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Occurrence and diversity of *Bacillus cereus* and moulds in spices and herbs

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ABSTRACT

Spices and herbs can contain toxin-producing bacteria and moulds, which can cause health problems for consumers and contribute to food spoilage and shelf-life reduction. The aims of the present work were (i) to determine the occurence and levels of *B. cereus* and moulds; (ii) to charactize the incidence and diversity of *B. cereus* emetic toxin (*ces*, *CER*), and diarrhoeal toxin-encoding genes (*entFM, nheA, hblC, cytK*) and toxigenic potential of Hbl toxin-producing *B. cereus* strains. Black ground pepper samples showed the most contamination with the highest concentration of *B. cereus* 2.49 $\log_{10}$ CFU/g. Moreover, cumin contained the most prominent mould concentration level of 3.6 $\log_{10}$ CFU/g. The most common moulds were *Aspergillus* and *Penicillium* spp. Compared to packaging type, all products acquired from the local market, except curry for *B. cereus*, exchibited high concentrations of *B. cereus* and moulds. Four genes were identified – 96% of *B. cereus* strains contained *entFM*, 94% *nheA*, 56% *hblC*, 42% *cytK*. None of the samples contained emetic toxin-encoding genes (*ces*, *CER*). Toxigenic potential of Hbl toxin was found in 72% of *B. cereus* strains. Different temperature, moisture levels and hygiene practices were observed at places of sale in local markets thus facilitating contamination and development of moulds. Moreover, the presence of *B. cereus* and its ability to produce toxins in spices and herbs, may suggest the need to establish microbiological criteria for mould and spore-forming bacteria in spices and herbs.

1. Introduction

Spices and herbs are derived from various anatomical parts of plants, such as leaves, rhizomes, stems, flowers, fruits, seeds, bulbs and bark. Despite their low nutritional value, they possess good sensory value and have a profound influence on the digestive system. Additives, such as essential acids, alkaloids, flavonoids may increase the activity of ferments as a result improves digestive processes (Garbowska, Pluta, & Rozanska, 2015; Yan, Meng, & Kim, 2012). Factors such as the country of origin, cultivation, harvesting, processing, packaging, transportation, storage and type of point of sale influence the quality of the product (Nei, Enomoto, & Nakamura, 2015). Spices and herbs are mostly grown and harvested in regions with warm and humid climate. Most spices are stored in dry conditions; however, contact with the air and increased humidity affects the quality of spices by increasing the probability of microbial spoilage (Kim, Sagong, Choi, Ryu, & Kang, 2012b).

Spices and herbs may contain various microorganisms, including spore-forming bacteria and moulds that can survive in low humidity conditions. Amongst the microorganisms that were most resistant to drying are the spore forming *Bacillus cereus*, *Clostridium perfringens*, *Clostridium botulinum*, and moulds, such as *Aspergillus* spp., and *Penicillium* spp., which are potential producers of the variety of toxins (Ainiza, Jinap, & Sanny, 2015; Schaarschmidt et al., 2016; Witkowska, Hickey, Alonso-Gomez, & Wilkinson, 2011). *B. cereus* can form endospores and survive in various stress conditions. Moreover, the elimination of *B. cereus* is challenging during the pasteurisation and sanitary procedures (Hertwig, Reineke, Ehlbeck, Knorr, & Schluter, 2015; Warda, Tempelaars, Abee, & Groot, 2016). *B. cereus* is a toxin producing human pathogen responsible for the diarrheal and emetic forms of food poisoning. Diarrheal type of illness is caused by at least 4 toxins: hemolysin *BL* (Hbl), nonhemolytic enterotoxin (Nhe), enterotoxin *FM* (entFM), cytotoxin *K* (CytK). Emetic type of disease is caused by the emetic toxin cereulide, which is produced in already contaminated food before its ingestion. Hemolysin BL (Hbl) also known for its three component structure, including binding component B and 2 lytic components L1 and L2, encoded by *hblA*, *hblD* and *hblC* genes,

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respectively. Rapid detection methods, such as *B. cereus* Enterotoxin-Reverse Passive Latex Agglutination (BCET-RPLA) test kit of Oxoid are commercially available and are used to detect L2 part of the Hbl toxin (Sastalla et al., 2013; Svensson, Monthan, Guinebretiere, Nguyen, & Christiansson, 2007).

European Union (EU) legislation has no definite microbiological standards for spices and herbs; however, the Codex Code of Hygienic Practice (CAC, 1995) states that spices and herbs should not contain any toxin-producing microorganisms in quantities that may raise health risks. Nonetheless, *Salmonella* spp. should be absent completely in 25 g of the sample (Codex Alimentarius Commission, 2014). The European Spice Association recommendations also call for a complete absence of *Salmonella* spp. in a 25 g sample. Additionally, the concentration in the same sample should not exceed the maximum levels of *Escherichia coli* $\leq 10^2$ CFU/g, *B. cereus* $\leq 10^4$ CFU/g and *C. perfringens* $\leq 10^3$ CFU/g and moulds $\leq 10^6$ CFU/g (European Commission (EC), 2004).

The aims of this study were (i) to determine the occurence and levels of *B. cereus* and moulds; (ii) to charactize the incidence and diversity of *B. cereus* emetic toxin (*ces*, *CER*), and diarrhoeal toxin-encoding genes (*entFM, nheA, hblC, cytK*) and toxigenic potential Hbl toxin-producing *B. cereus* strains.

2. Materials and methods

2.1. Sampling and sample preparation

A total of 60 samples composed of 5 different types of herbs (n = 14) and 15 different types of spices (n = 46) were collected from the local market and supermarket chain (Table 1). Whole retail packages of 50–100 g of spices (n = 40) were collected at the supermarket chain. In addition, 50–100 g of spice and herb samples (n = 20) were collected from the local market from opened retail bags and placed into sterile sampling bags. According to manufacturer's packaging instructions, the five pepper mix ingredients consisted of white, black, aromatic, pink and green peppers.

Ten grams of spice and herb samples were used for the detection of *Bacillus cereus* and moulds, which were weighed and diluted with 90 ml Maximum Recovery Diluent (Biolife, Milan, Italy) and homogenized for 30 s (Stomacher 400, Seward Limited, Worthing, England). Three repetitions were performed for each sample for the detection of *B. cereus*, and the identification and quantity of moulds.

Table 1
Type and counts of spice and herb samples.

Type of spice/herb	Botanical name	No. of samples
Spices		
Coriander	*Coriandrum sativum*	2
Turmeric	*Curcuma longa*	2
Curry	*Murraya koenigii*	4
Black ground pepper	*Piper nigrum*	11
Cumin	*Cuminum cyminum*	5
Clove	*Syzygium aromaticum*	4
Cinnamon	*Cinnamomum cassia*	7
Ground mustard	*Brassica juncea*	2
Powdered garlic	*Allium sativum*	1
Five-pepper mix	*Piper nigrum*	2
Ground chilli pepper	*Capsicum annum*	2
White pepper	*Piper nigrum*	1
Onion powder	*Allium cepa*	1
Sweet (bell) ground pepper	*Capsicum annum*	1
Ground cayenne pepper	*Capsicum annum*	1
Subtotal		46
Herbs		
Thyme	*Thymus vulgaris*	3
Basil	*Ocium basilicum*	3
Dill	*Anethum graveolens*	3
Rosemary	*Rosmarinus officinalis*	3
Parsley	*Petroselinum crispum*	2
Subtotal		14
Total		60

2.2. Detection of Bacillus cereus

B. cereus was detected according to the International organization for Standardization (Anonymous, 2004). The method included spread – plate technique on Mannitol Egg Yolk Polymyxin agar (Biolife, Milan, Italy) of 0.5 ml of 10^{-1} and 10^{-2} dilution on each plate. The samples were incubated for 18–24 h at 30 °C. Colonies characteristic to haemolysis determination were set on Sheep blood agar (Biolife, Milan, Italy), followed by 24 h incubation at 30 °C. Colonies with β haemolysis were identified using BBL Crystal Gram-Positive identification system (Becton and Dickinson, New Jersey, USA). The isolates were stored in Brain Infusion broth (BHI, Biolife, Milan, Italy) with added 30% of glycerol (Chempur, Piekary Śląskie, Poland).

2.3. Detection of moulds

Mould counts were determined according to International organization for Standardization (Anonymous, 2008) using the spread-plate technique on Dichloran Glycerol agar (DG – 18, Biolife, Milan, Italy). A total 0.1 ml of each 10^{-1} and 10^{-2} dilution were spread to DG - 18 agar and incubated for 96 h at 25 °C. The genuses of the moulds were identified by their macro- and micro-morphological characteristics according to Seifert, Morgan-Jones, Gams, & Kendrick, 2011, pp. 1–997.

2.4. DNA extraction of B. cereus

All isolated *B. cereus* strains were surface streaked on Tryptone Soy Agar (TSA, Biolife, Milan, Italy) and incubated for 18–24 h at 30 °C. DNA extraction was performed according to the PrepMan UltraSample Preparation (PN 4367554) protocol (PrepMan Ultra, Thermo Scientific, Waltham, USA). One hundred microliters of PrepMan Ultra sample preparation reagent was introduced into micro-centrifuge tubes. Isolated colonies were transferred to the tubes. The samples were vortexed for 10–30 s and incubated for a 10 min at 100 °C, followed by centrifugation for 3 min at 14 000 rpm. Acquired DNA concentrations were measured via spectrophotometer (Thermo Scientific, Waltham, USA) at 260/280 nm. Bacterial DNA of 10 ng/μl was used then for PCR (Biometra, Montgomery, USA).

2.5. PCR primers and PCR amplification for B. cereus

Emetic toxin gene (*ces, CER*) and enterotoxigenic toxin gene (*cytK, nheA, hblC, entFM*) oligonucleotide primers were used (Bioneer, Daejeon, Korea). *B. cereus* primer sequences and product size were used according to Kim et al., 2012a. A final concentration of 23 μl, consisting of 2 μl of DNA, 1× PCR buffer (Fermentas, Vilnius, Lithuania), distilled water, 1.5 mM MgCl2 (Fermentas, Vilnius, Lithuania), mix of 0.25 mM dNTP (Qiagen, Hilden, Germany), 0.3 μM of each primer and 1U Taq polymerase (Fermentas, Vilnius, Lithuania), was used for PCR amplification. The temperature cycling profile was initial denaturation at 95 °C for 10 min, followed by 30 cycles with denaturation at 94 °C, annealing at 54 °C for 1 min, elongation at 72 °C and with a final cycle at 72 °C for 5 min. DNase and RNase free water (SigmaAldrich, Munich, Germany) was used as a negative control. QiAxcel Advanced facility (Qiagen, Hilden, Germany) was used for gel electrophoresis according to the manufacturer's instructions.

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