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Short communication

Occurrence and behavior of *Bacillus cereus* in naturally contaminated ricotta salata cheese during refrigerated storage

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ABSTRACT

The present study shows the fate of *Bacillus cereus* in refrigerated ricotta salata cheese during shelf-life. 144 ricotta salata cheese belonging to nine naturally contaminated batches were stored refrigerated and analyzed at 24 h, 30, 60 and 90 days of storage. Total bacterial count, *B. cereus* spores and vegetative forms, intrinsic properties and composition were determined. The presence of spores was sporadic while the prevalence and the level of *B. cereus* vegetative cells decreased respectively from 83.3 % to 4.65 ± 0.74 cfu g^{-1} at the beginning of the observation period to 33.3 % and 1.99 ± 0.55 cfu g^{-1} after 90 days. No information is currently available on the fate of *B. cereus* in ricotta salata. The production process of ricotta salata includes steps such as whey heating followed by slow cooling of clots, which expose to the risk of spore germination and successive growth to levels compatible with toxins production. The prolonged refrigerated storage was not favorable to sporulation, explaining the successive death of vegetative cells. The present study demonstrate the potential risk of food poisoning as consequence of pre-formed emetic toxins in ricotta salata. Food safety of ricotta salata relies on the rapid refrigeration of the product during critical phases for cereulide production.

1. Introduction

The *Bacillus cereus* group includes Gram-positive rod shaped spore-forming bacteria, which are widely distributed in the natural environment. Within the group, *B. cereus sensu stricto* is the most important organism causing food spoilage and food-borne illness (Kramer et al., 1989). *B. cereus* causes two clinical forms of food-borne illness: the emetic and the diarrheal syndrome (Granum and Lund, 1997). A dose of 10^5–10^8 cells or spores per gram is generally considered necessary to cause illness (ICMSF, 1996; Granum and Lund, 1997). *B. cereus* is frequently isolated from raw milk and dairy products thus, representing a serious concern for the dairy industry (Svensson et al., 2006). Due to its ubiquitous nature and the extreme resistance of endospores to several harsh conditions (Nicholson et al., 2000), it is difficult to avoid the contamination of dairy products. *B. cereus* can enter the dairy chain mainly through raw milk contaminated at farm level (Heyndrickx, 2011). However, contamination may also arise from the food-processing environment (da Silva Fernandes et al., 2014). Dairy products have been seldom associated with human illness despite the frequent contamination with *B. cereus* (EFSA, 2005). Whey products processed at high temperatures and successively stored refrigerated are particularly exposed to the risk of *B. cereus* (Heyndrickx et al., 2002). The endospores are activated by whey heating applied during protein denaturation (>80 °C) and vegetative cells are then facilitated in their growth by the absence of competing microbiota, inactivated by the heat treatment (Scheldeman et al., 2006). *B. cereus* psychotropic strains can grow to temperature as low as 4–5 °C and during the refrigerated storage can reach levels potentially harmful for human health (Huck et al., 2007). Ricotta salata is a traditional dry and salted sheep's milk whey cheese produced in Sardinia (Italy). Technology and microbiological profile of ricotta salata have been previously described (Spanu et al., 2015). The attributed shelf-life of ricotta salata is generally up to several months under refrigerated storage (Casti et al., 2016). The present study was conducted following a case of large *B. cereus* contamination of ricotta salata occurred in one sheep's milk cheese-making plant operating in Sardinia. During the period September–October

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2014, a local food business operator observed the presence of *B. cereus* contamination in ricotta salata samples during routine microbiological testing conducted as part of their procedure based on HACCP principles. The mean level of contamination was 5.57 ± 0.15 $\log_{10}$ cfu g^{-1} in a batch. Although no food safety criteria for *B. cereus* are applicable to foodstuffs placed on the market during their shelf-life (EC Regulation No. 2073/2005), the food business operator as corrective action withdrew the entire batch of ricotta salata. The subsequent production batches positive for the presence of *B. cereus*, were destined to a durability study. The few published data existing on *B. cereus* contamination in ricotta salata produced in Sardinia reported a prevalence of ca. 15 % and a contamination level ranging from 1 to 3 $\log_{10}$ cfu g^{-1} (Cosentino et al., 1997; De Santis et al., 2008; Fadda et al., 2012). Despite the reported contamination levels are below the dose necessary to cause illness, they demonstrate that *B. cereus* in ricotta salata represents a potential concern for consumer's health. No published reports are currently available on the fate of *B. cereus* in naturally contaminated ricotta salata stored under refrigerated conditions. The aim of the present study was to describe the evolution of *B. cereus* in naturally contaminated ricotta salata during shelf-life and to assess the potential health risk associated with the microorganism survival or growth.

2. Materials and methods

2.1. Ricotta salata batches and samples

Ricotta salata batches used in the study were selected based on the natural occurrence of *B. cereus*. With this aim, during the period September–October 2014, ricotta salata production batches were tested on a daily basis for the presence of *B. cereus*. From each positive batch were randomly selected sixteen ricotta salata wheels. Samples were immediately vacuum packed in plastic bags, transported refrigerated to the laboratory and stored in cold room (4 ± 2 °C) until analyses were performed.

2.2. Experimental design

Ricotta samples were analyzed at four different times during the shelf-life. Sampling times were: within 24 h after the arrival of ricotta salata wheels defined as time zero (T_0), 30, 60 and 90 days after the production defined respectively as time 30 (T_{30}), time 60 (T_{60}) and time 90 (T_{90}). From each of the nine different batches and at each sampling time, two samples were used for microbiological analysis and two samples for physico-chemical determinations.

2.3. Microbiological analysis

Ricotta salata samples were analyzed for the determination of total aerobic mesophilic bacteria (ISO 4833, 2013) and enumeration of *B. cereus* (ISO 7932, 2004). The enumeration of both *B. cereus* vegetative cells and spores was determined, respectively before and after heating at 80 °C for 10 min by plating two 0.1 mL aliquot on selective chromogenic culture media such as Mannitol Egg Yolk Polymyxin agar (MYP, Biolife, Milan, Italy) and Polymyxin Pyruvate Egg-Yolk Mannitol Bromothymol Blue (PEMBA, Oxoid) agar. Samples were incubated at 30 °C in aerobic conditions for 24 h. From each positive sample were picked five presumptive *B. cereus* colonies, transferred onto Trypticase Soy Agar (TSA, Biolife) and incubated at 37 °C for 24 h. Each isolate was submitted to phenotypic identification and successively confirmed by PCR (Oh et al., 2012).

2.4. Intrinsic properties and composition

PH and a_W were measured using pH meter GLP22 (Crison Instruments SA, Barcelona, Spain) and water activity meter Aqualab 4 TE (Decagon, Pullman, WA, USA), respectively. Determination of centesimal composition (% of moisture, fat, protein, salt and total solids) was performed using the Near Infrared Transmittance (NIT) compositional analyzer (FOSS, Eden Prairie, MN, USA).

3. Results

3.1. Microbiological profile

The mean aerobic mesophilic counts ($\log_{10}$ cfu g^{-1}; $\bar{x}$ ± SD) of ricotta salata analyzed at T_0, T_{30}, T_{60} and T_{90} were 5.17 ± 1.39, 5.69 ± 0.54, 5.99 ± 0.67 and 5.62 ± 0.87, respectively. The prevalence of *B. cereus* vegetative cells and the mean contamination level decreased during the refrigerated storage ($P < 0.05$). At T_0, the prevalence was 83.3% with counts ranging from 3.45 $\log_{10}$ cfu g^{-1} to 6.20 $\log_{10}$ cfu g^{-1}, while at T_{90} the observed prevalence was 33.3% with counts ranging from 1.30 $\log_{10}$ cfu g^{-1} to 2.56 $\log_{10}$ cfu g^{-1} (Table 1). The mean reductions over time (ΔT) in *B. cereus* vegetative cells concentration ($\log_{10}$ cfu g^{-1}) were 0.38, 1.74 and 2.66 at T_{30}, T_{60} and T_{90}, respectively. The detection of *B. cereus* spores after heat activation was observed in two samples belonging to two different batches, one at T_{30} (2.30 $\log_{10}$ cfu g^{-1}) and one at T_{60} (2.0 $\log_{10}$ cfu g^{-1}), respectively. Out of 49 total positive samples (68.0 %) were isolated 245 presumptive *B. cereus* strains of which 101 were confirmed by molecular identification.

3.2. Physico-chemical characteristics

The pH values ranged between 6.23 and 6.67 at T_0 and between 5.30 and 6.32 at T_{90}, while a_W values ranged between 0.964 and 0.986 at T_0 and between 0.976 and 0.983 at T_{90}. The evolution of the mean centesimal composition values (%; $\bar{x}$ ± SD) at different sampling times is reported in Table 2.

4. Discussion

Despite raw milk is the main source of contsamination of dairy product with sporeformers, their level is generally low, $<1–10^2$ cfu mL^{-1} (Vissers et al., 2007). Seasonal variation has been reported with counts as high as 10^4 cfu mL^{-1} (Slaghuis et al., 1997; TeGiffel et al., 2002; Coorevits et al., 2008). The presence of *B. cereus* in ricotta salata is a rare finding, with maximum contamination level of ca. 3 $\log_{10}$ cfu g^{-1} (Cosentino et al., 1997; De Santis et al., 2008; Fadda et al., 2012; Spanu et al., 2012). The high level of contamination, up to 8.33 $\log_{10}$ cfu g^{-1}, observed in the present study and the large number of positive batches (nine) over a limited period of time (three months), should be considered as an event strictly associated with the late summer and early fall production period. Microbiological testing of each production batch, conducted on a regular basis in the frame of the food business operator's HACCP procedures, showed no occurrence of *B. cereus* contamination during the rest of the year. This could be explained with the typical sheep's milk breeding systems adopted in Sardinia. Milk production is seasonal, starting from December until July. The peak of milk production is concentrated between January and May, with a decrease between June and August, when the sheep start entering in the dry period. Cheese-making during the dry period relies on the milk available provided by flocks adopting the out-of-season breeding system. Poor pasture quality during this season determines a decline in milk yield and microbiological quality (Sitzia et al., 2015). Due to economic reasons, raw milk is picked and