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The fate of *Bacillus cereus* and *Geobacillus stearothermophilus* during alkalization of cocoa as affected by alkali concentration and use of pre-roasted nibs

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ABSTRACT

Alkalization is a step of cocoa processing and consists of the use of alkali and high temperature to improve the sensorial and technological qualities of cocoa. Intense food processing can select spores, which can compromise safety and quality of the final product. Thus, the aim of this study was to evaluate the fate of *B. cereus* and *G. stearothermophilus* spores during the alkalization of pre-roasted (Pr) nibs (held at 120 °C) and unroasted (Ur) nibs (held at 90 °C) using potassium carbonate (0, 2, 4 and 6% w/w). In all conditions, log-linear inactivation kinetics with a tail was observed. The inactivation rate (k_{max}) for *B. cereus* varied from 0.065 to 1.67 min^{-1}, whereas the k_{max} for *G. stearothermophilus* varied from 0.012 to 0.063 min^{-1}. For both microorganisms, the lowest k_{max} values were observed during Ur nibs alkalization. The carbonate concentration increase promoted k_{max} values reduction. The highest tail values were observed for *G. stearothermophilus* in Ur nibs alkalization, reaching 3.04 log spores/g. Tail formation and low k_{max} values indicated that cocoa alkalization does not cause significant reductions on bacterial spore population. Therefore, the microbiological control should be primarily ensured by the raw material quality and by avoiding recontamination in the cocoa chain.

1. Introduction

Due to their composition, bacterial spores are structures of great resistance to physical and chemical agents, such as extreme conditions of pH and temperature, the presence of antimicrobial compounds or low water activity (Furukawa et al., 2009; Wells-Bennik et al., 2016). Due to this extreme resistance to adverse environmental factors, the spore-forming bacteria are regularly reported as causative agents of food spoilage (Lücking et al., 2013; Piskernik et al., 2016; Yang et al., 2011) and foodborne disease outbreaks (Andersson et al., 1995; Carlin et al., 2000; Feng et al., 2015; Lund et al., 2000). *Bacillus cereus* and *Geobacillus stearothermophilus* stand out among the pathogenic and spoilage spore-forming microorganisms of importance in foods (André et al., 2017; Pereira and Sant'Ana, 2018; Wells-Bennik et al., 2016).

B. cereus is capable of producing toxins (emetic and diarrheic toxins) associated with pathological syndromes in human beings (Abee et al., 2011). Furthermore, it can also be associated with food spoilage (Peña et al., 2014; Warda et al., 2015). Despite being commonly associated to foods subjected to mild thermal processes, such as pasteurization (Hayrapetyan et al., 2016), *B. cereus* can also survive to more intense heat processes, especially when the physical properties of food difficult heat transfer (Nascimento et al., 2012). *G. stearothermophilus* is a thermophilic bacterium that can survive to thermal processing of several low acid foods due to the high thermal resistance of its spores (Furukawa et al., 2009; Zhou et al., 2013). *G. stearothermophilus* spores that survive thermal processing of low acid foods can germinate and outgrow during their storage when temperatures exceed 35–40 °C. *G. stearothermophilus* growth results in "flat sour" spoilage, i.e., product acidification without any gas production occurs (Kakagianni et al., 2016). Raw food materials are one of the most important sources of spore-forming bacteria such as *B. cereus* and *G. stearothermophilus* (Carlin, 2011; Pereira and Sant'Ana, 2018).

Ingredients obtained from cocoa, such as cocoa powder, have a diverse microbiota, including spore-forming bacteria (Gabis et al., 1970; Lima et al., 2012, 2011). The counts of spores during the production of cocoa ingredients may vary along the production chain, and the quantification of the effects of processing operations on spore-forming bacteria need to be characterized for better management of

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microbiological safety and quality (Pereira and Sant'Ana, 2018).

Alkalization (or "dutch process") is a unit operation employed during cocoa processing (Afoakwa et al., 2008) that combines the addition of an alkaline agent, usually potassium or sodium carbonate, and heating (Afoakwa et al., 2008; Bispo et al., 2005). This process aims at reducing fermented almonds acidity, increasing the solubility of cocoa powder and contributing to the taste and flavor of cocoa-based products. Furthermore, alkalization provides products with different colors and tonalities, such as light brown, red or even dark black (Gültekin-Özgüven et al., 2016; Li et al., 2012; Miller et al., 2008; Minifie, 1999). The type of alkalis and the alkalization conditions, including alkali concentration (1–6%), temperature (60 °C to 120 °C) and process time (30–150 min) are highly diverse and dependent on the desired sensorial and technological properties of final products (Bispo, 1999; Bispo et al., 2005; Li et al., 2014; Minifie, 1999). Alkalization can be applied to the cocoa liquor, pie, cocoa powder, unroasted or pre-roasted nibs (Afoakwa, 2010; Taş and Gökmen, 2016). When alkalization is employed to cocoa nibs, their pH can increase from 5.5 to 6.8–8.0 at the end of alkalization (Morr and Richter, 1999). All these conditions observed during the alkalization process could select the most resistant micro-organisms, such as spore-forming bacteria, which may further cause several problems in foods produced from cocoa (Witthuhn et al., 2011). However, for the improvement of the quality and microbiological safety of products obtained from cocoa, data about the influence of cocoa processing steps on spore-forming microorganisms must be available. Therefore, in this work, the fate of *B. cereus* e *G. stearothermophilus* spores inoculated in unroasted (Ur) and pre-roasted (Pr) cocoa nibs subjected to alkalization processes at different concentration of alkali were quantified.

2. Materials and methods

2.1. B. cereus and G. stearothermophilus strains and preparation of spore suspensions

G. stearothermophilus strains (n = 5) belonged to standard culture collections, but no information was available on their primary sources: Coleção de Cultura do Genêro *Bacillus* e Gêneros correlatos (CCGB) 792 [Laboratório de Fisiologia Bacteriana (LFB) - Fundação Oswaldo Cruz (FIOCRUZ) 792]; CCGB 793 [LFB FIOCRUZ 793]; CCGB 794 [LFB FIOCRUZ 794]; Instituto Adolfo Lutz (IAL) 2128 and American Type Culture Collection (ATCC) 7953TM. The *B. cereus* strains (n = 5) employed were CCGB FIOCRUZ 432 and CCGB FIOCRUZ 436 (isolated from white chocolate), CCGB FIOCRUZ 511 and CCGB FIOCRUZ 512 (isolated from chocolate fortified with vitamins), and CCGB FIOCRUZ 540 (isolated from a chocolate drink).

2.2. Preparation of suspensions of spores of B. cereus and G. stearothermophilus

Each strain was grown on Nutrient Broth (Kasvi, Curitiba, Brazil) supplemented with 5 mg/L of manganese sulfate (NB-Mn) incubated at 30 °C (*B. cereus*) and 55 °C (*G. stearothermophilus*) for 24 h. Then, a volume of 2 mL of NB-Mn of each previous culture was transferred to Roux bottles (37 per strain) containing Nutrient Agar (Kasvi, Curitiba, Brazil) supplemented with 5 mg/L of manganese sulfate (NA-Mn). The Roux bottles were incubated at 30 °C (*B. cereus*) and 55 °C (*G. stearothermophilus*)(Peña et al., 2014; Pflug, 1999). Malachite green staining of the cultures was regularly employed using an optical microscope under a 1000 × magnification to check the presence of spores. Spores were harvested using a sterile glass stick and sterile distilled water when representing > 90% of the cell population (Spinelli et al., 2010), i.e., between 23 and 36 days for *B. cereus* and 21 and 40 days for *G. stearothermophilus*. The harvested spore suspensions were centrifuged at 4000 × *g* at 4 °C for 20 min (Oteiza et al., 2014), suspended again in sterile distilled water for washing. The procedures of washing and centrifugation were repeated twice, and resulting pellets were suspended in sterile distilled water. Spore suspensions of each strain were heat shocked (70 °C for 15 min for *B. cereus* and 110 °C for 10 min for *G. stearothermophilus*), and their concentrations were determined by enumeration on NA (Bennett et al., 2001; Olson and Sorrells, 2001). Aliquot volumes of the spore suspensions were frozen (− 20 °C) separately for subsequent experimental use. A cocktail with equal concentrations of the five *B. cereus* or *G. stearothermophilus* strains (approx. 10^8 spores of each strain per mL) was prepared before experiments by mixing a specific volume of each of the five strains of each species.

2.3. Cocoa nibs

Two types of cocoa nibs (pre-roasted and unroasted nibs) were donated by a cocoa powder processing plant located in the state of Bahia, Brazil. Pre-roasted (Pr) cocoa nibs were produced from seeds that were fermented, roasted at 110–140 °C for 10–60 min and broken to obtain cocoa flakes (nibs). Pr cocoas nibs are subsequently subjected to the alkalization step. Unroasted (Ur) nibs are produced from fermented and broken seeds, being submitted to alkalization step and, further roasted. Thus, in the laboratory, both Pr and Ur nibs were submitted to alkalization process to mimic the effect of different industry practices on inactivation of *B. cereus* and *G. stearothermophilus* spores, *i.e.*, roasting prior and after alkalization. Before the experiments, spores of *B. cereus* (Bennett et al., 2001) and thermophilic aerobic bacteria (Olson and Sorrells, 2001) were enumerated in the cocoa nibs. Populations in Ur and Pr nibs of *B. cereus* were lower than < 2 log spores/g (limit of quantification), and populations of thermophilic spore-formers were lower than 3.5 log spores CFU/g.

2.4. Inoculation of cocoa nibs with B. cereus and G. stearothermophilus spores

Approximately 150 g samples of cocoa nibs were dispensed into sterile plastic bags. Then, cocoa nibs were separately inoculated with the spore suspensions (item 2.2) of *B. cereus* or *G. stearothermophilus* using a micropipette. The volume of suspension used was sufficient to reach a final concentration of 10^6 spores/g of cocoa nibs. The nibs were homogenized and dried on sterile trays at 30 °C for 15 min, so their water activity returned to the original value (i.e., approximately 0.6 for Ur nibs and 0.3 for Pr nibs).

2.5. Alkalization process

2.5.1. Alkalization conditions and agent

Alkalization conditions were employed to simulate industrial practices. Alkalization of Ur and Pr cocoa nibs were conducted at 90 °C and 120 °C, respectively (Minifie, 1999). The alkalization agent was potassium carbonate (Dinâmica, Diadema, Brazil), at 0 (control), 2, 4 and 6% (w/w) (Bispo, 1999). The alkalization solutions were prepared by dissolving potassium carbonate in sterile distilled water to reach the desired concentrations. Thus, a total of 16 experiments were conducted to assess the inactivation kinetics of *B. cereus and G. stearothermophilus* spores in unroasted and pre-roasted nibs at 90 °C and 120 °C. The experiments were replicated twice.

2.5.2. Alkalization equipment and experiments

The alkalization process was simulated in a jacketed stainless steel reactor coupled to a thermostatic oil bath (model N3, HAAKE, Karlsruhe, Germany). A mechanical stirrer (model 0250M2, Quimis, Diadema, Brazil) set to 200 rpm was used to homogenize the nibs with the alkalization agent. All the equipment used in the experiments were previously sterilized in an autoclave at 121 °C for 15 min. The alkalization process began with the addition of alkalization solution on the inoculated cocoa nibs in a proportion of 1:2 (alkali solution: nibs; w/w), as employed by cocoa industries (Minifie, 1999). The process time

started when nibs were transferred to a previously heated reactor. Samples of inoculated and alkali-treated nibs were collected at appropriate time intervals (according to the tested microbial species or alkali concentration) for microbial enumeration, pH and a_w.

2.6. Enumeration of B. cereus and G. stearothermophilus spores

The counts of *B. cereus and G. stearothermophilus* spores during alkalization processes studied were determined in 10 g of samples. The samples of cocoa nibs were stomached in 90 mL of sterile distilled water for 30 s (Stomacher® 400 circulator, model BA7021, Seward, London, England). The counts of *B. cereus* spores were done after application of heat shock (70 °C/15 min, followed by cooling in a water and ice bath), and plating onto Mannitol Egg Yolk Polymyxin agar (MYP, Oxoid, Basingstoke, UK) (Bennett et al., 2001). For enumeration of *G. stearothermophilus* spores, a heat shock at 110 °C/10 min (followed by cooling in water and ice bath) was applied. The aliquots were inoculated on Dextrose Tryptone agar (DTA), formulated with the following ingredients: tryptone (10 g/L, Acumedia, Lansing, USA), dextrose (5 g/L, Dinâmica, Diadema, Brazil), agar-agar (15 g/L, Oxoid, Hampshire, England) and 1% bromocresol purple solution (4 mL/L, Dinâmica, Diadema, Brazil) (Olson and Sorrells, 2001). MYP and DTA plates were incubated at 30 °C/24 h and 55 °C/48 h, respectively. Colonies were counted, and the results were expressed as log (base 10) spores/g.

2.7. pH and a_w measurement throughout the alkalization process

The pH was determined using a previously calibrated pHmeter (model K39-2014B, KASVI, China) (Instituto Adolfo Lutz, 2008). The a_w measurement was done using AquaLab equipment (model CX-2, Decagon Devices, Washington, USA). The pH and a_w measurements were done in triplicate for each of the samples collected in the different processes (replicates). Both analyses were performed at room temperature.

2.8. Inactivation kinetics of B. cereus and G. stearothermophilus spores during cocoa nibs alkalization

The add-in for Microsoft Excel GinaFit was used to fit inactivation models to the data obtained in this study (Geeraerd et al., 2005). Spore surviving populations (log spores/g) were plotted as a function of time. Survival curves were fitted using the model of Geeraerd (Geeraerd et al., 2000) (equation (1)) since kinetics presented log-linear inactivation followed by tail formation.

$$logN(t) = \log\left(\left(10^{logN_0} - 10^{\log(N_{res})}\right) \times e^{(-k_{max} \times t)} + 10^{\log(N_{res})}\right) \quad (1)$$

Where ***t*** is time [minutes]; **logN(t)** is the survivor population [log (base 10) spores/g] at time "*t*"; $\mathbf{logN_0}$ is the initial population [log spores/g]; $\mathbf{logN_{res}}$ is the residual population [log spores/g]; and $\mathbf{k_{max}}$ is the constant inactivation rate [min^{-1}].

For each microorganism and condition studied, two inactivation curves were individually fitted, and the inactivation kinetic parameters were obtained.

To evaluate the effect of carbonate concentration on the inactivation rate, $logk_{max}$ *vs*. carbonate concentration graphs were generated.

The time to reach 3 log reductions (t_{3D}) for each condition studied was estimated by solving equation (1) for the case in which N(t) = N (t_{3D}) = $N_0 \times 10^{-3}$, resulting in the following equation (2):

$$t_{3D} = \frac{-1}{k_{max}} \times \ln \frac{(10^{-3} \times N_0) - N_{res}}{N_0 - N_{res}} \quad (2)$$

The maximum logarithmic reduction (γ_{max}) observed in each process, considering the effect of tail formation, was calculated with equation (3):

$$\gamma_{MAX} = log\,(N_{res}) - log\,(N_0) \quad (3)$$

Finally, the time to *B. cereus* or *G. stearothermophilus* spore populations become approximately N_{res}, $t_{(N \approx Nres)}$ [minutes], was obtained by solving equation (1) for logN (t) = logNres + 0.1, resolved by the following formula (equation (4)):

$$t_{(N \approx N_{res})} = \frac{-1}{k_{max}} \times \ln \frac{N_{res} \times 0.259}{N_0 - N_{res}} \quad (4)$$

2.9. Statistical analysis

The mean and the mean absolute deviation of the inactivation kinetic parameters obtained for each condition and microorganism were calculated. Analysis of variance (ANOVA), followed by Scott-Knott and t tests were employed using Assistat version 7.7 (Campina Grande, Brazil) (Silva and Azevedo, 2002). Significant differences were found when $p \leq 0.05$ (Granato et al., 2014; Nunes et al., 2015).

3. Results

3.1. Variation of pH and a_w throughout the nibs alkalization

Fig. 1 shows the variation of pH and a_w as a function of the time of alkalization. The pH values for Pr nibs and Ur nibs before the alkalization processes were 5.19 and 5.15, respectively. The pH of nibs increased to 8.69, 9.31, and 9.50 immediately after addition of K_2CO_3 at concentrations of 2, 4, and 6% w/w, respectively and only slightly varied during the further process, with a tendency to decrease (Fig. 1a and c). Ur and Pr nibs had initial a_w values of 0.596 and 0.318, respectively. In the processes conducted with Pr which have a shorter duration (t ≈ 40 min), a_w remained > 0.80 (Fig. 1d), regardless of K_2CO_3 concentration. In the alkalization with a long time process, however, a_w tremendously decreased to 0.10 because of the long period of dehydration of cocoa nibs (Fig. 1b and d). Considering usual process times, a_w of Ur nibs was 0.932 after 60 min and a_w of Pr nibs was 0.934 after 30 min.

3.2. Inactivation kinetics of B. cereus and G. stearothermophilus spores throughout the nibs alkalization

The inactivation curves obtained for *B. cereus* and *G. stearothermophilus* spores during the alkalization of Ur nibs (process temperature = 90 °C) and Pr nibs (process temperature = 120 °C) using different concentrations of K_2CO_3 can be seen in Figs. 2 and 3, respectively.

In all treatments, regardless of temperature and alkali content, the spores of *B. cereus* and *G. stearothermophilus* showed death kinetics characterized by a log-linear inactivation followed by a tail. These inactivation curves were satisfactorily described by the model of Geeraerd (Geeraerd et al., 2000). All adjusted models presented coefficients of determination $R^2 > 0.95$ (Table 1). The fitted models presented low values of root mean square error (RMSE), which ranged from 0.09 to 0.33 for the *G. stearothermophilus* inactivation curves and from 0.18 to 0.31 for the *B. cereus* inactivation curves. The k_{max}, $logN_{res}$, t_{3D} (time to 3 decimal reductions) values in each experimental condition are presented in Table 1.

3.3. Influence of alkalization conditions on k_{max} values

The k_{max} of *B. cereus* during the alkalization of Ur nibs (90 °C) ranged from 0.065 to 0.108 min^{-1} depending on the K_2CO_3 concentrations while during the alkalization of Pr nibs (120 °C), k_{max} were higher (0.61–1.67 min^{-1}). The k_{max} of *B. cereus* was significantly higher ($p < 0.05$) in nibs processed at 120 °C than in nibs in processed at 90 °C nibs. Regarding *G. stearothermophilus,* the k_{max} values (Table 1)

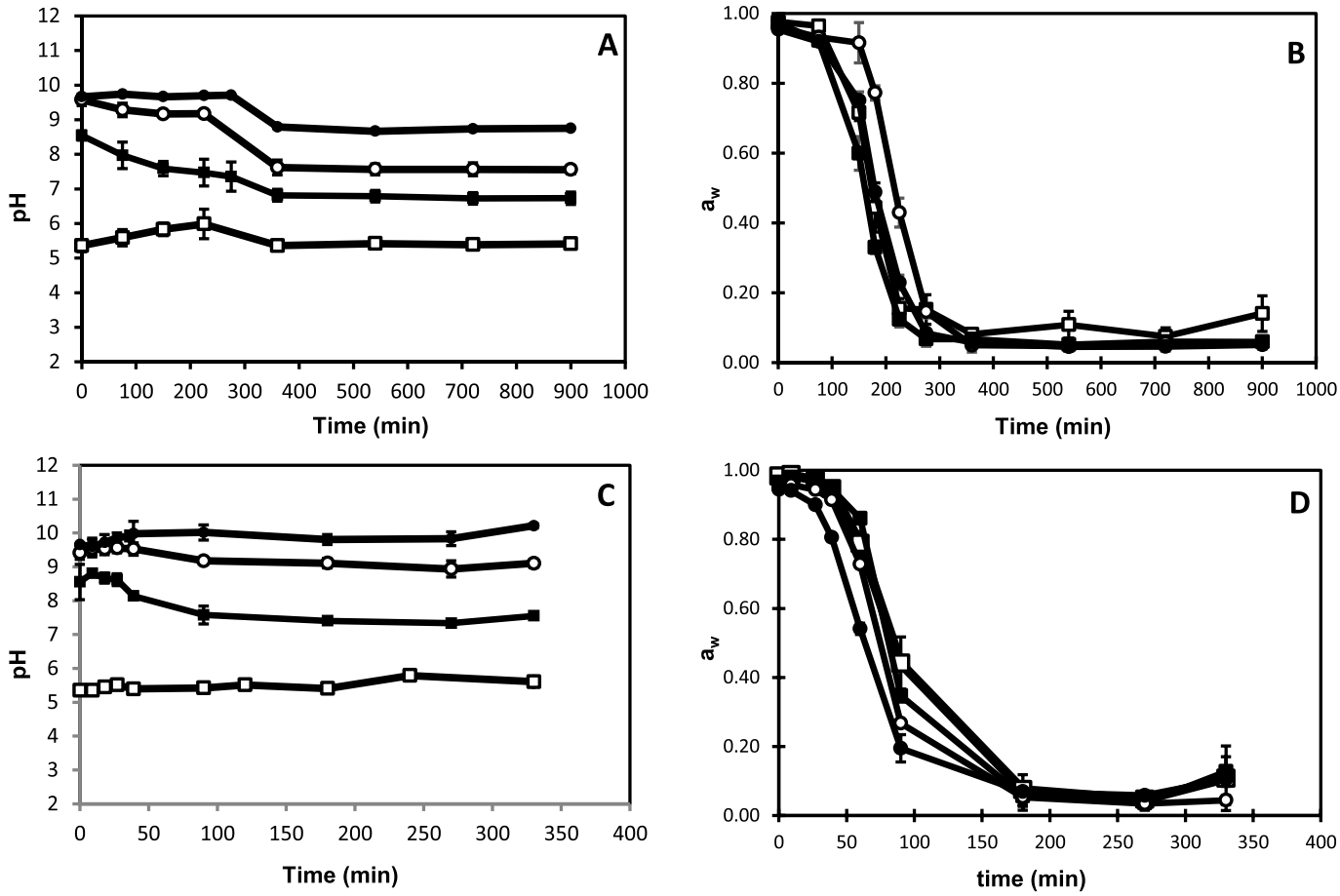

Fig. 1. Variation of intrinsic factors of cocoa nibs during different alkalization conditions. (A) pH and (B) water activity of Ur nibs during alkalization processes at 90 °C. (C) pH and (D) water activity of Pr nibs during alkalization processes at 120 °C. The symbols represent the average of data from two independent replicates of alkalization with 0% (□), 2%(■), 4%(○) and 6%(●) of K_2CO_3. Standard deviation is represented by error bars.

ranged from 0.012 to 0.021 min^{-1} in the alkalization of Ur nibs. In the alkalization of Pr nibs, k_{max} values ranged between 0.046 and 0.063 min^{-1}. Similarly, for each K_2CO_3 concentration, k_{max} was higher (~3.7 times) in Pr nibs than in Ur nibs ($p < 0.05$).

In order to evaluate the influence of the alkalizing agent concentration on the inactivation parameters of bacterial spores, the alkalization process was carried out at different concentrations (2, 4 and 6% of K_2CO_3, w/w). Additionally, a negative control (0% K_2CO_3) was also evaluated. According to the Scott-Knott test, the variation of the K_2CO_3 concentration in the processing of Pr nibs did not cause a significant change in the k_{max} for both microorganisms ($p > 0.05$). On the other hand, the increase in the K_2CO_3 concentration in the processing of Ur nibs increased the k_{max} values of *B. cereus* (from 0.065 to 0.098 min^{-1}) ($p < 0.05$), as can be seen in Table 1.

Comparing the k_{max} values of the spores in the processes containing K_2CO_3 to the negative control (0% K_2CO_3), there was a reduction in the thermoresistance of *G. stearothermophilus* spores, while the opposite behavior occurred for *B. cereus* spores (Fig. 4). However, when the K_2CO_3 concentration increased, the k_{max} in all the experiments also increased even if slightly. For *G. stearothermophilus*, the increase in K_2CO_3 concentration in processes conducted at 90 °C increased the k_{max} by 1.8 times, while at 120 °C this increment was 1.4 fold. It is noteworthy that the k_{max} profile concerning the K_2CO_3 concentration was the same at both temperatures (Fig. 4). Regarding *B. cereus*, the increase of the K_2CO_3

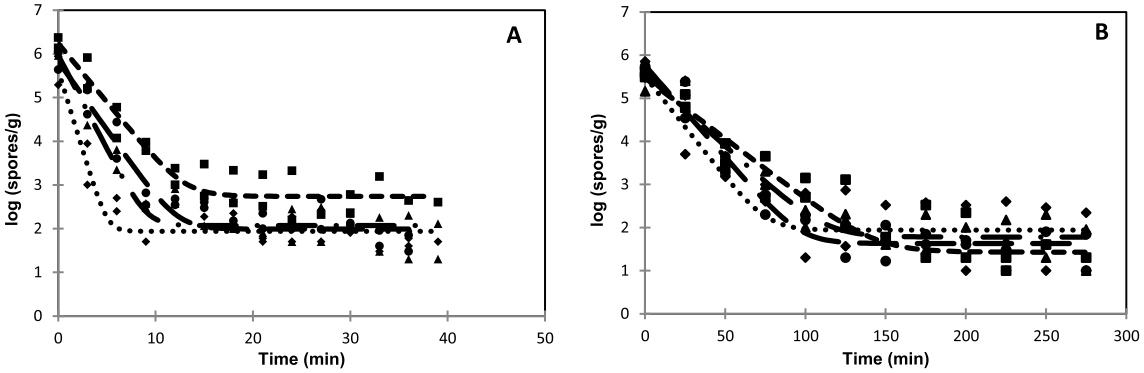

Fig. 2. Inactivation curves of *B. cereus* spores on different alkalization conditions. (A) Pr nibs alkalization at 120 °C. (B) Ur nibs alkalization at 90 °C. The symbols represent observed individual data from two independent replicates of alkalization with 0% (♦), 2% (■), 4%(▲) and 6% (●) w/w of K_2CO_3. The lines represent the fitted Geeraerd model using average parameters from each fitted replicate for 0% (·····), 2% (– – – –), 4% (– · – ·) and 6% (— —) of K_2CO_3.

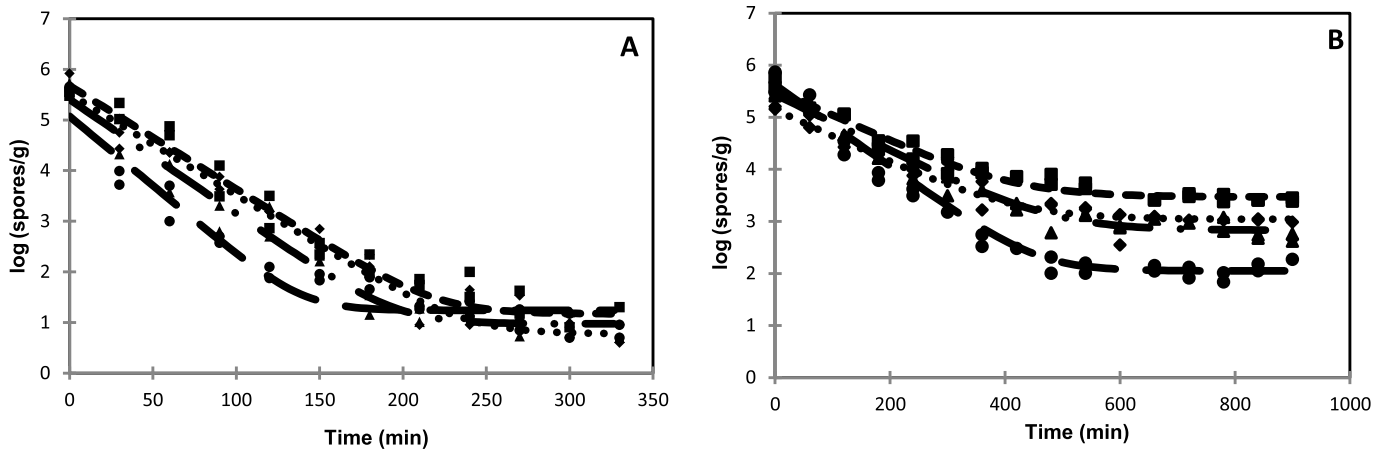

Fig. 3. Inactivation curves of *G. stearothermophilus* spores on different alkalization conditions. (A) Pr nibs alkalization at 120 °C. (B) Ur nibs alkalization at 90 °C. The symbols represent observed individual data from two independent replicates of alkalization with 0% (♦), 2%(■), 4%(▲) and 6% (●) (w/w) of K_2CO_3. The lines represent the fitted Geeraerd model using average parameters from each fitted replicate for 0% (·····), 2% (– – – –), 4% (– · – ·) and 6% (— —) of K_2CO_3.

concentration from 2 to 6% tended to increase the k_{max} values: ~1.5 and ~1.2 fold, respectively, to alkalization of Ur and Pr nibs.

3.4. Influence of alkalization conditions on $logN_{res}$ values

For *B. cereus* spores, the $logN_{res}$ parameters, namely the level of the residual population, varied between 1.44 and 2.74 ($p > 0.05$) under the different conditions. Only for *G. stearothermophilus* spores, all $logN_{res}$ values were significantly higher ($p < 0.05$) in the alkalization at 120 °C (2.07–3.47 log spores/g) in comparison to the alkalization at 90 °C (0.77–1.24 log spores/g) in the same carbonate concentration. Thus, for this microorganism, the increase in temperature in the alkalization of nibs reduced ~1.8 logs of $logN_{res.}$

In relation to the influence of the K_2CO_3 concentration on $logN_{res}$, only significant reduction ($p < 0.05$) was observed on $logN_{res}$ values of *G. stearothermophilus* at Ur nibs processing, since the K_2CO_3 concentration range between 2% and 6% (pH around 6.8 and 8.8, respectively) reduced 1.40 log cycles in $logN_{res}$ value.

Comparing the parameters between both microorganisms studied, the k_{max} values of *G. stearothermophilus* were lower in all experimental conditions than those found for *B. cereus* under the same conditions. As for $logN_{res}$, in the alkalinization at 90 °C, *G. stearothermophilus* presented the highest $logN_{res}$ values, while at 120 °C these values became less than obtained to *B. cereus*. These data indicate the $logN_{res}$ values are temperature-dependent to *G. steartothermophilus*, but not to *B. cereus.*

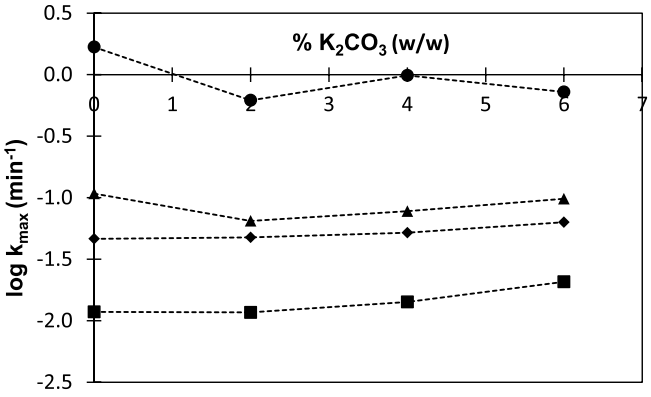

Fig. 4. k_{max} of spores on alkalization as a function of potassium carbonate concentration. *B. cereus* on (●) Pr nibs alkalization and (▲) Ur nibs alkalization; and *G. stearothermophilus* on (♦) Pr nibs alkalization and (■) Ur nibs alkalization.

3.5. Time to 3 logarithmic reductions and estimated reductions in conventional alkalization

The inactivation of *B. cereus* and *G. stearothermophilus* spores was also characterized by the time required to cause 3 logarithmic

Table 1
Inactivation parameters[1] of *B.cereus* and *G. stearothermophilus* during cocoa nibs alkalization.

Microorganism	$[K_2CO_3]$ (%w/w)	Pre-roasted nibs alkalization (T = 120 °C)					Unroasted nibs alkalization (T = 90 °C)				
		k_{max} (min^{-1})	t_{3D} (min)	Log N_{res}	R^2	RMSE[2]	k_{max} (min^{-1})	t_{3D} (min)	Log N_{res}	R^2	RMSE[2]
B. cereus	0.0	$1.67^{a,A}$ ± 0.64	6.9	$1.94^{a,A}$ ± 0.25	0.9476	0.31	$0.108^{a,B}$ ± 0.003	62.3	$1.94^{a,A}$ ± 0.69	0.9749	0.22
	2.0	$0.61^{a,A}$ ± 0.04	11.8	$2.74^{a,A}$ ± 0.30	0.9800	0.18	$0.065^{d,B}$ ± 0.002	108.2	$1.44^{a,A}$ ± 0.17	0.9707	0.27
	4.0	$0.98^{a,A}$ ± 0.07	7.2	$2.07^{a,A}$ ± 0.36	0.9768	0.22	$0.078^{c,B}$ ± 0.001	94.2	$1.74^{a,A}$ ± 0.31	0.9708	0.25
	6.0	$0.72^{a,A}$ ± 0.02	9.8	$1.99^{a,A}$ ± 0.01	0.9736	0.24	$0.098^{b,B}$ ± 0.002	73.1	$1.64^{a,A}$ ± 0.23	0.9859	0.19
G. stearothermophilus	0.0	$0.046^{a,A}$ ± 0.002	149.8	$0.77^{a,B}$ ± 0.11	0.9847	0.23	$0.012^{a,B}$ ± 0.002	> 900[3]	$3.04^{b,A}$ ± 0.02	0.9878	0.09
	2.0	$0.047^{a,A}$ ± 0.003	146.9	$1.17^{a,B}$ ± 0.35	0.9879	0.21	$0.012^{a,B}$ ± 0.001	> 900[3]	$3.47^{a,A}$ ± 0.01	0.9818	0.11
	4.0	$0.052^{a,A}$ ± 0.000	132.5	$0.97^{a,B}$ ± 0.04	0.9829	0.23	$0.014^{a,B}$ ± 0.002	> 900[3]	$2.86^{c,A}$ ± 0.03	0.9736	0.16
	6.0	$0.063^{a,A}$ ± 0.006	112.6	$1.24^{a,B}$ ± 0.19	0.9602	0.33	$0.021^{a,B}$ ± 0.003	362.7	$2.07^{d,A}$ ± 0.02	0.9924	0.13

[1]These values represent the mean ± standard deviation for each parameter estimated using data from two independent experiments. Different lowercase letters in the same column indicate significant differences in the inactivation kinetic parameters at different potassium carbonate concentrations at the same temperature and for the same spore-forming bacteria according to Scott knott ($p < 0.05$). Different capital letters in the same row, for the same inactivation kinetics parameter, indicate significant differences between the two alkalization processes tested according to the teste t ($p < 0.05$).
[2]Root Mean Square Error.
[3]At these conditions, the models did not estimate the time to reach three log reduction (t_{3D}), i.e., the difference between N_0 and N_{res} was < 3 log spores/g.

Table 2
Maximum number of logarithmic reductions (γ_{max}), time for tail formation ($t_{N \approx Nres}$) and logarithmic reductions after conventional alkalization processes using the parameters of the models generated in this study as input.

Microorganism	[K_2CO_3] (%w/w)	Pre-roasted nibs alkalization (T = 120 °C)			Unroasted nibs alkalization (T = 90 °C)		
		γ_{max}	$t_{(N-Nres)}$ (min)	γ_{30min} [a]	γ_{max}	$t_{(N-Nres)}$ (min)	γ_{60min} [b]
B. cereus	2.0	−3.50	15	−3.50	−4.04	165	−1.69
	4.0	−3.98	11	−3.98	−3.77	130	−2.01
	6.0	−3.82	14	−3.82	−4.15	114	−2.49
G. stearothermophilus	2.0	−4.53	250	−0.62	−2.12	547	−0.29
	4.0	−4.44	221	−0.68	−2.60	522	−0.37
	6.0	−3.89	165	−0.82	−3.55	464	−0.54

[a] Logarithmic reduction in an alkalization of pre-roasted nibs after 30 min at 120 °C.
[b] Logarithmic reduction in an alkalization of unroasted nibs after 60 min at 90 °C.

reductions (t_{3D}). For the alkalization process of Ur nibs inoculated with spores of *G. stearothermophilus* conducted at 0, 2 and 4% of K_2CO_3, t_{3D} values were much higher than the duration of the experiment, because of a high level of residual population. For the experiment conducted at 6% of K_2CO_3, the t_{3D} value was 362 min. In the processing of Pr nibs, the values ranged from 113 to 150 min. The t_{3D} values of *B. cereus* in the Ur nibs were between 62 and 108 min, whereas in the processing of Pr nibs, t_{3D} values were in the range of 7 and 12 min.

According to the adjusted models, the reduction achieved during the process times performed in industrial condition would be a relatively low (Table 2). For the more resistant microorganism, *G. stearothermophilus*, the reduction would be between 0.30 and 0.54 log cycle after 60 min of Ur nibs alkalization and between 0.62 and 0.82 log cycle after 30 min of Pr nibs alkalization. The maximum possible logarithmic reduction is obtained when the tail region is reached. In general, the times required for tail formation are longer than the commercial process times. Therefore, during the industrial process, the inactivation was restricted to the linear log region.

4. Discussion

Ensuring food safety and preventing food spoilage depend on an in-depth knowledge of the microbial ecology of foods and the dynamics of microorganisms as affected by food formulations and unit operations during food processing. Pasteurization and commercial sterilization are among the most frequently applied and therefore studied unit operations. Many quantitative data describing microbial behavior during these processes are available (Cerf and Condron, 2006; Mtimet et al., 2015; Oliveira et al., 2018; Sant'Ana et al., 2009). Nevertheless there still a lack of data regarding the impact of several other unit operations employed during food processing on microorganisms. As an example, the impact of unit operations employed during cocoa pre-processing, in particular, alkalization, on spore-forming bacterias hahaves not been reported yet (Pereira and Sant'Ana, 2018).

Alkalization is generally done with potassium carbonate (K_2CO_3). K_2CO_3 has alkalizing properties due to dissociation in aqueous solutions into potassium and carbonate ions. Carbonate ions have basic character and increase pH of cocoa nibs from 5.2 to values > 8.0 when added. Color changes seem to be associated with enzymatic darkening and formation of dark-colored polymeric compounds. Although it may have an additional effect, alkalization is not aimed at microbial destruction, which is mainly due to the high temperature, which can reach up to 120 °C (Minifie, 1999).

The alkalization temperature (90 °C and 120 °C) and K_2CO_3 concentrations varying from 2 to 6% were studied during simulated processes at a laboratory scale. Spores of *B. cereus* and *G. stearothermophilus* were used as targets to represent the fate of mesophilic and thermophilic spore-forming bacteria frequently found in cocoa (Gabis et al., 1970; Lima et al., 2011; Witthuhn et al., 2011). All inactivation curves were log-linear with a tail. The highest k_{max} values for both *B. cereus* and *G. stearothermophilus* were found in Pr nibs which is held at 120 °C; however, this process was found to be particularly detrimental for *B. cereus*, which is much less heat resistant than *G. stearothermophilus* (Furukawa et al., 2009). For instance, $D_{110°C}$ (decimal reduction time at 110 °C) values from 5.2 to 8.2 min have been reported for *B. cereus*, and $D_{110°C}$ values from 48.4 to 115.4 min for *G. stearothermophilus* (Sadiq et al., 2016).

G. stearothermophilus presented lower k_{max} values compared to *B. cereus* in all conditions studied, but seemed to be more sensitive than *B. cereus* to the addition of K_2CO_3. For *B. cereus,* the addition of 2–6% of K_2CO_3 in the system caused protection of the spores (thermoresistance increase when compared with control experiment), and this fact may be observed by the slightly reduced k_{max}. This phenomenon was observed with *B. cereus* spores probably because the addition of carbonate promoted an increase of pH, which was more acidic in the control experiment. Even though the addition of 2% of K_2CO_3 has resulted in pH change to values near the neutrality (which resulted in better stability of *B. cereus* spores), the increase of K_2CO_3 concentration from 2 to 6% led to increasing of k_{max} values for both microorganisms. This increase in kmax occurred probably because higher pH values would approach very alkaline conditions which also favored microbial inactivation. Despite some trend in the increase in k_{max} with the increase in alkali concentration, in particular for *G. stearothermophilus*, the overall effect was in most instances at the limit of statistical significance, and practically of marginal importance for the resistance of spores.

Studies regarding the effects of high pH values or alkalizing agents on thermal inactivation of bacterial spores at high pH values are scarce and have been done in model systems (Duncan et al., 1972; Pinho et al., 2015; Setlow et al., 2002). This is because the food industry rarely employs alkaline conditions (pH > 8.0) for processing. The inactivation of spore-forming microorganisms at high NaOH concentration (1M) and 24 °C seems to be caused by the inactivation of lytic enzymes from the cortex, which presents an important role during the germination process (Setlow et al., 2002). High concentrations of alkali allied to high temperatures increased the k_{max} of *G. stearothermophilus* spores (Pinho et al., 2015). Another hypothesis for the inactivation in alkaline medium suggests the removal of soluble coat proteins, resulting in significant damages to the lysis system, which is also necessary for spore germination (Duncan et al., 1972). The model generated in buffer solution indicates that the thermoresistance of *B. cereus* spores tends to increase at neutral pH (lower D value found was at pH 7.0) (Couvert et al., 1999). In a model proposed by Gaillard et al. (1998), the thermal resistance of spores increased as a_w decreased. Thus, the low a_w reached during the alkalization process may be one of the contributing factors for the formation and maintenance of the tails. The small variation of the inactivation parameters for both microorganisms, even with such a marked variation of pH, confirms the great resistance of the studied spores to combined high temperature and pH values.

In addition to k_{max} values, another critical parameter describing the behavior of *B. cereus* and *G. stearothermophilus* during cocoa alkalization

is the $logN_{res}$. In this region of the curve, the count of microorganisms remains constant as a function of the process time. High $logN_{res}$ values indicate that a fraction of the spore population has (much) higher capacity of survival (Geeraerd et al., 2005, 2000). The existence of subpopulation (N_{res}) could be explained by different theories (Cerf, 1977). The subpopulation is intrinsically more resistant to the imposed inactivation conditions because of the existing genetic variability within cells. It is, therefore, a permanent resistance for these individuals. Different phenomena may also justify the existence of N_{res}: clump formation, subpopulation not accessible, or adapted to heat (acquired resistance). Fujikawa and Itoh (1996) showed that the tail might be maintained for long periods in an inactivation experiment (Fujikawa and Itoh, 1996). The study indicated that the lower the initial population, the lower the $logN_{res}$. Therefore, the $logN_{res}$ value is a function of the initial population.

The tail observed in all conducted processes shows that spore-forming bacteria can withstand the alkalization process, regardless of the time, temperature, and alkali concentration applied. The increase in temperature tends to reduce the $logN_{res}$ level because the probability of resistant cells will be reduced (Casolari, 1988). This could be verified in the processes with *G. stearothermophilus* spores inactivation. The low $logN_{res}$ values of *B. cereus* even in the alkalization done at 90 °C is a result of the lower heat resistance of this bacterium in comparison to *G. stearothermophilus*.

G. stearothermophilus spores present higher $logN_{res}$ values (thus, greater resistance) than *B. cereus* during alkalization employing temperature of 90 °C. However, the significant reduction of $logN_{res}$ with an increase of carbonate only for this microorganism indicates that the variation of the carbonate concentration exerts a more significant influence on the $logN_{res}$ variation of *G. stearothermophilus* than *B. cereus*. Therefore, in milder processes (90 °C), working with higher carbonate concentrations (6%) leads to a significant reduction in the $logN_{res}$ of this thermophilic spores.

From the industrial standpoint, the alkalization of cocoa nibs is performed at shorter times compared to the ones adopted in the current study. It is noteworthy that the conditions adopted herein were used to obtain inactivation of the spores. During industrial processing, the time usually varies from 30 to 60 min, depending not only on the alkalization conditions but also on the coloring and flavor required (Bispo, 1999). The milder processes (Ur nibs treated at 90 °C) tend to last longer (60 min) than the alkalization of Pr nibs (process conducted at 120 °C per maximum of 30 min), precisely because of the temperature difference applied (Minifie, 1999). According to the adjusted models, once highly contaminated cocoa is used, the final product may present a high thermophilic spore count, since the reduction in any process would not exceed one cycle log. Within the hypothetical processes evaluated, the greatest reductions would occur for *B. cereus* in Pr nibs alkalization (between 3.50 and 3.98 logarithmic reductions). This reduction was, however, limited by the tail formation, which occurs in ~15 min of the process. In other processes, tail formation occurs at longer times compared to industrial process times: For *B. cereus*, this time can reach up to 165 min (Ur nibs alkalization), while for *G. stearothermophilus*, 250 min (Pr nibs alkalization) or still 547 min (Ur nibs alkalization). It is evident that the alkalization of Pr nibs, under industrial process conditions, lead to more inactivation of both microorganisms when compared to the alkalization of Ur nibs, because the temperature is higher in the former than in the latter. Although the processing of Pr nibs occurs in shorter times, higher temperatures favor thermal inactivation. Also, the increase in K_2CO_3 concentrations favors an increase in the k_{max}.

The characterization of the cocoa powder microbiota indicates bacilli as the main contaminants, which may include spores of *B. cereus* and *G. stearothermophilus* (Gabis et al., 1970). Many samples may contain spores with high thermal resistance that can survive after drastic thermal processes (such as UHT) and contaminate cocoa powder-based products (Witthuhn et al., 2011). The presence of thermophilic aerobic spores in cocoa powder been reported in the literature (> 3.0 log spores/g) (Lima et al., 2011), and data indicate that the aerobic spore-forming bacteria counts in cocoa nibs were significantly reduced after alkalization process about 2 log cycles (Lima et al., 2012). As the alkalization process does not aim to serve as a microbiological control step but rather to improve sensory and technological features, the process time cannot be adjusted to achieve a certain log reduction of spore-forming bacteria. Despite this, other stages of cocoa processing, such as roasting (which also occurs at high temperatures), may also contribute to the reduction of microbial load. However, it becomes clear that the quality control of the raw materials is key to guarantee the quality and safety of the cocoa powder produced.

5. Conclusions

If on the one hand, the thermal treatment conditions and alkali concentration such as those employed during alkalization lead to a reduction in microbial load, on the other hand, select heat-resistant microorganisms, such as spore-forming bacteria. According to industrial conditions evaluated, the highest reduction for *B. cereus* would occur in Pr nibs alkalization with 4% of K_2CO_3 (3.98 cycles log), whereas for *G. stearothermophilus*, the highest would occur in the Pr nibs alkalization with 6% of K_2CO_3 (0.82 log cycles). Therefore, the Pr nibs alkalization (which occurs at a higher temperature) is better for reducing the spores population. Increasing potassium carbonate concentration may increase the inactivation rate of spores or reduce the level of residual population in a few cases. Thus, it is essential to control the microbiological quality of raw material.

The simulation of the behavior of spore-forming bacteria such as *B. cereus* and *G. stearothermophilus* under industrial conditions, as proposed in the current work, is of great importance because these findings may assist food industry to further understand and control their occurrence during processing of cocoa ingredients. Furthermore, these data are valuable for the food industry to estimate the population of spores present in the final product based on the initial contamination, which is crucial for designing and guaranteeing the effectiveness of thermal processing of cocoa-based products.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.01.009.

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