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Survival variability of 12 strains of *Bacillus cereus* yielded to spray drying of whole milk

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ABSTRACT

The variability in spore survival during spray drying of 12 *Bacillus cereus* strains was evaluated. *B. cereus* spores were inoculated on whole milk (7.2 ± 0.2 $\log_{10}$ spores/g dry weight) and processed in a spray-dryer. Twelve independent experiments were carried out in triplicate. The spore count was determined before and after each drying process, based on the dry weight of whole milk and powdered milk. Then, the number of decimal reductions (γ) caused by the spray drying process was calculated. *B. cereus* strains presented γ values ranging from 1.0 to 4.7 log10 spores/g dry weight, with a high coefficient of variation (CV) of 46.1%. Cluster analysis allowed to group *B. cereus* as sensitive (strains 511, 512, 540, 432 and ATCC 14579), intermediate (strains B18, B63, and B86) and resistant strains (strains B3, B94, B51 and 436). Three strains (one of each group) were selected for further investigation and characterization of their physicochemical and molecular (proteomics) differences. Fourier transform infrared spectroscopy (FTIR), and differential scanning calorimetry (DSC) were used to determine physicochemical characteristics and glass transition temperature (T_g), respectively. No differences in signs among the three strains were found in spectra ranging from 900 to 4000 cm^{-1}. The endothermic peak ranged between 54 and 130 °C for strain 540; between 81 and 163 °C for strain B63; and between 110 and 171 °C for strain 436. However, they showed different T_g: 88.82 °C for strain 540; 114.32 °C for strain B63; and 122.70 °C for strain 436. A total of eleven spots were identified by mass spectrometry, with the spore coat protein GerQ, sporulation protein YtfJ (GerW), and peptidyl-prolyl cis-trans isomerase being found in at least two strains. Altogether, the results suggested that the high survival variability of *B. cereus* spores to the spray drying process seems to be mainly associated with different T_g and protein content. The study highlights the importance of quantifying the effects of this unit operation over the target microorganisms. These data may be relevant for the development of effective measures aiming to control the occurrence of *B. cereus* in milk powder as well as to reduce spoilage or safety issues associated with the presence of this bacterium in foods, particularly those formulated with milk powder.

1. Introduction

The *Bacillus cereus* group or *B. cereus* sensu lato encompasses several species (such as *Bacillus cereus*, *B. mycoides*, *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. pseudomycoides* and *B. cytotoxicous*) with considerable phenotypic and genotypic similitude (Okinaka and Keim, 2016; Spanu, 2016). *Bacillus cereus* is a mobile Gram-positive rod, spore-forming species widely distributed in nature (Carlin, 2011; Spanu, 2016). In addition to the pathogenic potential, it is also responsible for the production of lipases and proteases that cause coagulation, gelation, and bitterness in dairy products. *Bacillus* species, including *B. cereus*, are widely prevalent in the milk and dairy products production chain (Spanu, 2016).

The primary sources of milk and dairy products contamination by *B. cereus* are soil, animal feces, and water (Carlin, 2011; Magnusson et al., 2007). Once present in raw milk, *B. cereus* spores may have access to the dairy processing facilities and persist in these environments due to their adhesive ability and the formation of biofilm on surfaces, pipes, and equipment (Carlin, 2011; Kumari and Sarkar, 2014; Peña et al., 2014). Due to their high prevalence in raw material and persistence in the production facilities (Shaheen et al., 2010), this species is commonly recovered from processed dairy products, for example, pasteurized

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milk, UHT milk and milk powder (Becker et al., 1994; Larsen and Jørgensen, 1997; Lin et al., 2017; Owusu-Kwarteng et al., 2017; Reyes et al., 2007; Sadiq et al., 2016).

Several studies report on the prevalence and counts of *B. cereus* in milk subjected to pasteurization (Larsen and Jørgensen, 1997) and UHT milk (Lin et al., 2017). Some studies, however, have reported on the occurrence of *B. cereus* in powdered milk (Becker et al., 1994; Kumari and Sarkar, 2014; Reyes et al., 2007; Sadiq et al., 2016), ranging from 0.3 to 10^4 spores/g. Despite the reports on the occurrence of *B. cereus* in dried milk, data on the impacts of the spray drying process on spores of *Bacillus cereus* in powdered milk are very scarce (Alvarenga et al., 2018)

During spray drying of milk, the air is heated over 150 to 270 °C at the inlet of the process (Bhandari et al., 2008; Schmitz-Schug et al., 2013). The particle comes into contact with hot air for 0.2 to 55 s, which typically culminate in the inactivation of most microorganisms (Jeantet et al., 2008; Kieviet and Kerkhof, 1995; Schmitz-Schug et al., 2013, 2016; Silva et al., 2011; Usui et al., 1985; Zbicinski et al., 2002). However, several phenomena associated with the mass transfer (such as moisture reduction) can minimize the deleterious effects on microorganisms exposed to the high temperatures during spray drying (Bhandari, 2013). The combination of this phenomenon with the removal of water may result in higher spore counts in the dried product. (Alvarenga et al., 2018; Cal and Sollohub, 2010; Sadiq et al., 2016).

Spore-forming bacteria tend to dominate the microbiota of dehydrated products, including those obtained by spray drying processes (Becker et al., 1994; Beuchat et al., 2013; in't Veld et al., 1993). The concentration of bacterial spores in powdered milk may be critical considering the widespread use of this product as an ingredient in food formulations that will be processed and stored under different conditions. This fact can be even more critical considering that individuals with varying susceptibility to pathogens will consume these products.

Although powdered milk does not provide conditions for germination of *B. cereus* spores when this product is rehydrated for consumption or used in the formulation of other food, favorable conditions for the germination and outgrowth of *B. cereus* can be provided (Reyes et al., 2007). Counts $\sim > 10^6$ CFU/g or ml were reported in outbreaks in which *B. cereus* (emetic and diarrheal syndrome) was implicated (Griffiths and Schraft, 2017).

Knowing the spore load in the ingredients to be used in food formulations is crucial for designing and guaranteeing the efficiency of thermal processes (Gauvry et al., 2017). Therefore, it becomes clear that it is vital to computing the effect of unit operations, such as the spray drying process, on spore-forming bacteria present in milk. Nonetheless, due to the heterogeneity associated with strains and even individual cells (Kakagianni et al., 2017; Trunet et al., 2017), significant variability in spores' survivability can be observed. The characterization of this variability is critical to the design and control of processes in the food industry. The lack of consideration of variability in the resistance of spores could allow the survival of more resistant strains and result in spoilage of the product or occurrence of disease outbreaks (den Besten et al., 2017a,b; Lianou and Koutsoumanis, 2013). This study was carried out to determine the variability in survival of 12 *B. cereus* strains during the spray drying process of whole milk. The physicochemical and molecular (proteomics) of three strains was also investigated to gain insights regarding the difference in their survival when subjected to spray drying.

2. Material and methods

2.1. Strains and preparation of spore suspensions

Twelve strains of *B. cereus*, donated by the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) were used in this study. The strains used were isolated from dairy goods (n = 5), cocoa derivatives (n = 2), meat products (n = 1), ready-to-eat meal (n = 1), starch derivatives (n = 2) and standard strain (n = 1). The pure cultures were stored at −80 °C in nutrient broth with 20% (w/w) glycerol until the time of use.

The preparation of the spore suspensions followed procedures described in detail in Martinez et al. (2016), Peña et al. (2014), Pflug (1999) and Alvarenga et al. (2018). For the spray drying processes, each spore suspension was diluted in PBS [phosphate buffered saline] (Dynamic Diadema, Brazil) and then inoculated into 500 g of whole milk separately at 7.2 (± 0.2) $\log_{10}$ spores/g dry weight.

2.2. Spray drying process

A spray dryer model SD 1.0 (LabMaq, Stream Black, Brazil) (Fig. S1 - Supplementary material) with a nominal drying capacity of 1.0 L/h, dimensions 1800 mm (height with casters) 500 mm (width), 800 mm (length) was used in this study. The dimensions of the drying chamber were 500 mm (length) and 150 mm (width), the spray nozzle of the double fluid type with a 1.2 mm diameter orifice. Before and after each drying process, the spray dryer was cleaned, disinfected and decontaminated as described by Alvarenga et al. (2018).

2.3. Drying process

The spray dryer was fed by a peristaltic pump (PS I, LabMaq, Stream Black, Brazil) with a maximum flow of 1.0 L/h coupled to a silicone hose [internal ∅ 2.38 mm, external ∅ 5.56 mm, wall thickness 1.58 mm] (Versilic SPX-50, St. Gobain, Beaverton, USA). The drying parameters employed, simulating the industrial drying conditions and considering the limitations of the equipment, were: inlet air temperature (190 °C ± 2 °C), drying air flow rate (84 m^3/h), feed rate (7×10^{-4} m^3/h), compressed air flow (2.4 m^3/h), compressed air pressure (0.25 MPa), and outlet temperature (110 °C ± 5 °C). The inlet and outlet temperatures were monitored during the drying processes through a thermocouple (PT 100, LabMaq, Ribeirão Preto, Brazil). The powdered milk was collected in a sterile screw cap reagent bottle with a nominal capacity of 250 mL (Borosilicate 3:3, Laborglass, São Paulo, Brazil) coupled to the cyclone screw outlet. The drying processes lasted on average 1 h, considering the time required to reach the equilibrium condition, an inlet temperature of 190 °C stable for 15 min.

Three independent drying processes were conducted for each of the 12 strains studied resulting in 36 drying processes. The control experiments consisted of two drying processes using non-inoculated milk. The powdered milk recovered after each drying process was analyzed and the concentration of *B. cereus* spores per gram of powder was determined.

An infrared balance (Gehaka IV3100, São Paulo, Brazil) was employed to find the content of solids in the samples. The solid content in the whole milk before the drying process was 10.7 g of solids/100 g of wet basis and the powdered milk after the drying process presented 94.2 g of solids/100 g of wet basis. The amount of milk powder recovered was ~23 g (± 3.0 g). The measurement of water activity (Aqualab, 4TEV, Decagon Devices, Pullman, USA) indicated values of 0.995 ± 0.001 and 0.402 ± 0.011 for whole milk (before drying) and for milk powder, respectively.

2.4. Enumeration of B. cereus spores

B. cereus spores counts in milk were determined prior and subsequently to each spray drying process following the adapted methodology of Bennett et al. (2015). Before spray drying, a sample of 10 g of milk was exposed to heat shock at 80 °C for 30 min (optimum condition to recover spores before the drying process, according to preliminary experiments). After spray drying, 1 g of powdered milk was diluted in 9 mL of sodium citrate buffer (Dinâmica, Diadema, Brazil). After that, they were submitted to heat shock for 20 min at 75 °C (optimal heat shock for spores' recovery after drying, according to preliminary experiments not shown) (Alvarenga et al., 2018).

After each shock, decimal dilutions were prepared in sodium citrate