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Influence of food matrix on outgrowth heterogeneity of heat damaged *Bacillus cereus* spores

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ABSTRACT

Spoilage of heat treated foods can be caused by the presence of surviving spore-formers. It is virtually impossible to prevent contamination at the primary production level as spores are ubiquitous present in the environment and can contaminate raw products. As a result spore inactivation treatments are widely used by food producing industries to reduce the microbial spore loads. However consumers prefer mildly processed products that have less impact on its quality and this trend steers industry towards milder preservation treatments. Such treatments may result in damaged instead of inactivated spores, and these spores may germinate, repair, and grow out, possibly leading to quality and safety issues. The ability to repair and grow out is influenced by the properties of the food matrix. In the current communication we studied the outgrowth from heat damaged *Bacillus cereus* ATCC 14579 spores on Anopore membrane, which allowed following outgrowth heterogeneity of individual spores on broccoli and rice-based media as well as standard and mildly acidified (pH 5.5) meat-based BHI. Rice, broccoli and BHI pH 5.5 media resulted in delayed outgrowth from untreated spores, and increased heterogeneity compared to BHI pH 7.4, with the most pronounced effect in rice media. Exposure to wet heat for 1 min at 95 °C caused 2 log inactivation and approximately 95% of the spores in the surviving fraction were damaged resulting in substantial delay in outgrowth based on the time required to reach a maximum microcolony size of 256 cells. The delay was most pronounced for heat-treated spores on broccoli medium followed by spores on rice media (both untreated and treated). Interestingly, the increase in outgrowth heterogeneity of heat treated spores on BHI pH 7.4 was more pronounced than on rice, broccoli and BHI pH 5.5 conceivably reflecting that conditions in BHI pH 7.4 better support spore damage repair. This study compares the effects of three main factors, namely heat treatment, pH of BHI and the effect of food matrix highlighting the impact of different (model) food recovery media on outgrowth efficiency and heterogeneity of non-heat-treated and heat-damaged *B. cereus* spores.

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1. Introduction

Bacterial spores are widely present in the environment and are often identified as a source of contamination in the food industry. Highly resistant dormant spores that survived processing treatments can be present in final products and may germinate and grow out leading to foodborne illness or (premature) spoilage. The resistance of spores is lost when germination is initiated under nutrient rich conditions or when spores are exposed to specific physical or chemical triggers (Løvdal et al., 2011; Setlow, 2003). However, spores may remain dormant for years until the initiation of germination, a phenomenon that makes the eradication and control of spores difficult for food producing industries.

The control of spores is further complicated by the tendency to use less intense preservation and processing strategies such as the use of milder heat treatments in combination with secondary mild preservation hurdles. However, reduction of the heat treatment intensity may lead to a subpopulation of spores that are sublethally damaged rather than inactivated. Such spores may eventually grow out after repair of the damage. This phenomenon conceivably contributes to increased heterogeneity in the population resulting in less accurate prediction of spore outgrowth behaviour. Knowledge on the behaviour of individual spores and its variability could assist in more accurate prediction of spore behaviour for shelf life prediction and refinement of risk assessments.

Spore germination is a relatively fast process that can be triggered by the presence of nutrients including sugars, single amino acids or combinations thereof (Abee et al., 2011; Hornstra et al., 2006; Løvdal et al., 2011; Setlow, 2003; van der Voort et al., 2010). Non-nutrient germination triggers have been described including chemical components such as pyridine-2,6-dicarboxylic acid chelated with calcium and the cationic surfactant dodecylamine. In addition, lytic enzymes such as lysozyme

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and high hydrostatic pressure treatments may trigger germination (Shah et al., 2008).

The sequential events in germination and outgrowth can be highly heterogeneous, as the precise fine tuning of the process is related to several intrinsic spore characteristics that may differ between individual spores depending on the sporulation conditions (Ramirez-Peralta et al., 2012). Individual spores may vary in sensitivity to heat and other processing treatments (Stringer et al., 2011) and in superdormancy (Ghosh and Setlow, 2010). Also the food matrix composition may have a considerable impact on the germination and outgrowth efficiency of spores. Commonly applied preservation strategies used by food industry to control spores (and vegetative cells) include the reduction of the water activity by using elevated concentrations of salt or sugar, or lowering of pH with organic acids (Rajkovic et al., 2010), which might affect heat resistance (Mazas et al., 1999; Moussa-Boudjemaa et al., 2006; Samapundo et al., 2014). In addition, stress conditions encountered during spore dormancy such as heat treatment, UV and disinfectant treatment can cause sublethal damage to spores that may increase variability in spore behaviour, especially in combination with non-optimal outgrowth conditions such as low pH, presence of salt or other inhibitory compounds that originate from food. As a result, spore germination and outgrowth can be significantly affected.

The sporeformer *Bacillus cereus* has been associated with food spoilage (Andersson et al., 1995) and food-borne disease (Stenfors Arnesen et al., 2008). Depending on the type of toxin produced, *B. cereus* can cause two distinct syndromes. The emetic syndrome is caused by ingestion of the preformed heat stable toxin cereulide whereas the diarrheic syndrome is caused by enterotoxins secreted by vegetative cells present in the small intestine. Symptoms are usually mild and self-limiting, but in rare instances they can lead to life-threatening situations (Dierick et al., 2005; Ehling-Schulz et al., 2004; Granum, 2005; Kotiranta et al., 2000; Schoeni and Wong, 2005; Stenfors Arnesen et al., 2008). Effective heat preservation strategies are required to prevent *B. cereus* proliferation. A number of studies focussed on germination and/or outgrowth heterogeneity after heat treatment but the conditions used were relatively mild that either lead to heat activation or to marginal inactivation such as 10 min at 90 °C (*B. cereus*), 10 min at 95 °C (*Bacillus subtilis*) or 20 s at 80 °C (*Clostridium botulinum*) and laboratory media of optimal compositions were used to monitor outgrowth (Pandey et al., 2013; Stringer et al., 2009; van Melis et al., 2014). In this study, we focus on conditions that result in a 1.5–2 log inactivation and a severe heat damage in the surviving spores. Outgrowth capacity and heterogeneity of individual non-heated and heat-damaged *B. cereus* ATCC 14579 spores was subsequently assessed using the Anopore approach described previously (den Besten et al., 2007, 2010; van Melis et al., 2014) with meat-based BHI, and food-matrices based on rice and broccoli, to mimic conditions that may be encountered in food processing.

2. Materials and methods

2.1. Strain and sporulation conditions

B. cereus ATCC 14579 obtained from the American Type Culture Collection (ATCC) was cultured in Brain Heart Infusion broth (BHI; Beckton Dickinson, Le Point de Claix, France) at 30 °C with aeration at 200 rpm. Spores were prepared in a nutrient-rich, chemically defined sporulation medium designated MSM medium that was described previously (Garcia et al., 2010). One ml of an overnight-grown pre-culture was used to inoculate 100 ml of sporulation media in 500 ml flasks and incubated at 30 °C with aeration at 200 rpm. Sporulation was monitored during 2–3 days by phase contrast microscopy until the release of over 99% of spores from the mother cell. Spores were harvested by 15 min centrifugation at 5000 rpm at 4 °C (5804R, Eppendorf, Germany) and washed with chilled phosphate buffer (100 mM, pH 7.4) containing 0.1% Tween80 to prevent spore clumping. Spores were washed twice a day over a period of 2 weeks with phosphate buffer with gradually decreasing Tween80 concentration until a final concentration of 0.01% (further referred as suspension buffer). Spores cleared from vegetative cells and debris were stored at 4 °C and used for a maximum of six months.

2.2. Heat treatment

Hundred twenty μl of a spore suspension containing approximately 10^8 spores/ml in suspension buffer were transferred into capillary tubes (Micropipettes 200 μl max, Blaubrand intraMARK, Germany) and heat-sealed at both ends. The capillary tubes were placed in an oil bath (Julabo MC-12, Germany) set at 95 °C for 1 min, followed by immediate cooling in ice-cold water. The heat-treated spore suspension was recovered from the capillary tubes and decimally diluted in suspension buffer. Fifty μl of appropriate dilutions were plated in duplicate on BHI pH 7.4 and BHI pH 7.4 supplemented with 5.5% salt as previously used to estimate the degree of damage by Cazemier et al. (2001). Plates were incubated at 30 °C and colonies were counted after 24, 48 h and a week (no increase in colony counts after a week). For the surviving population, the percentage of damaged spores was calculated by the following formula:

$$\%\ Damaged\ spores = \frac{(\text{Number of cfu's BHI}) - (\text{Number of cfu's BHI 5.5\% NaCl})}{(\text{Number of cfu's BHI})} * 100.$$

2.3. Food based media used in this study

Rice based media were prepared by boiling ready-to-cook pouches filled with 125 g rice produced by the manufacturer (Lassie B.V, The Netherlands) in demineralised water (5:32 w/v) for 45 min. The rice bags were removed and 1.5% (w/v) Bacteriological Agar was added and boiled twice to dissolve the agar before pouring into petri dishes. The final pH of the prepared rice medium was 7.2. Broccoli based media were prepared by mixing sterile 5% (w/v) agar solution with Olvarit broccoli baby food (4 month baby food (60% broccoli, 17% rice, 17% water, 6% apple juice)) (Nutricia, The Netherlands) puree to reach a final concentration of 1.5% (w/v) agar. The final pH of the prepared broccoli medium was 5.8. BHI acidified with HCl to pH 5.5 was selected to simulate mild acid stress. The water activity of the three media ranged from 0.993 to 0.995 and was comparable to the water activity of BHI pH 7.4, while the addition of 5.5% salt to BHI pH 7.4 resulted in a drop to 0.958. All media were prepared one day before the experiments.

2.4. Anopore

Anopore strips (8 by 36 mm by 60 μm, 0.2-μm-diameter pore size, pore density of up to 50%; Whatman, the Netherlands) were prepared as described previously (den Besten et al., 2007, 2010) and were placed on agar based media. Both heat treated and untreated spores were diluted in suspension buffer, and were spotted on the Anopore strips and incubated at 30 °C. At regular sampling times (30 min after spotting, followed by 30 or 60 min intervals after outgrowth), individual Anopore strips were transferred onto an agarose pad consisting of a microscope slide covered with a 1-mm-thick film of 1% (w/v) solidified low-melting-point agarose (Invitrogen, The Netherlands) dissolved in demineralized water containing 1 μM of the fluorescent reporter dye SYTO-9 (Invitrogen, The Netherlands). Following 10 min staining in the dark, the Anopore strip was transferred for 10 min to an agarose pad without SYTO-9 dye, to reduce background signals and placed under a fluorescence microscope as described previously (van Melis et al., 2014) and routinely for each time point 100 to 1000 events (spores or microcolonies) were imaged. For comparative purposes time points 30 min, 4.5, 5.5 and 6.5 h for untreated spores are presented, with an additional time point of 3.5 h for BHI pH 7.4 presented in

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