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Quantification and differentiation of *Bacillus cereus* group species in spices and herbs by real-time PCR

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ABSTRACT

Spores of *Bacillus* (*B.*) *cereus* group species are frequent contaminants in foodstuffs including spices and herbs. However, the distribution of individual *B. cereus* group species is unknown as standard cultural methods are insufficient for differentiation. Real-time PCR is an alternative method to detect, differentiate and quantify *B. cereus* group species in food.

In our study we applied a combination of previously described real-time PCR assays to detect and quantify the *B. cereus* group (excluding *B. cytotoxicus*) with simultaneous discrimination of *B. pseudomycoides* and *cry1*-positive *B. thuringiensis* as well as differentiation of *B. weihenstephanensis* from *B. cereus* group species with non-rhizoid colony morphology. For testing food matrices, which can also include PCR inhibiting substances, an internal amplification control was included. In total, five DNA extraction kits were tested on pure spore suspensions to select the one with the best recovery rate when coupled to real-time PCR. The Qiagen DNeasy Blood & Tissue Kit performed best with a limit of detection (LOD) of approximately 100 cfu/ml for spores of each *B. cereus*, *B. weihenstephanensis*, *B. thuringiensis* and *B. pseudomycoides* strain. However, applied to allspice, paprika, pepper and oregano artificially contaminated with *B. cereus* spores the LOD was $\geq 10^5$ cfu/g. In contrast, using the open-formula cetyltrimethylammonium bromide (CTAB) method LODs of 10^2 to 10^3 cfu/g were achieved for paprika, pepper and oregano. For allspice, the LOD was 10^6 cfu/g.

Our quantitative multiplex real-time PCR coupled to DNA extraction by the CTAB method provides a sensitive culture independent technique with the potential to quantitatively detect and differentiate *B. cereus* group species in several spices and herbs.

1. Introduction

The term *Bacillus* (*B.*) *cereus* is often used as a synonym for the so called *B. cereus* group, also referred to as *B. cereus* sensu lato (s.l.) or presumptive *B. cereus*. The *B. cereus* group comprises seven closely related spore forming species with varying pathogenic potential: *B. anthracis* is known to cause anthrax whereas *B. cereus* sensu stricto (s.s.) and *B. cytotoxicus* are associated with foodborne diseases. *B. thuringiensis* is commercially used as a biological insecticide. *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis* are considered as causes for food spoilage rather than foodborne diseases (Ehling-Schulz & Messelhausser, 2013). However, except for *B. pseudomycoides*, cytotoxicity could be attributed to all *B. cereus* group species to a varying extent (Ceuppens et al., 2011; Guinebretiere et al., 2010).

B. cereus (s.s.) is able to produce one or more enterotoxins causing diarrhoea, namely non-haemolytic enterotoxin, haemolysin BL and cytotoxin K. Some strains are capable of producing an additional heat stable toxin called cereulide, causing emesis. Spores of *B. cereus* are ubiquitous in soil and can be transferred to raw plant materials through soil contaminations (e.g. rain splash) (Pedersen, Damgaard, Eilenberg, & Hansen, 1995), colonisation of the plant (Bizzarri & Bishop, 2008) or during drying processes on the ground (Sagoo et al., 2009). Due to their high resistance to physical and chemical stress such as low water activity, excessive pH values or heat stress (Blackburn & McClure, 2009) spores of this organism can survive in different kinds of food, among them also spices and herbs. According to the European Food Safety Authority (EFSA) spores of *B. cereus* are a frequent contaminant in spices and herbs (EFSA, 2005, 2013). When added to foods with favourable water

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content and pH *B. cereus* spores may germinate and the cells may proliferate under ambient temperature conditions. Accordingly, various foodborne disease outbreaks could be linked to spices contaminated with *B. cereus* (EFSA, 2009, 2011, 2013; WHO, 2014).

Routine testing for *B. cereus* in food including spices and herbs is based on cultural detection methods according to ISO 7932:2005 and ISO 21871:2006 (Anonymous, 2005, 2006). However, these methods are only suitable to detect presumptive *B. cereus*, meaning that no reliable differentiation between the *B. cereus* group species is possible, especially with regard to *B. cereus* (s.s.), *B. weihenstephanensis*, *B. thuringiensis*, and *B. cytotoxicus*. Consequently, our knowledge about the true prevalence of individual *B. cereus* group species is very limited. Hence, also the contribution of individual species to foodborne diseases is uncertain. This constitutes the need for new approaches to detect, differentiate and quantify members of the *B. cereus* group in food.

A non-cultural approach to detect and refine *B. cereus* (s.l.) species in food is based on DNA extraction followed by polymerase chain reaction (PCR). Using this approach two aspects need to be considered: firstly, the resistance of the spore envelope against chemical or physical breakdown and secondly, substances in the food interfering with the DNA extraction or the subsequent PCR. A number of studies are available, which compare DNA extraction techniques for their suitability to extract DNA from spores of *Bacillus* species in water, food or environmental samples. Most of them refer to *B. anthracis* (Dauphin, Moser, & Bowen, 2009; Dineen, Aranda, Anders, & Robertson, 2010; Mertens et al., 2014; Thomas et al., 2013), but some also to *B. cereus* (s.s.) (Dineen et al., 2010; Dzieciol, Fricker, Wagner, Hein, & Ehling-Schulz, 2013). Yet, there is no data available giving evidence that spores of different *B. cereus* group species react similar towards DNA extraction. Moreover, the culture independent detection of *B. cereus* spores in the heterogeneous and complex matrix of spices and herbs was not investigated in any of the studies.

Several PCR methods are described with different foci towards the taxonomy or characterisation of *B. cereus* species. For example Dzieciol et al. (2013) published a quantitative multiplex real-time PCR to detect all seven species of the *B. cereus* group based on *gyrB*, the gene encoding for gyrase B, while simultaneously distinguishing emetic and non-emetic strains based on the presence of the *ces* gene. For species differentiation Oliwa-Stasiak, Kolaj-Robin, and Adley (2011) used real-time PCR to quantitatively detect the whole *B. cereus* group (except *B. cytotoxicus*) targeting the *motB* gene. To differentiate *B. pseudomycoides* and *B. weihenstephanensis* from the *B. cereus* group they used a hypothetical 217-bp gene sequence of *B. pseudomycoides* (*bpm*) and *motB* polymorphism in *B. weihenstephanensis*, respectively. Wielinga et al. (2011) applied a multiplex real-time PCR method to target *B. anthracis* based on the lambda pro-phage type 3 sequence (PL3) and *B. thuringiensis* DNA (used as internal amplification control) based on the Crystal protein encoding *cry1* gene, respectively. Krause et al. (unpublished results) combined these protocols to establish a 4-plex real-time PCR to detect the whole *B. cereus* group (except *B. cytotoxicus*) and simultaneously differentiate *B. pseudomycoides*, *B. weihenstephanensis* and *cry1*-positive *B. thuringiensis* strains in enriched cultures from isolates. Through the exchange of one primer pair and probe as described by Wielinga et al. (2011) *B. anthracis* can be discriminated additionally by this 4-plex PCR.

The aim of our study was to establish a quantitative method capable to detect and differentiate individual *B. cereus* group species in spices and herbs. For this, the above mentioned 4-plex real-time PCR was added up to a 5-plex real-time PCR by incorporation of an internal amplification control (IAC). Five DNA extraction kits were compared for their suitability to extract DNA from *B. cereus* (s.l.) spores. After that we used the most promising kit (DNeasy Blood & Tissue Kit) to prove if spores of different *B. cereus* group species (*B. cereus*, *B. thuringiensis*, *B. pseudomycoides* and *B. weihenstephanensis*) are equally sensitive towards DNA extraction and if the multiplex PCR is similarly efficient for the different target species. Subsequently we applied the DNeasy Blood & Tissue Kit to samples of allspice, paprika, pepper and oregano artificially contaminated with spores of *B. cereus*. To increase the sensitivity for *Bacillus* spores in spice and herb matrices, we further adapted an open-formula DNA-extraction technique – the so called CTAB (cetyltrimethylammonium bromide) method based on ISO 21571:2013-08 (Anonymous, 2013a) and modifications according to Minarovičová et al. (in this issue). Using this method the simultaneous differentiation and quantification of spores of four *B. cereus* group species was demonstrated on the example of artificially contaminated pepper.

2. Material and methods

2.1. Bacillus cereus (s.l.) strains, culture conditions and spore production

The strains used in this study were the following: emetic *B. cereus* (s.s.) strain DSM 4312, *B. thuringiensis* strain DSM 2046, *B. pseudomycoides* strain DSM 12442, *B. weihenstephanensis* strain DSM 11821 and *B. cereus* (s.s.) strain ATCC 14579. Strains were kept at −80 °C as glycerol-stock.

Production of spore suspension was based on a modified protocol of Cronin and Wilkinson (2008). Hundred microliters of an overnight culture in nutrient broth were plated on nutrient agar (Sifin, Berlin, Germany) supplemented with 40 mg/l $MnSO_4$ and 100 mg/l $CaCl_2$ and incubated for eight to ten days at 30 °C. Then, 5 ml sterile distilled water was added to the surfaces of the plates and spore material was scrapped with a spatula. The resulting spore suspension was centrifuged at 3800 × *g* and washed three times with sterile distilled water. Finally, the spore pellet was diluted in phosphate buffered saline (PBS, 8 g/l NaCl, 0.2 g/l KH_2PO_4, 1.44 g/l $Na_2HPO_4 \times 2H_2O$, 0.2 g/l KCl, pH 7.4). Only samples with spore-purity of at least 95%, which was checked by light microscopy, passed to further experiments. Spore concentrations based on colony forming units were determined by plating on polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA; Oxoid, Wesel, Germany) and counting colonies after incubation for 24 h at 37 °C or 30 °C in case of *B. weihenstephanensis* strain DSM 11821.

2.2. DNA extraction from pure spore/cell suspensions (comparison of kits and species)

To evaluate which DNA extraction method is suitable for DNA extraction from *B. cereus* (s.l.) spores in spices and herbs, five DNA extraction kits have been selected based on literature data: 1) the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany); 2) the MasterPure Gram Positive DNA Purification Kit (Epicentre Technologies, Madison, WI, USA); 3) the Invisorb Spin Plant Mini Kit (STRATEC Molecular, Berlin, Germany); 4) the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) and 5) the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). Criteria for the kit choice were: suitability for lysis of spores, applicability for difficult matrices and removal of PCR inhibiting substances as well as differing approaches between the kits with regard to the cell lysis and DNA extraction principle (summarized in Table 2). Most commercial kits apply chaotropic solid-phase extraction (SPE) using spin columns (Kuchta, 2006) as does the DNeasy Blood & Tissue Kit as well as the UltraClean and the PowerSoil DNA Isolation Kit. In contrast, the Invisorb Spin Plant Mini Kit

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