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Identification and characterization of aerobic spore forming bacteria isolated from commercial camel's milk in south of Algeria

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ABSTRACT

The aim of this study was to evaluate aerobic spore-forming species contamination levels in raw and pasteurized camel's milk. The isolated strains were also identified by 16S rDNA sequencing and characterized for their heat resistance. Camel milk (8 samples of which 4 were pasteurized) were collected from farms and markets from different regions in south east Algeria's (Laghouat, Ghardaïa and Ain Aminas). Aerobic spore counts had 2 log CFU/mL in the raw samples. The 16S rDNA sequencing enables the identification of a selection of 20 isolates: *Bacillus licheniformis* (Five strains), *Bacillus cereus* (Nine strains), *Bacillus subtilis* (Three strains), *Bacillus pumilus* (One strain), *Bacillus sonorensis* (One strain), *Geobacillus stearothermophilus* (One strain). All *B. cereus* strains belonged to the phylogenetic group III of *B. cereus*, according to the classification proposed by Guinebretière et al. (2008), and supposedly represent potential strains of pathogenic *B. cereus*.

The heat resistance (D-value i.e. the decimal reduction time) and heat sensitivity (z_T values i.e. the temperature increase that lead to a ten-fold reduction of the D value) of spores of *B. cereus*, *B. licheniformis*, *B. pumilus*, *B. sonorensis*, *B. subtilis* were determined. The $D_{100\,°C}$-values were ranged 1.09–9.55 min whereas the z-values 6.45–8.50 °C.

1. Introduction

The dromedary camel (*Camelus dromedaries*) most prevailing and fast growing livestock in arid and semi-arid areas (Yakhchalim and Cheraghi, 2007; Silanikove and Koluman, 2015). Out of the 25 million camels worldwide (FAOSTAT, 2013), more than 250000 camels' are located in Algeria, The daily milk yield of Maghrebin she camels varies from 2.0 L/d (under intensive management) (El-Hatmi et al., 2004) to 6 and 12 L/d (under more favorable conditions) (Abdalla et al., 2015). The total production within 25 countries as estimated more than 1.3×10^6 tons with a highest quantity (1.0×10^6 tons) in Somalia and the lowest (30 tons) in the Russian federation (FAOSTAT, 2013). The average production of camel's milk in Algeria amounted in the year 2011–12 271 tons (FAOSTAT, 2013). Camel milk represents an important component of the human nutrition in arid and semi-arid regions. It is known for its high nutritional value. The vitamins content (e.g. vitamin C) is 3 folds higher than in cow milk and 1.5 times higher than in human milk (Khan et al., 2004). The iron content is 10 times higher than in cow milk (Akweya et al., 2012). Wernery et al. (2003) reported that in camel's milk, proteins are more heat resistant than those of cow's milk. Camel's milk is frequently claimed for its therapeutics proprieties, especially for elderly persons and immunocompromised patients, thus person groups which are susceptible to microbiological risks (e.g., Mullaicharam, 2014). However, modernization of camels dairy industry is still in infant stages: For instance, in Algeria it is often consumed as raw products. It is sold at informal markets packaged in second-hand water bottles or in the "Gerba" (large goatskin bag), served at the sale moment in non sterile plastic bags or package provided by the consumers. Only few farmers are selling the milk in sealed bottles Pasteurized camel milk is sold in only few countries including Saudi Arabic, United Arab Emirates, Kazakhstan and Mauritania (Wernery et al., 2003). In Algeria, none

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of these products are commercialized except one irregular small manufacturing site which pasteurizes milk situated at Ghardaïa (600 km from Algiers). The non-treated camel's milk may contain a lot of spoiling and/or pathogenic bacteria as *Staphylococcus aureus*, *E. coli* and *Streptococcus* spp. (Ahmad et al., 2011; Amena et al., 2011; Elhaj et al., 2014). Besides, several studies confirmed the presence of lactic acid bacteria in Algerian camel's milk (Karam and Karam, 1994; Benmechernene et al., 2013). However, only few data were concerned with spore-forming bacteria in camel milk, which can represent a risk for consumers (Stenfors-Arnesen et al., 2008). Their presence in camel milk should be considered, as the commercial sterility requirements are not achieved.

The objective of this study is to evaluate the contamination level with spore-forming bacteria of camel's milk by aerobic and to identify isolated strains by sequencing the 16S rDNA and to characterize their heat resistance.

2. Material and methods

2.1. Material

The raw and pasteurized camel's milk samples were collected during March 2013 from three different regions in the south of Algeria (Laghouat, Ghardaïa and Ain Aminas). The samples were kept in their commercial packages and information related to these samples is indicated in Table 1. The counting of spore-forming bacteria in camel's milk was carried out in the laboratory at Laghouat University.

The milk samples, which were purchased in the morning and transported in an isotherm bag at 4 °C to the laboratory, were analyzed immediately for bacterial spores counting. The milk samples from Ghardaïa, Ain Amenas (W. Illizi), which were transported at 4 °C to the laboratory were frozen at −18 °C, before quantifying the aerobic spores in the samples.

2.2. Enumeration and isolation of Bacillus ssp

10 mL of each sample were transferred into a sterile tube and heated at 80 °C for 10 min. Then, 1 mL of each sample was homogenized with 9 mL of Tryptone Salt buffer (TS buffer, Biokar Diagnostics, Beauvais, France) and serial of decimal dilutions were made in TS buffer for each sample. 0.5 mL of each tube dilution was streaked on Mueller Hinton Agar (BD Difco 211436). The Mueller Hinton Agar was choosed for its starch content, which improves the recovery of spore-forming bacteria. The total number of aerobic spore was enumerated after incubation at 30 °C for 24 h for all samples. In parallel, thermophilic aerobic spore counts was performed for 2 samples of pasteurized milk (L47c, L47d), after incubation at 55 °C for 48 h. A selection of 20 colonies with distinct morphology were randomly selected and isolated on nutrient agar (Fluka, 70148, Buch/Switzerland).

The obtained isolates were checked for catalase activity and examined microscopically after Gram staining. The Gram positive and catalase positive bacteria were purified and stocked at 4 °C on nutrient agar. Spore was produced as described by Ziane et al. (2014). The final pellet of spores was suspended in a minimal volume of sterile distilled water to obtain 10^{10} spores per milliliter and was stored at 4 °C.

2.3. Identification of isolates by rDNA 16S sequencing

Studied strains were cultivated overnight in nutrient broth at 37 °C, or 55 °C. 1.5 mL from this suspension was used to extract bacterial genomic DNA according to Sambrook et al. (1989). Genomic DNA was obtained after alkaline cell lysis and genomic DNA extraction with phenol/chloroform/isoamylol solution (P2069, Sigma-Aldrich, Milan, Italy).

The amplification of 16S rDNA partial gene sequences by PCR was carried out using both universal bacterial primers, the forward primer 27f (5′-GAGTTTGATCMTGGCTCAG-3′) and the reverse primer 1492r (5′-GNTACCTTGTTACGACTT-3′ (Weisburg et al., 1991). The PCR products were resolved in 1% agarose gel (w/v) with an electric field at 5 V/cm. The samples were coloured with ethidium bromide (0.5 μg mL^{-1} as final concentration) in Tris-borate-EDTA buffer (TAE, 40 mM Tris-acetate (Tris (93349, Sigma-Aldrich,Milan, Italy) and glacial acetic acid (695092 Sigma-Aldrich, Milan, Italy), 10 mM EDTA (E9884, Sigma-Aldrich, Milan, Italy), pH 8,2). The PCR reactions were performed with a "Flexigine" thermal cycler (Techne, Staffordshire, ST15 OSA, UK). The Direct sequencing of the PCR fragments was performed by GATC Biotech (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 strain identity (Altschul et al., 1990). The recognized 16S rDNA gene sequence were checked following a cluster analysis using the MEGA 5 software after an alignment with ClusterW in the aim to confirmed the blast result. It consist to align the 16S rDNA gene sequence of isolates strains with almost of database sequence at the NCBI than established the phylogenetic tree.

2.4. Discrimination of Bacillus cereus phylogenetic group based on panC gene sequencing

Discrimination of *B. cereus* phylogenetic groups was performed using *panC* partial gene sequence according to the procedure described by Guinebretière et al. (2008, 2010). Briefly, the PCR amplification of *panC* was carried out using a first set of primers for the determination of group I to VI, *i.e.* 5′ TYGGTTTTGTYCCAACRATGG 3′ and 5′ CATAATCTACAGTGCCTTTCG 3′, as forward and reverse primers respectively. The PCR was carried out in a CFX96 (Bio-Rad, CFX96, Marnes-la-Coquette, France) 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; Guinebretière et al., 2008).

To establish strain identity, gene sequences were compared to available database using a BLAST online tool with the following link http://symprevius.eu/software/.

2.5. Heat resistance evaluation

The heat resistance of 9 spore's suspensions were determined using capillary method as described by Gaillard et al. (1998) at three temperatures, which ranged from 90 °C to 105 °C, depending on the strain. Survival spore enumeration was performed by inclusion of 0.5 mL of the heated spore solution in 15 mL of nutrient agar (Biokar Diagnostics, Beauvais, France). The plates were incubated at 37 °C (mesophilic bacteria) and 55 °C (thermophilic bacteria) for 24 h. After the incubation the number of unit forming colonies (CFU) was determined.

The classical decimal reduction times was applied as follow (Eq. (1)):

$$\mathrm{Log}N = \mathrm{log}N_0 - \left(\frac{t}{D}\right) \quad (1)$$

N_0: represents the initial number of cells, N: the number of cells at time t, D parameter represents the time necessary to inactivate the 90% of the population. The influence of the temperature on bacterial