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Assessment of the of acid shock effect on viability of *Bacillus cereus* and *Bacillus weihenstephanensis* using flow cytometry

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ABSTRACT

The present work studies the effect of acid shock on cell viability of *Bacillus cereus* and *Bacillus weihenstephanensis*. Experiments were performed by means of flow cytometry (FCM) combined with fluorescent labelling. Propidium iodide, for membrane evaluation, and carboxyfluorescein diacetate, for esterase activity were used for differential staining of both strains. pH values of 3.4, 3.8 and 4.2 were selected on the basis of previous screening tests. FCM analysis showed to clearly discriminate between different populations, viable and damage cells, leading to successful assessment of acid shock effect on *B. cereus* and *B. weihenstephanensis* vegetative cell viability. Cellular viability decreased at lower pH values and longer exposure times, being *B. weihenstephanensis* more acid resistant than *B. cereus*. Reliability of FCM analysis for detection of *B. cereus* and *B. weihenstephanensis* vegetative cells was compared with classical viable plate count techniques (VPC). Although FCM and VPC data were not directly correlated and classical analytical methods show higher reliability, FCM analysis provides high-speed information at real time on damage at single cell level, whereas VPC only gives an indication of cells able to grow at a certain time. These data could help to establish more accurately the potential risk associated to foods or processing conditions where pH is involved, taking damage into consideration.

1. Introduction

Recent changes on eating habits have enhanced consumption of refrigerated processed food which are a favourable cold niche for *B. cereus* and *B. weihenstephanensis* (Stenfors Arnesen, Fagerlund, & Granum, 2008). Both species are able to survive mild heat treatments and then grow at low temperature increasing the probability of spoilage incidents and outbreaks caused by these microorganisms. In this context, a thorough understanding of *Bacillus* pathogenicity and the identification of suitable food preservatives will be essential to prevent food spoilage and toxin production.

It is well known that pH is a key parameter to control bacterial growth. Low-pH foods are widely used to ensure microbiological quality and stability by adding organic acids. Besides, adaptation and survival in low pH environments are factors of particular interest in food safety and to engineer probiotic Bacilli (Ter Beek & Brul, 2010). The risk of *B. cereus* food poisoning is strongly determined by their resistance to low pH since the diarrhoeal syndrome is caused by spores and vegetative cells ingested with food. These cells subsequently produce enterotoxins in the intestine after surviving to the acid environment of human stomach (Kotiranta, Lounatmaa, & Haapasalo, 2000).

The efficacy of low pH has been generally assessed using classical plate count methods which are supposed to be accurate under optimal conditions, but may underestimate the numbers of viable bacteria due to biases introduced by selected incubation conditions, as well as by different environmental factors (Paparella et al., 2008). Besides, development of new detection methods is needed for resolution at single cell level to make the data applicable for predictive modelling and risk assessment approaches capable of being implemented in the food industry (Ter Beek & Brul, 2010). In this regard, flow cytometry (FCM) offers many potential advantages over conventional techniques. It has been proved to be faster than growth based methods, allowing both the measurement of heterogeneity within a population and the analysis of individual microorganisms, and providing real-time information on physiological status of the cell. Indeed, FCM has been used as a powerful, reliable and fast tool to assess the resistance and survival of *B. cereus* vegetative cells to acid and osmotic stress, antibiotics, food processing treatments and process equipment sanitation regimes (Cronin &

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Wilkinson, 2010). Moreover, previous studies reveal this technique as an effective method of verifying the viability of microorganisms present in milk, wine and water (Gunasekera, Attfield, & Veal, 2000; Malacrinò, Zapparoli, Torriani, & Dellaglio, 2001; Porter, Diaper, Edwards, & Pickup, 1995) or the antimicrobial activity of essential oils (Muñoz, Guevara, Palop, Tabera, & Fernández, 2009; Paparella et al., 2008).

The present research aims to characterize the physiological behaviour of *B. weihenstephanensis* and *B. cereus* vegetative cell populations after exposure to acid shock by using flow cytometric analysis (FCM) in combination with fluorescent techniques. To discriminate cells in different physiological states, two specific labels, propidium iodide (PI) and carboxyfluorecein diacetate (cFDA), were successfully used as previously reported by Biesta-Peters, Mols, Reij, and Abee (2011). Both of them selectively stain different structures allowing differentiation of viable and damaged cells. PI is a membrane impermeant dye which binds to DNA by intercalating between the bases when membrane integrity is lost (Ananta, Heinz, & Knorr, 2004). cFDA is a lipophilic dye able to diffuse inside the cells where it is cleaved by unspecific esterases converting in a membrane-impermeant fluorescent compound, carboxyfluorescein (cF), which is retained in viable cells with intact cytoplasmic membrane (Petit, Denis-Gay, & Ratinaud, 1993). Viable cells stained with cFDA emitted green fluorescence, whereas DNA of membrane-damaged cells binds PI emitting red fluorescence. Likewise, reliability of FCM analysis for detection and quantification of *B. cereus* and *B. weihenstephanensis* vegetative cells was assessed in comparison with classical techniques (VPC).

2. Materials and methods

2.1. Bacterial strains

Two bacterial strains were used in this study, *B. weihenstephanensis* KBAB4 kindly provided by the National Institute of Agronomical Research (INRA, Avignon, France) and *B. cereus* CECT 148 (which corresponds to the type strain *B. cereus* ATCC 14579) selected from Spanish Type Culture Collection (CECT, Valencia, Spain). These microorganisms were sporulated in Fortified Nutrient Agar (FNA) (Mazas, González, López, González, & Martín-Sarmiento, 1995). To prepare the spore suspensions, the agar surface was inoculated with 0.2 mL of a 24 h culture grown at 30 °C in Brain Heart Infusion broth (BHI, Scharlau, Barcelona, Spain) After 5 days of incubation at 30 °C more than 90% of sporulation rate was achieved, as determined by a phase contrast microscopy (Leica, Wetzlar, Germany).

Spores were collected by flooding the agar plates with 2 mL of sterile distilled water, scratching the surface with a spatula. After harvesting, spores were washed four times by centrifugation at 3000 ×*g* for 10 min and resuspended in sterile distilled water. The concentration of spores in the final suspension was adjusted at 10^9 spores mL^{-1} with sterile distilled water. The spore suspensions were stored at −20 °C in 30% glycerol until use. Spores were activated 10 min at 80 °C before each experiment to reduce heterogeneity in germination. This ensures that, once growth starts, the subsequent vegetative cells would have the same physiological state.

2.2. Acid shock treatment

B. weihenstephanensis KBAB4 and *B. cereus* ATCC 14579 were revitalized by two consecutive subcultures in pH 7.4 ± 0.2 BHI at 30 °C with shaking (200 rpm) up to stationary phase before inducing an acid tolerance response (ATR) (Jobin, Clavel, Carlin, & Schmitt, 2002). Next, 50 mL of each bacterial suspension was concentrated by centrifugation at 3000 ×*g* for 10 min and pellets were resuspended in 10 mL of BHI to obtain a final concentration of 10^8 CFU mL^{-1}. To study the effect of an acid shock on viability of cells, 1 mL of concentrated bacterial culture was inoculated in 4 mL of BHI acidified with HCl 1 M to final pHs of 3.4, 3.8 and 4.2 and incubated at 30 °C (pH values were selected on the basis of previous bacterial growth studies made in BHI acidified to different pH ranging from 3 to 5). At different time points (0 min, 30 min, 2 h and 24 h), samples were taken for flow cytometry analysis and bacterial growth was measured by viable plate counts (VPC). Hence, an experimental design consisting in 3 pH values (3.4, 3.8 and 4.2) and 4 sampling times (0 min, 30 min, 2 h and 24 h) for each microorganism was developed.

2.3. Flow cytometry measurement

Flow cytometry was performed using a FACSCalibur Flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser. The software used for data acquisition was BD CellQuest Pro (BD Biosciences). The sheath fluid used for all experiments was FACSFlow™ (BD Biosciences), filtered through a 0.2 μm Millipore Millex-GN filter (Millipore Corporation, Bedford, USA). Fluorescent beads (Calibrite™ three colours calibration beads; BD Biosciences) were used as an internal standard for scatter and fluorescence. The flow cytometry (FCM) analyses were performed with the following detector settings: FSC, E00; SSC, 400; FL1, 530; and FL3, 680 using logarithmic gains. Green fluorescence was recorded at 530/30 nm bandpass filter in the FL1 channel and red fluorescence at >650 nm in the channel FL3. For this experiment a commercial solution of PI 1 mM (Molecular Probes, Oregon, USA) and a stock solution 1 mM of cFDA (Sigma Aldrich Chemie) were performed in phosphate buffered saline and were used to stain *Bacillus* cells. Both fluorescent dyes were stored at −20 °C until use.

To study viability and damage of vegetative cells of *B. weihenstephanensis* KBAB4 and *B. cereus* ATCC 14579 incubated at different pH (3.4, 3.8 and 4.2), FCM samples were prepared with each culture diluted in prefiltered FACSFlow to obtain an approximate concentration of 10^6 CFU mL^{-1}. Then, 1 mL of these samples was stained with 1 μL of fluorescent dye, cFDA or PI, to get a final dye concentration of 1 μM. Samples were vigorously mixed and incubated in the dark in the incubator at 30 °C temperature for 15 min prior to analysis. Samples were adjusted to an event rate of 200–700 cells per second and a total of 10,000 events were registered per sample. Experiments were performed in triplicate in different days with different cultures to confirm reproducibility.

Data were analysed with the software package BD CellQuest Pro (BD Biosciences). Fluorescence collected in channel FL1 and FL3 was analysed using FCM-derived dot plots of green or red fluorescence versus SSC. To discriminate bacteria from artefacts a gate in the dot-plot of FS vs. SS was determined. Quantitative assessment of each bacterial subpopulation was performed by counting the number of events included inside the corresponding gate (Paparella et al., 2008). Data were displayed as dual parameter fluorescence density plots.

2.4. Test of suitability of fluorescent dyes for *B. cereus* and *B. weihenstephanensis* differential staining

A staining protocol for *B. cereus* and *B. weihenstephanensis* was tested to estimate viability and to determine the cause of viability loss. For this purpose suitability of PI and cFDA for differential staining of damaged and viable cells, respectively, was assessed for the microorganisms studied. To inactivate the cells, samples were heated at 80 °C for 10 min and then labelled with PI. It was shown that heat treated bacteria took up this intercalating dye indicating that inactivation treatment was coincident with physical compromise of the plasmatic membrane. In parallel, cell inactivation was confirmed by plating treated samples onto BHI agar (BHIA, Scharlau). Counts revealed a decrease in bacterial population of 7–8 $\log_{10}$ cycles (for both microorganisms coincident with a marked upshift in red fluorescent towards the FL3 channel). Non-heat treated *B. cereus* and *B. weihenstephanensis* cells (viable cells) stained with cFDA exhibited a large signal in the green channel reflecting the