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

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

Carlo Spanu*[1], Christian Scarano[1], Vincenzo Spanu[1], Carlo Pala[1], Daniele Casti[1], Sonia Lamon[1], Francesca Cossu[1], Michela Ibba[1], Gavino Nieddu[2], Enrico P. L. De Santis[1]

[1]*Department of Veterinary Medicine, University of Sassari, Via Vienna 2, 07100, Sassari, Italy*

[2]*Cooperativa Allevatori Ovini Formaggi Soc. Coop. Agricola, Loc. "Perda Lada" Fenosu, 09170, Oristano, Italy*

*Corresponding author. Tel.: +39 079 229454; fax.: +39 079 229458. *E-Mail address*: cspanu@uniss.it (C. Spanu); Via Vienna 2, 07100, Sassari, Italy.

Abstract

The present study shows the fate of *B. 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% and 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 cerulide production.

Keywords: *Bacillus cereus*; whey cheese; sheep's milk

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 and Gilbert, 1989). *B. cereus* causes two clinical forms of foodborne 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 and Scheldeman, 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 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.

2.4. 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).

2.5. 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 contamination of dairy product with sporeformers, their level is generally low, <1-10^2cfu 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 transformed every three or five days instead that daily. In addition, during winter and spring periods sheeps mainly graze on grass pasture, while during the summer and fall periods on stubble with concentrate and feedstuff supplement, which may increase the risk of transferring spores into raw milk. Total bacterial count and yield records of the milk used to make the ricotta salata used in the present study, were obtained by the food business operator. Data confirmed differences in milk yield and microbiological quality over the milking season. In the period from January to June, the total bacterial count (geometric mean) of raw milk was ca. 140,000 cfu mL^{-1} with an average production of 2,150,000 l, while in the out-of-season period the total bacterial count was ca. 1,100,000 cfu mL^{-1} with an average milk yield of 135,000 l. Therefore, the production of ricotta salata during the out-of-season period was characterized by risk factors increasing the likelihood of a high initial level of *B. cereus* contamination in the product. The greater relative decrease in *B. cereus* vegetative cells was observed after 60 days (-1.36 $\log_{10}$ cfu g^{-1}) and after 90 days (-0.92 $\log_{10}$ cfu g^{-1}) of refrigerated storage. Since psychrotrophic strains can grow at temperature as low as 4-5°C, contamination was likely due to mesophilic strains which minimum growth temperature is 15°C (ICMSF, 1996). Growth and survival characteristics of *B. cereus* vary widely between strains and depend upon a complex series of interacting factors such as temperature, pH, water activity (NaCl concentration), nutrients and presence of competitive microbiota. *B. cereus* is generally a poor competitor in unpasteurized products (Andersson, Ronner, & Granum, 1995). The high total bacterial count (ca. 6 log), combined with the decrease of pH values (from 6.49 to 5.63) observed over time, suggest the possible presence of contaminants from the whey or the environment that may have exerted a possible competitive action. A previous study, conducted on vacuum packed ricotta salata, showed a mean aerobic mesophilic bacteria count ($\log_{10}$ cfu g^{-1}) after 2 and 4 months of refrigerated storage ranging from 7.56±0.85 and 4.57±0.62 on the rind and from 3.64±0.71 and 2.95±0.65 on the inner paste, respectively (Spanu et al., 2013). At the beginning of the ricotta salata storage *B. cereus* is mainly present in its vegetative form, as consequence of heat activation of spores occurred during whey heating. The successive phase of pressing of the warm clots, expose the product to temperature at risk for the growth of the vegetative forms to levels compatible with the emetic toxin (cerulide) production. The reduction in *B. cereus* vegetative cells count over the storage period suggests the death of the microorganism rather than sporulation, since no grow was observed in samples analyzed after pasteurization. The sporulation is a complex process which occurs as response to stress such as starvation, high cell density (10^6-10^7 cfu g^{-1}) or DNA damage and it is regulated by hundreds of genes (Eichenberger et al., 2003; Piggot and Hilbert, 2004). Borge et al. (2001) concluded that vegetative cells are unlikely to develop endospores in refrigerated media. The high levels of ricotta salata contamination with *B. cereus* observed in the present study indicates that this product could represents a risk for human health due to the potential presence of pre-formed emetic toxins in the product. The low contamination level observed in the product after long refrigerated storage could lead to the wrong conclusion that the product is safe, while cerulide still persists. In fact, cerulide is highly resistant to heat, low pH, and proteolytic activity of pepsin and trypsin (Kramer and Gilbert, 1989; Rajkovic et al., 2008). Further investigation is needed in order to assess whether the origin of the contamination is from ingredients, processing environment or from packaging materials and to determine the pathogenicity of the strains.

5. Conclusion

Ricotta salata production process includes critical phases such as heat coagulation and slow cooling of clots, which support the activation of *B. cereus* spores and the successive growth of vegetative cells, in the absence of competing microbiota. The present investigation demonstrates that the level of *B. cereus* vegetative cells in naturally contaminated ricotta salata decreases during refrigerated storage, while the presence of spores is a rare finding. The control of *B. cereus* in ricotta salata relies on one hand on limiting the level of spores in raw milk, and as consequence in the whey, and on the other hand in preventing germination and successive growth of vegetative cells.

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Table 1. Evolution of *B. cereus* ($\log_{10}$ cfu g^{-1}; mean±SD) in nine batches of vacuum-packed Ricotta salata stored refrigerated until 90 days after production.

Batch	T_0	T_{30}	T_{60}	T_{90}
1	4.51±0.00^{a} (n = 1/2)	2.79±0.79^{a} (n = 2/2)	N.D. (n = 2/2)	N.D. (n = 2/2)
2	4.37±0.37^{a} (n = 2/2)	5.04 ±0.00^{a} (n = 1/2)	2.94±1.00^{a} (n = 2/2)	N.D. (n = 2/2)
3	4.68±0.24^{a} (n = 2/2)	4.49±0.20^{a} (n = 2/2)	3.26±0.93^{ab} (n = 2/2)	1.30±0.00^{b} (n = 1/2)
4	4.50±0.33^{a} (n = 2/2)	4.68±0.20^{a} (n = 2/2)	2.15±0.00^{b} (n = 1/2)	1.78±0.68^{b} (n = 2/2)
5	4.66±0.35^{a} (n = 2/2)	3.85±0.21^{a} (n = 2/2)	N.D. (n = 2/2)	N.D. (n = 2/2)
6	4.88±0.00^{a} (n = 1/2)	3.81±0.74^{a} (n = 2/2)	3.45±0.16^{a} (n = 2/2)	N.D. (n = 2/2)
7	4.0±0.00^{a} (n = 1/2)	3.62±0.00^{b} (n = 1/2)	2.38±0.00^{a} (n = 2/2)	N.D. (n = 2/2)
8	6.19±0.14^{a} (n = 2/2)	4.83±0.43^{b} (n = 2/2)	3.78±0.00^{bc} (n = 1/2)	2.56±0.00^{c} (n = 1/2)
9	3.77±0.46^{ab} (n = 2/2)	5.43±0.89^{b} (n = 2/2)	2.46±1.67^{a} (n = 2/2)	2.26±0.00^{a} (n = 2/2)
total	4.65±0.74^{a} (n =15/18)	4.27±0.90^{a} (n =16/18)	2.91±0.84^{b} (n =12/18)	1.99±0.55^{c} (n =6/18)

The sampling time (T_0, T_{30}, T_{60} and T_{90}) were respectively the day of packaging and 30, 60 and 90 days after the production. Means in the same row with different superscript letters are significantly different ($P < 0.05$); values within brackets indicate the prevalence of positive samples. N.D = data not definable, below the detection limit of the method.

Table 2. Intrinsic properties (mean ± SD) and composition (% ± SD) evolution during storage of ricotta salata wheels.

	sampling times			
parameter	T_0	T_{30}	T_{60}	T_{90}
pH	6.49±0.10[a]	6.18±0.10[b]	5.73±0.14[c]	5.63±0.28[c]
a_W	0.978±0.001[ab]	0.976±0.002[a]	0.976±0.002[a]	0.980±0.001[b]
% moisture	58.28±2.91[a]	58.56±3.29[a]	57.41±2.99[ab]	56.23±2.55[b]
% total solids	41.72±2.93[a]	41.44±3.26[a]	42.59±2.99[ab]	43.77±2.49[b]
% fat	23.74±3.92[a]	23.13±4.28[a]	23.32±3.73[a]	23.39±3.86[a]
% protein	12.65±1.12[a]	12.83±1.16[a]	13.01±0.84[a]	13.36±1.14[a]
% salt	2.60±0.30[ab]	2.73±0.24[b]	2.49±0.28[c]	2.01±0.49[d]

The sampling time (T_0, T_{30}, T_{60} and T_{90}) were respectively the day of packaging and 30, 60 and 90 days after the production. Means in the same row with different superscript letters are significantly different ($P < 0.05$).