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Identification, heat resistance and growth potential of mesophilic spore-forming bacteria isolated from Algerian retail packaged couscous

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ABSTRACT

Aims: In this study, species taxonomy, heat resistance and growth potential of mesophilic aerobic spore-forming *Bacillus* strains isolated from commercially available couscous were determined.
Methods and results: Aerobic spore-forming bacteria were isolated from three Algerian retail couscous samples heated at 80 °C for 10 min. Plate counting of spore-forming bacteria showed a mean concentration of 20 CFU/g. By monitoring 16S gene sequencing, ten *Bacillus* strains were identified, belonging to 3 different species: five *Bacillus licheniformis* strains, four *Bacillus cereus* group strains *sensu lato* and one *Bacillus subtilis* strain. According to the *panC* gene sequencing, the four *B. cereus* strains were assigned to the group IV (mesophilic and heat resistant group, associated with cases of foodborne illness). *B. cereus* cells growth kinetics in moistened couscous semolina showed a specific growth rate of 0.33 h^{-1} at 30 °C, confirming their growth ability in this media.
The heat resistance (δ value *i.e.* the first decimal reduction time) and heat sensitivity (z_T values *i.e.* the temperature increase leading a ten-fold reduction of the δ value) of spores of *B. cereus* and *B. subtilis* strains were determined using Weibull and Bigelow models, respectively. $\delta_{100\,°C}$ values are ranged from 0.14 to 7.90 min and estimated z_T values ranged from 7.52 °C to 10.38 °C. Moreover, the estimate four decimal reduction times at 90 °C (*t*4D) of spores of isolated *B. cereus* strains were from 0.58 h to 3.73 h.
Conclusions: B. cereus strains isolated from retail packaged couscous semolina are resistant to heat treatment both during the industrial food process and can easily grow in moistened couscous semolina. These observations could explain numerous *B. cereus* outbreaks associated with couscous semolina consumption.

1. Introduction

Wheat flour and different related food products such as pasta, semolina or couscous are frequently consumed. Couscous or K'sksou is a popular traditional food in Southern Europe and in North Africa, especially for Algerian people who prepare it for daily meals and collective celebrations. According to Derouiche (2003, 125 pp.) the consumption of couscous reaches 9.21 kg per year and per inhabitant in the East of Algeria. Moreover, couscous is eaten at least once a week in Constantine (Algeria) by more than 50% of the population (Benlacheheb, 2008, 175 pp.). According to CODEX STAN 202-1995, couscous is the product prepared from durum wheat semolina (*Triticum durum*) which has undergone physical processing such as cooking and drying. The elements of couscous are bound by adding drinking water.

Couscous semolina is considered as safe food, its low water activity limits bacterial or fungal development. It does not need any particular storage condition for its commercialization, transport and packaging. The microbial risks associated with durum wheat

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semolina appear and increase according to culinary customs and waiting time before consumption.

Bacterial spores are contaminants frequently found in the wheat flour or meal at low concentrations (10^2spores/g) (Aydin, Peter Paulsen, & Smulders, 2009; Berghofer, Hocking, Miskelly, & Jansson, 2003; Rogers, 1978; Spicher, 1986). Valerio et al. (2012) have analysed bacterial spore contaminations in durum wheat semolina. They have identified bacterial species such as *Bacillus amyloliquefaciens* (56.1% of isolates) which may lead to food alterations by enzyme productions. Other species frequently present such as *Bacillus cereus* (18.9% of isolates) that may represent health risks. *B. cereus* is the cause of many foodborne outbreaks. In France this bacteria specie is the fourth cause of identified foodborne outbreak and between 2006 and 2010 (1156 outbreak occurrences distributed in 62 consumption origins linked to *B. cereus*), 50% of these outbreak occurrences were linked to the consumption of starchy foods (38% with semolina or couscous) (Cadel Six et al., 2012).

Food poisoning outbreaks associated with couscous consumption have also been detected in North Africa (Aoued, Benlarabi, & Soulaymani-Bencheikh, 2010; Belomaria et al., 2007; Benkadour, 2002). Furthermore, several cases may not be declared, especially when related to self-healing. In Algeria, the microbiological specifications did not report any research on *Bacillus* species for cereal grains, milling and pasta products (JORADP, 1998). Spore-forming bacteria are not cited among the tested bacteria that can survive in dry food and present a high risk for consumers.

In industrial processes, couscous preparation consists in shearing the wheat and then sifting the dry wheat to store it in its separate forms. The grains formed are steam-cooked; this heat treatment (95°C–98 °C) how long does not completely inactivate bacterial spores. The cooked granules are dried to decrease the humidity to 12% then cooled and packaged (Yousfi, 2002, 141 pp.). Home preparation consists in hydrating couscous semolina and then steam heating. The temperature used during home preparation activates spore-forming bacteria which can grow and produce toxins in couscous, especially during long waiting time after preparation combined with temperature abuse.

The aim of the present study was to isolate, identify, and determine the heat resistance and growth potential of aerobic mesophilic spore-forming bacteria isolated from Algerian retailed packaged couscous.

2. Materials and methods

2.1. Bacterial strain isolation

In May 2011, 10 different precooked packaged couscous from three main Algerian brands (coded AB, EH and CM) were collected in 3 different retail markets to isolate mesophilic bacterial spores. The studied couscous was packed in hermetic plastic packaging and was sampled two months after its commercialization.

The plastic surfaces were sterilized with ethanol before opening the packets. The grains of couscous were mixed in sterile saline. All samples were heated at 80 °C for 10 min to eliminate vegetative cells. After cooling, 0.5 mL of sample was inoculated on a nutrient Agar plate then incubated at 37 °C for 24 h–72 h in aerobic conditions. Ten strains of mesophilic aerobic *Bacillus* species have been isolated and identified.

2.2. Strain identification

The isolated strains were identified following rRNA 16S sequencing and the affiliation of *B. cereus* groups was determined by *panC* gene sequencing.

2.2.1. DNA extraction

Studied strains were cultivated in nutrient broth at 37 °C overnight. 1.5 mL of this suspension was used to extract bacterial genomic DNA according to Sambrook, Fritsch, and Maniatis (1989). Briefly genomic DNA was obtained after alkaline cell lysis and genomic DNA extraction with phenol/chloroform/isoamylol solution.

2.2.2. 16S rRNA gene sequence

The PCR amplification of 16S rRNA partial gene sequences was carried out using both universal bacterial primers, the forward primer 27f (5′-GAGTTTGATCMTGGCTCAG-3′) and the reverse primer 1492r (5′-GNTACCTTGTTACGACTT-3′) (Weisburg, Barns, Pelletier, & Lane, 1991). The PCR products were resolved in 1% agarose gel (w/v) in the presence of ethidium bromide (0.5 μg/mL as final concentration) in TAE buffer with an electric field at 5V/cm. PCR reaction were performed with a "Flexigine" thermal cycler (Techne). Direct sequencing of the PCR fragments was performed at the company AGCT (Heidelberg, Germany). 16S gene sequences were further used to search for nucleotide–nucleotide matches in the BLAST database at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) in order to establish an initial strain identity (Altschul, Gish, Miller, Myers, & Lipman, 1990).

2.2.3. panC gene sequence

The PCR amplification of *panC* partial gene was carried out using 5′ GAGGCGAGAGAATACGGAATACG 3 and 5′ GCCCATTTGACTCGGATCCACT 3', as forward and reverse primers respectively (Candelon, Guilloux, Ehrlich, & Sorokin, 2004; Guinebretière et al., 2008) with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 15 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, and a final extension of 7 min at 72 °C (Candelon et al., 2004).

To establish the strain identity, sequencing analyses were performed at the following homepage https://www.tools.symprevius.org/Bcereus/(Guinebretière et al., 2010).

2.3. Heat resistance evaluation

Heat resistance of studied strains was carried out by the capillary method after spore preparation.

2.3.1. Spore preparation

Bacterial spore suspensions have been realised for the 10 strains of isolated mesophilic bacteria. First, cells were precultivated at 37 °C for 24 h in Brain Heart Infusion (Difco, Le Pont de Claix France). A volume of 0.5 mL was spread on nutrient Agar plates supplemented with 40 mg/L of $MnSO_4$ and 100 mg/L of $CaCl_2$. The plates were further incubated at 30 °C during 4 days until the maximum spore production was obtained (Baril et al., 2012). Spores were then collected by scraping the Agar surface and suspended in 20 mL of sterile distilled water. The spore suspension was washed three times with sterile distilled water followed by a centrifugation step at 10,000 g for 15 min. The washed pellet was suspended in water/ethanol (1V/1V) at 4 °C for 12 h in order to eliminate the vegetative form. Spores were harvested by centrifugation at 10,000 g then washed three times. The final pellet was suspended in a minimal volume of sterile distilled water to obtain 10^{10} spores per millilitre. The spore concentration was estimated by enumeration on nutrient Agar and kept at 4 °C for two months before thermal treatment.

2.3.2. Heat treatment

A volume of 30 μL of spore suspension was diluted in 3 mL of Brain Heart Infusion (Difco, Le Pont de Claix France). Then, the capillary tubes were filled with 100 μL of this dilution and heat