils and their possible applications as food preservatives (Lachowicz et al., 1998; Canillac and Mourey, 2001; Burt, 2004; Ponce et al., 2004; Moreira et al., 2005; Valero and Giner, 2006; Cava et al., 2007). The antimicrobial properties of several vegetable ingredients (herbs, fruits and spices) have also been recognised and used since ancient times for food preservation and medicine (Zaika, 1988; Conner, 1993); however few of these ingredients are actually used in the food industry with purposes other than flavouring.

The effects of different natural antimicrobials and essential oils have already been studied in *Bacillus cereus* (Periago and Moezelaar, 2001; Delgado et al., 2004; Falcone et al., 2005; Valero et al., 2006; Majhenic et al., 2007). This microorganism is an ubiquitous, spore-forming, facultative anaerobic rod. Many authors have documented the presence of *B. cereus* in raw and processed meat, vegetable, rice, eggs and dairy products (Dufrenne et al., 1994; Hassan and Nabbut, 1996; Carlin et al., 2000; Valero et al., 2002; Collado et al., 2006). As *B. cereus* spores are resistant to mild heat treatments, the use of natural antimicrobial compounds could be an additional control measure of the growth of surviving spores under conditions of refrigerated storage abuse temperature or cold chain brake.

Cocoa has numerous uses in the food industry. It is most commonly used to make chocolate, but it is also used as flavouring in drinks, cookies, ice cream and other products. Cocoa polyphenols (Wollgast and Anklam, 2000), and particularly flavan-3-ols (Aron and Kennedy, 2008), have been reported to exhibit several health benefits by acting as antioxidant, anti-carcinogen, cardio-preventive, antiviral and neuro-protective agents. Cocoa polyphenols have also shown an antimicrobial effect *in vivo* (Smullen et al., 2007), specifically 3-flavonol (Aron et al., 2008) which seems to be the compound responsible for this activity. Nevertheless, its antimicrobial activity has not been extensively studied and there are only a few studies regarding the antimicrobial capacity of cocoa powder when used as a food ingredient (Gabis and Langlois, 1967; Busta and Speck, 1968). Moreover, no systematic studies have been carried out to calculate how it affects microbial growth rates and lag phase parameters.

In the present work, the effect of this food ingredient on the growth of *B. cereus* spores in reference media as a model was studied and kinetic parameters were deduced by fitting the results to a mathematical model.

Materials and Methods

Bacterial Spore Preparation

Bacillus cereus (ATCC 14579) type strain was provided by the Spanish Type Culture Collection and used as target microorganism.

Spores were obtained using the sporulation medium described by Mazas et al. (1995) slightly modified, according to Ferrer et al. (2009). Flasks containing 500 ml of sporulation medium were inoculated with 1 ml of an overnight culture of *B. cereus* in nutrient broth, and incubated at 32 ºC. Sporulation development was checked daily using phase contrast microscopy.

After four days, when the spore crop contained approximately 95 % of phase-bright spores, spores were harvested and washed with sterilised distilled water by repeated centrifugation at 2500 g. Spores were then resuspended in sterilised distilled water at a final concentration of 1 x 10^5 spores/ml and stored at 4 ºC until use.

Flavonol-rich Cocoa Powder

The cocoa powder used for this experiment was CocoanOX (Natraceutical S. A., Valencia, Spain), a flavanol-rich cocoa powder produced from unfermented, blanch-treated, non-roasted cocoa beans using the procedure described by Tomás-Barberán et al. (2007). Comparison with conventional cocoa powder showed that the flavanol-rich powder contained eight times more epicatechin and procyanidin B2 than the conventional cocoa powder. In addition, this flavanol-rich cocoa was demonstrated to have a high bioavailability in humans when it was administered in a milk drink (Tomas-Barberán et al., 2007). Total polyphenol content and the corresponding flavan-3-ols profile of the product used in this study were presented in Table 1.

TABLE 1: *Total polyphenols (mg/g) and flavan-3-ols (mg/g) of the flavanol-rich cocoa powder used in this study.*

Total Polyphenols (mg/g)[1]	**17.454 ± 0.0132**
Total flavan-3-ols[2]	**45.843 ± 0.251**
(+) Catechin	5.570 ± 0.096
(–) Epicatechin	20.834 ± 0.032
Procyanidin B2	18.128 ± 0.069
Procyanidin B1	1.351 ± 0.073

The results are expressed on a dry basis as mean ± SD (n=2). [1]Spectrophotometric method Folin-Ciocalteu. Results expressed as catechin equivalent. [2]DAD-HPLC.

Growth Curves of *B. cereus* Spores

100 µl of the stored *B. cereus* spore suspension were inoculated in 50 ml flasks containing 20 ml of sterilized mixture of BHI broth (Scharlau Chemie, S. A., Barcelona, Spain) and flavonol-rich cocoa powder at different concentrations (0, 1.5, 2.5 and 5 %) to a final concentration of 5 x 10^2 spores/ml. Inoculated vials were incubated at 32, 20 and 7 ºC. Temperatures of 20 and 7 ºC were chosen to simulate cold chain break and refrigerated storage abuse temperatures, respectively. Samples from each vial were analysed at specific time intervals (0, 1, 2, 3, 6, 10, 24, 27, 30 h).

Serial decimal dilutions of the samples were made in peptone water (Scharlau Chemie, S. A., Barcelona, Spain) and poured plated in duplicate with BHI agar (Scharlau Chemie, S. A. Barcelona, Spain). All plates were then incubated at 32 ºC for 24 h. All experiments were carried out in triplicate.

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Mathematical Modelling

Growth data at the different temperature and cocoa powder concentration combinations were modelled using the Modified Gompertz Equation (Zwietering et al., 1990) (Equation 1), in order to obtain growth kinetic parameters (maximum specific growth rate, μ_{max}, and lag phase, λ) by using the Statgraphics Centurion version XV (Statpoint Inc., Herndon, Virginia, USA).

$$y(t) = y_0 + (y_{max} - y_0) \cdot \exp\left\{-\exp\left[1 + \mu_{max} \cdot e\left(\frac{\lambda - t}{y_{max} - y_0}\right)\right]\right\} \quad (1)$$

Statistical Analyses

All statistical analyses were performed using the statistic software the Statgraphics Centurion version XV. The effects of temperature and cocoa powder concentrations were studied by applying multifactor ANOVA. In the cases where the effects were significant, the values were compared using Fisher's least significant difference (LSD) test ($p<0.05$).

Results and Discussion

The growth of *Bacillus cereus* at different flavonol-rich cocoa powder concentrations (0, 1.5, 2.5 and 5 %) and different incubation temperatures which mimic abuse or cold break temperatures (32, 20 and 7 °C) was studied. Figure 1 shows an example of *B. cereus* growth curve at the most favourable incubation temperature (32 °C) and different flavonol-rich cocoa powder concentrations. The flavonol-rich cocoa powder appeared to have a bacteriostatic effect on *B. cereus* at lower concentrations, showing a bactericidal effect at 5 %, as can be seen by the decrease in the population observed when increasing the added concentration of flavonol-rich cocoa powder. Table 2 shows the *B. cereus* population (log N/N_0) after 24 h incubation, when the populations had already reached stationary phase, at the different temperatures and flavonol-rich cocoa powder concentrations. Significant effects ($P<0.001$) were observed for both, temperature and flavonol-rich cocoa powder concentration, showing a decrease in *B. cereus* population when increasing the flavonol-rich cocoa powder and/or decreasing the temperature.

The experimental data for the growth of *B. cereus* spores with and without the ingredient were fitted to the modified Gompertz equation (1). The Gompertz equation has been extensively used by researchers to fit a wide variety of growth curves from different micro-organisms (McKellar and Lu, 2004). Chorin et al. (1997) already used this equation to model *B. cereus* growth in BHI broth obtaining a good fitness. Table 3 shows mean values and standard deviation for the growth parameters (maximum specific growth rate, μ_{max}, and lag phase, λ) obtained in the present work for *B. cereus* at the different studied conditions. Temperature and concentration had significant effects ($P<0.001$) on the μ_{max}, which decreased when increasing the cocoa powder concentration or decreasing the incubation temperature. Regarding λ, only the temperature showed a significant effect ($P<0.001$) lengthening the lag phase duration, while the effect of increasing the concentration were only significant at 32 °C ($P<0.05$), but not at 20 °C where the temperature effect seemed to be more important.

These results obtained in these experiences were interesting as they indicate the ability of flavonol-rich cocoa powder in combination with low temperatures to control the growth of *B. cereus* spores. The decrease in the growth rate caused by cocoa powder would present an advantage in the case of cold chain break represented by 20 °C, as this would add an extra microbiological control hurdle to the foodstuffs (Leistner, 2000). Moreover, it would be useful in under-developed tropical countries, as it seemed to have a good effectiveness at higher temperatures.

There is a general agreement on the antimicrobial activity of plant polyphenols (Proestos et al., 2005), which suggests that the high content of flavonols present in the cocoa powder used in this study must be the responsible for its antimicrobial activity. Similar effects against *B. cereus* have been reported for other polyphenol rich food and

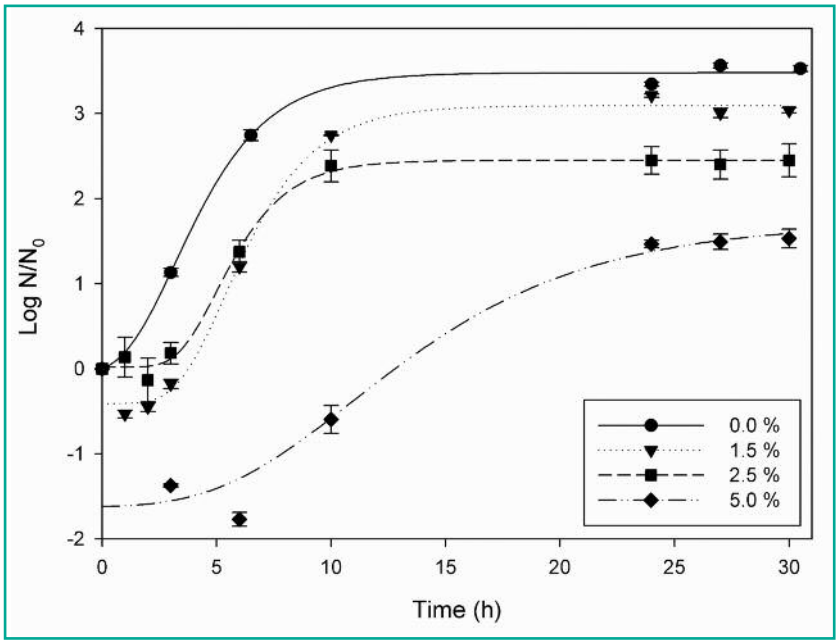

FIGURE 1: *Growth of B. cereus spores at 32 °C in BHI supplemented with various concentrations of flavonoid-enriched cocoa powder. Symbols represent mean values and standard deviation of the experimental data and lines represent adjusted growth curbs.*

TABLE 2: *Mean values and standard deviation of B. cereus population in reference media at the different temperatures and flavonoid-ekriched cocoa powder concentrations after 24 h incubation.*

Cocoa powder concentrations (%)	*B. cereus* population (log N/N_0) 32 °C	20 °C	7 °C
0.0 %	3.34 ± 0.03 a^1	2.87 ± 0.11 a^2	0.37 ± 0.20 a^3
1.5 %	3.21 ± 0.02 a^1	1.32 ± 0.04 b^2	–0.81 ± 0.01 b^3
2.5 %	2.45 ± 0.16 b^1	1.40 ± 0.07 b^2	–1.06 ± 0.13 b^3
5.0 %	1.47 ± 0.04 c^1	–2.12 ± 0.04 c^3	–0.99 ± 0.10 b^2

a–c: Values with different letters indicate significant differences between concentrations at the same temperature. $^{1–3}$: Values with different numbers indicate significant differences between temperatures at the same concentration.

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TABLE 3: *Mean values and standard deviations of the maximum specific growth rate (μ_{max}) and lag phas (λ) e at the different studied conditions.*

Cocoa powder concentrations (%)	Maximum specific growth rate (μ_{max}, h^{-1})	Lag phase (λ, h)	R^2
32 °C			
0.0 %	0.574 ± 0.026 b^1	0.921 ± 0.131 c^2	0.9974
1.5 %	0.630 ± 0.009 a^1	3.579 ± 0.137 b^2	0.9910
2.5 %	0.523 ± 0.020 c^1	3.304 ± 0.197 b^2	0.9931
5.0 %	0.328 ± 0.005 d	4.637 ± 1.276 a	0.9999
20 °C			
0.0 %	0.283 ± 0.024 a^2	8.872 ± 0.907 a^1	0.9921
1.5 %	0.185 ± 0.012 b^2	5.713 ± 0.626 c^1	0.9967
2.5 %	0.141 ± 0.037 b^2	7.300 ± 0.094 b^1	0.9886
5.0 %	ng	ng	
pT	***	***	
pC	***	ns	

ng: no growth. a–d: Values with different letters indicate significant differences between concentrations at the same temperature. ^{1}–^{2}: Values with different numbers indicate significant differences between temperatures at the same concentration. pT, pC: Effect of temperature and concentrations on μ_{max} and λ. ***: Significant p<0.001. ns: not significant.

herbs, as is the case of Tunisian pome fruit (Fattouch et al., 2008), finger millet (Viswanath et al., 2009), Chinese green tea (Si et al., 2006) and *Salvia* spp. (Ozkan et al., 2010). In order to confirm which compounds were responsible of antimicrobial activity in Chinese green tea, Si et al. (2006) applied bioassay guided fractionation technique. These authors found that two compounds derived from catechin and epicatechin (specifically epicatechin gallate and epigallocatechin gallate) were the most active antimicrobials. The mechanism of antimicrobial activity of these compounds has been attributed to its effects on cell wall components (Ikigai et al., 1993) and related enzymes (Blanco et al., 2003; Zhang and Rock, 2004). However, Si et al. (2006) observed by electron microscopy analysis that most *B. cereus* cells appeared to be locked in the process of division during the treatment, which suggested polyphenol treatments might interfere with the bacterial cell division. Catechin and epicatechin were two of the compounds present in the flavonol-rich coca powder, and therefore they could be partly responsible for the observed decrease in the growth rate when increasing the ingredient concentration.

Conclusion

This is the first attempt to systematically analyse the effect of cocoa on *Bacillus cereus* spores in order to obtain kinetic parameters accounting for this effect. The results indicated that flavonol-rich cocoa powder had a bactericidal effect at high concentrations and only bacteriostatic at lower concentrations. This food ingredient could be used as an additional control measure in combination with other physical preservation treatments and in the case of cold chain break. However, further studies in foods are required in order to assess the efficiency of this compound as a preservative when in contact with the food matrix. It would also be important to know whether a synergistic effect of cocoa powder and another physical control measure will take place.

Natural antimicrobials may contribute to extend food shelf-life and improve safety by reducing the presence of pathogens, such as *B. cereus, Listeria* spp., *Salmonella* spp. or *Staphylococcus* spp. Considering various research works carried out by different authors, protection for some types of food may be provided through different combinations of naturally existing compounds such as chitosan extracts from shellfish, bacteriocins produced by bacteria and plant components such as essential oils.

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Address for correspondence:
Prof. Dr. Antonio Martínez López
Instituto de Agroquímica y Tecnología de Alimentos
Avda. Agustín Escardino, 7.
46980 Paterna (Valencia)
Spain
conaml@iata.csic.es

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