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Tracking spore-forming bacteria in food: From natural biodiversity to selection by processes

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ABSTRACT

Sporeforming bacteria are ubiquitous in the environment and exhibit a wide range of diversity leading to their natural prevalence in foodstuff. The state of the art of sporeformer prevalence in ingredients and food was investigated using a multiparametric PCR-based tool that enables simultaneous detection and identification of various genera and species mostly encountered in food, *i.e. Alicyclobacillus*, *Anoxybacillus flavithermus*, *Bacillus*, *B. cereus* group, *B. licheniformis*, *B. pumilus*, *B. sporothermodurans*, *B. subtilis*, *Brevibacillus laterosporus*, *Clostridium*, *Geobacillus stearothermophilus*, *Moorella* and *Paenibacillus* species. In addition, 16S rDNA sequencing was used to extend identification to other possibly present contaminants. A total of 90 food products, with or without visible trace of spoilage were analysed, *i.e.* 30 egg-based products, 30 milk and dairy products and 30 canned food and ingredients. Results indicated that most samples contained one or several of the targeted genera and species. For all three tested food categories, 30 to 40% of products were contaminated with both *Bacillus* and *Clostridium*. The percentage of contaminations associated with *Clostridium* or *Bacillus* represented 100% in raw materials, 72% in dehydrated ingredients and 80% in processed foods. In the last two product types, additional thermophilic contaminants were identified (*A. flavithermus*, *Geobacillus* spp., *Thermoanaerobacterium* spp. and *Moorella* spp.). These results suggest that selection, and therefore the observed (re)-emergence of unexpected sporeforming contaminants in food might be favoured by the use of given food ingredients and food processing technologies.

1. Introduction

Bacterial sporeformers are characterised by the capacity to form endospores that can resist extreme conditions, including pressure, extreme heat or cold, drought, starvation, biocides, UV radiations and may remain dormant for centuries (Nicholson et al., 2000; Moeller et al., 2008). Although metabolically inactive, spores are still able to sense their surroundings and rapidly respond to the presence of appropriate nutrients by germinating and resuming vegetative growth. When germination is initiated the spore extreme resistance is rapidly lost and cells can easily be inactivated by heat treatment or various biocides.

Sporeforming bacteria belong to the phylum of the Firmicutes and new insights into genetic diversity have yield taxonomy rearrangements. Today, the *Bacillus* genus is split into 18 genera, among which *Alicyclobacillus*, *Aneurinibacillus*, *Brevibacillus*, *Geobacillus*, *Lysinibacillus*, *Paenibacillus*, *Ureibacillus*, *Virgibacillus* and *Sporosarcina* (Fritze, 2004) while similar rearrangements have been proposed for *Clostridium sensu lato*. Several misnamed anaerobic *Clostridium* species have been reallocated to existing genera such as *Caloramator*, *Eubacterium*, *Filifactor*, *Moorella*, *Thermoanaerobacter*, and *Thermoanaerobacterium* (Collins et al., 1994; De Vos and Stackebrandt, 2009).

Sporeforming bacteria are commonly found in soil, involved in organic matter decay, and are natural inhabitants of the gastrointestinal tract of insects and many warm-blooded animal species. Hong et al. (2009) determined that *Bacillus* sporeformer concentration is about 10^6 spore/g in soil and 10^4 spore/g in human faeces and suggested possible adaptation as gut commensals besides the well known intestinal anaerobic species related to *Clostridium* spp. Endospore forming bacteria exhibit a wide range of phenotypic and genotypic characteristics with aerobes, facultative anaerobes or obligate anaerobes, psychrotrophic, psychrophilic, mesophilic, thermotolerant and thermophilic strains. *Bacillus* or related species commonly dominate the culturable soil microflora and are thus encountered in animals, vegetables, food, natural or man-made environment.

Sporeforming bacteria development in food is responsible for food spoilage and food poisoning, two major issues leading to high economical losses. The majority of food poisoning cases are linked to heat treated foods subjected to temperature abuse during storage and handling, which result in spore germination, multiplication and food consumption with hazardous levels of cells or toxins. The incidence of foodborne toxigenic *B. cereus*, *C. perfringens*, *C. botulinum* and other

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neurotoxigenic species such as *C. baratii* and *C. butyricum* is reported elsewhere and does not enter the scope of this study (Arnesen et al., 2008; van Immerseel et al., 2007; Lindstrom et al., 2006; Meng et al., 1997; Peck, 2006). In food industry, hygiene management and (thermal) processing largely contribute to lower sporeforming bacteria contamination in foodstuff ensuring quality and safety of final products. However, concerns about spore contamination in minimally processed or chilled food have increased over the past few years, as treated food may still contain viable spores that can germinate and outgrow during storage even at low temperature (Guinebretiere et al., 2003; Peck, 2006; Ranieri et al., 2009). Similarly, the emergence and description of highly heat resistant endospores (HRS) surviving commercial sterilisation and ultrahigh temperature (UHT) processing of milk have been isolated from UHT milk of several countries (Foschino et al., 1990; Klijn et al., 1997). In contrast with *Bacillus sporothermodurans* strains isolated from farm environment, strains isolated from heat-treated milk and characterised as HRS have been suggested to have a clonal origin, underlining the importance of environmental conditions encountered during sporulation on native spore resistance, survival and germination capacities (Brown, 2000; Nicholson et al., 2000; Nicholson and Law, 1999; Guillaume-Gentil et al., 2002; Scheldeman et al., 2002). Such (re)-emergence of unintended extremely resistant contaminants might foreshadow their huge adaptation capacity and resembles emergence of antibiotic resistant hospital strains in man-made environment free of competitive vegetative microflora (Hoffmaster et al., 2004).

Sporeforming bacteria presence and persistence in food industries are thus real issues as (i) the ubiquitous nature of sporeformers makes it basically impossible to prevent their presence in raw food and ingredients, (ii) pasteurisation and food industry processes inactivate vegetative competitive flora but fail to kill HRS spores, (iii) spore adhesive characteristics enhance their persistence in industrial plants, (iv) harsh conditions encountered in feed or food ingredients processing and packaging technologies might enhance adaptation or selection of extremely resistant endospores. The lack of fast and simple diagnosis tool to detect sporeformers and the limited knowledge about their prevalence and diversity are the main hurdles to control spores entering the food chain.

The aim of this study was to track sporeforming-bacteria and evaluate their occurrence and prevalence in a wide range of raw materials, ingredients and food samples using a multi-parametric PCR-based method dedicated to rapid detection and identification of major sporeformers implicated in food spoilage and food poisoning outbreaks.

2. Materials and methods

2.1. Sporeformer bacterial counts

For enumeration of foodborne microorganisms, 10 g of each food sample was homogenised in a 1/10 dilution with buffered peptone water (Biokar Diagnostics, Beauvais, France) using an EasyMix® blender (AES, Bruz, France) under aseptic conditions. Total spore counts and bacterial counts were determined, respectively, with or without preliminary heat treatment (80 °C, 10 min) in a Huber Ministat oil bath (Bioblock Scientific, Illkirch, France). Mesophilic and thermophilic bacterial counts were determined after a 48–72 h incubation on agar media at 37 °C and 55 °C, both in aerobic and anaerobic conditions. PCA (plate count agar) and RCM (reinforced clostridial medium) agar media were reconstituted and supplemented with 0.2% starch (Merck KGaA, Darmstadt, Germany) in order to favour spore germination (all media from Biokar Diagnostics, Beauvais, France).

2.2. Food enrichment and DNA extraction

Prior to GeneDisc® Plate molecular identification, an enrichment step was carried out according to a previously established protocol (Postollec et al., 2010). Briefly, for each of the 90 products, a total of 25 g of food was sampled according to standard guidelines, and homogenised in 100 ml RCM broth. Food samples were provided by industrial partners, *i.e.* 30 egg-based products (5 whole egg, 7 egg white, 3 yolk, 10 egg powder and 5 pasteurised egg), 30 milk and dairy products (5 raw milks, 4 UHT milks, 12 milk powders, 3 UHT creams, 6 cheeses) and 30 canned foods and ingredients (10 dehydrated vegetables, 2 spice mixes, 5 texturing agents, 13 canned ready-to-eat foods). Visible food alteration was observed for three out of 13 processed dairy products (23% spoiled end products) and nine out of 14 canned foods (64% spoiled end products). For fermented matrices, samples were submitted to preliminary heat treatment to inactivate lactic flora. Samples were then divided into four tubes which were incubated in different conditions optimised for the germination and growth of either aerobic, anaerobic, mesophilic or thermophilic species. The enrichment step was systematically validated and molecular identification of the cultivable isolates was achieved by 16S rDNA gene sequencing, as described below. After overnight incubation, 10 ml of each enrichment tube was grouped into a single sample, centrifuged, and DNA was extracted after 15 min at 100 °C using chelex resin-based lysis tubes (Pall GeneSystems, Bruz, France). After centrifugation, the supernatant containing DNA was collected (200 µl) and stored at −20 °C until use.

2.3. Recovery of enrichment isolates

The food enrichment step was validated by streaking on PCA, RCM and Mossel agar (Biokar Diagnostics, Beauvais, France). PCA plates were incubated 48–72 h at both 30 °C and 55 °C in aerobic conditions, RCM plates were incubated at both 37 °C and 55 °C in anaerobic jars using Genbox anaer generators (Biomerieux, Craponne, France) and Mossel plates were incubated at 30 °C in aerobic conditions for the detection of *B. cereus* group characteristic colonies. Depending on the features of recovered colonies, five to 10 colonies were identified by 16S *rRNA* gene sequencing to estimate the composition of dominant cultivable populations.

2.4. Multiparametric real time PCR analysis

Real time PCR-based biochip assays referred as GeneDisc® Plate device (Pall GeneSystems, Bruz, France) were used to allow simultaneous detection of several genera (GeneDisc® Plate 1 "sporeformer genera") or targeted *Bacillus* species (GeneDisc® Plate 2 "*Bacillus* spp.") from food sample enrichments. Details on GeneDisc® Plate specificity is reported elsewhere (Postollec et al., 2010) but tools' inclusivity and exclusivity were validated with 176 DNA extracts obtained from isolates of *B. cereus* group (*Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*), 11 *Bacillus* species (*Bacillus amyloliquefaciens*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus licheniformis*, *Bacillus macroides/simplex*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus sonorensis*, *Bacillus sporothermodurans*, *Bacillus subtilis*), 9 *Clostridium* species (*Clostridium baratii*, *Clostridium beijerinckii*, *Clostridium bifermentans*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium tyrobutyricum*, *Clostridium perfringens*, *Clostridium sordelli*, *Clostridium sporogenes*), 3 *Brevibacillus* species (*Brevibacillus laterosporus*, *Brevibacillus agri*, *Brevibacillus parabrevis*), 4 *Paenibacillus* species (*Paenibacillus amylolyticus*, *Paenibacillus odorifer*, *Paenibacillus polymyxa*, *Paenibacillus macerans*), two *Moorella* species (*Moorella thermoacetica*, *Moorella thermoautotrophica*), one *Anoxybacillus* and one *Geobacillus* species (*Anoxybacillus flavithermus*, *Geobacillus stearothermophilus*). These disposable PCR devices are preloaded with specific primers and dual-fluorescence dye-labelled probes, targeting the sporeformer genera and species mostly encountered in food, *i.e. Alicyclobacillus*, *A. flavithermus*, *Bacillus*, *B. cereus–B. thuringiensis*, *B. licheniformis*, *B. pumilus*, *B. sporothermodurans*, *B. subtilis*, *B. weihenstephanensis–B. mycoides*, *Brevibacillus laterosporus*, *Clostridium*, *Geobacillus stearothermophilus*, *Moorella* and *Paenibacillus*. A final volume of 10 µl, containing Master Mix and 1/10 diluted food DNA extracts, was loaded on the biochips and analysed with a dedicated GeneDisc® cycler automated system (Pall GeneSystems, Bruz, France). Amplification curves were obtained using a single pre-