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Evaluation of psychrotrophic behavior and lipolytic and proteolytic activity of *Bacillus cereus* isolated from refrigerated dairy products

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ABSTRACT. The present study evaluated the incidence of *B. cereus* in samples from 85 refrigerated dairy products through inoculation in selective medium and confirming according to biochemical proofs. Confirmed strains were then inoculated in specific growth medium at 7, 10 and 30°C to test the psychrotrophic behavior, and lipolytic and proteolytic activity. *B. cereus* was found in 15 of the 85 samples (17.6%); and 12 of them (80%) were let to grow up at 10°C for 7 days. When incubated at 7°C for 10 days, four strains (26.7%) grew up. All isolated *B. cereus* strains have shown proteolytic activity at 30°C, and 5 (33%) also presented lipolytic activity. At 10°C for 7 days, six strains (40%) showed proteolytic activity, and only one (6.7%), lipolytic activity. The temperature of 7°C for 10 days inhibited the production of proteases and lipases by the strains. The present work confirmed the presence of *B. cereus* strains with psychrotrophic, proteolytic and lipolytic activities in dairy products, which represents a potential risk for products stored under refrigeration.

Keywords: lipase, protease, *Bacillus cereus*, refrigeration.

Avaliação do comportamento psicrotrófico e atividade lipolítica e proteolítica de *Bacillus cereus* isolado de produtos lácteos refrigerados

RESUMO. *Bacillus cereus* é um micro-organismo termodúrico, psicrotrófico e patogênico. O presente trabalho avaliou a incidência de *B. cereus* em 85 amostras de produtos lácteos refrigerados por inoculação em meio seletivo e confirmação por provas bioquímicas. As estirpes confirmadas foram inoculadas em meio de crescimento específico e incubadas a 7, 10 e 30°C para avaliação do comportamento psicrotrófico, atividade lipolítica e proteolítica. *B. cereus* foi isolado em 15 amostras (17,6%), sendo que 12 (80%) se multiplicaram a 10°C por sete dias; a 7°C por dez dias quatro estirpes (26,7%) apresentaram crescimento. Todas as cepas de *B. cereus* apresentaram atividade proteolítica a 30°C e cinco (33%) apresentaram atividade lipolítica. A 10°C por sete dias seis cepas (40%) apresentaram atividade proteolítica e somente uma (6,7%) apresentou atividade lipolítica. A temperatura de 7°C por dez dias inibiu a produção de lipases e proteases. O presente trabalho confirmou a presença de estirpes de *B. cereus* psicrotróficas, proteolíticas e lipolíticas em produtos lácteos, representando um potencial perigo para os produtos armazenados em refrigeração.

Palavras-chave: lipase, protease, *Bacillus cereus*, refrigeração.

Introduction

Bacillus cereus is a gram-positive rod-cell, aerobic and spore-forming bacterium. It is an environmental microorganism and its optimum temperature for multiplication is between 25 and 37°C, while psychrotrophic thermoduric strains can grow from 3 to 75°C (DROBNIEWSKI, 1993; DUFRENNE et al., 1995).

This is a thermoduric microorganism because its spores can survive to the thermal processes used in food industry (AIRES et al., 2009; GAILLARD et al., 1998; MAZAS et al., 1999). Some strains have psychrotrophic behavior, being able to multiply at refrigeration temperatures (GARCIA-ARMESTO; SUTHERLAND, 1997; LARSEN; JORGENSEN, 1999).

The combination of psychrotrophic and thermoduric properties in the same microorganism represents a great potential to spoil food (MATTA; PUNJ, 1999; MEER et al., 1991). In addition, *B. cereus* is responsible for two types of food borne diseases: the diarrhea syndrome, caused by an enterotoxin produced *in vivo*, and the emetic syndrome, related to a preformed toxin in food (AGATA et al., 1995; OMBUI et al., 1997; WIJNANDS et al., 2006).

The presence of *B. cereus* in dairy products and processed milk could be related to raw material contamination, survival of the spores to the heating

process and the post-processing contamination (CHRISTIANSSON et al., 1999; GIFFEL et al., 1997; REZENDE-LAGO et al., 2007; SCHRAFT et al., 1996; SVENSSON et al., 2004).

The presence of *B. cereus* has been reported in raw and pasteurized milk, UHT milk, powder milk, fermented milk, ice cream and others dairy products in several countries (BARTOSZEWICZ et al., 2008; CHRISTIANSSON et al., 1999; REYES et al., 2007; REZENDE-LAGO et al., 2007; VIDAL-MARTINS et al., 2005; WONG et al., 1988), as well as strains of *B. cereus* with psychrotrophic behavior (GARCIA-ARMESTO; SUTHERLAND, 1997; GIFFEL et al., 1997; SVENSSON et al., 2004; VAISANEN et al., 1991; ZHOU et al., 2010).

B. cereus usually synthesizes several enzymes, among them proteases and lipases, responsible for giving undesirable sensory attributes to dairy products (ALMEIDA et al., 2000; CHEN et al., 2004; JANSTOVÁ et al., 2006).

The proteases can cause gelatinization in UHT milk (VIDAL-MARTINS et al., 2005), inducing the formation of bitter taste, and decrease the powder milk solubility (CELESTINO et al., 1997) and also reduce the cheese production yield (HICKS et al., 1986). The lipases role on the milk fatty acids causes the formation of off flavor in pasteurized milk, UHT milk, milk powder and cheese (FROMM; BOOR, 2004; SORHAUG; STEPANIAK, 1997).

Lipolysis induces the formation of bad taste in dairy products due to the hydrolysis of milk triglycerides. Short-chain fatty acids such as butyric acid, caproic acid, and caprylic acid confers strong and spicy taste, on the other hand, the medium-chain fatty acids such as capric acid and lauric acid have greater participation in the formation of "soapy taste" (CHEN et al., 2003).

This study aimed to evaluate the incidence of *Bacillus cereus* in traded dairy products, verifying the psychrotrophic behavior, proteolytic and lipolytic activities of isolated strains.

Material and methods

We evaluated 85 samples from different refrigerated dairy products of regular Brazilian brands, from April to June of 2009. Those samples consisted of 22 cream cheeses, 17 pasteurized milk, 17 pasteurized milk creams, 12 butter, 8 white cheeses, 5 fresh cheese and 4 other dairy products. All samples were stored at 4°C till the laboratory analyses.

For the research of *B. cereus*, it was performed selective enrichment in tryptone soy broth (TSB) supplemented with polymyxin B, followed by incubation at 30°C for 24-30 hours. The selective plating agar was made with mannitol-egg and yolk-polymyxin B (MYP). After incubation at 30°C for 18-40 hours, characteristic colonies of *B. cereus* (pink, with a precipitation halo around the colony) were subjected to biochemical identification (BENNET; BELAY, 2001).

The biochemical identification of *B. cereus* was done by the evidence of anaerobic use of glucose, decomposition of tyrosine, Voges-Proskauer test, reduction of nitrate to nitrite, and lysozyme resistance (BENNET; BELAY, 2001).

All isolated colonies confirmed as *B. cereus* by the biochemical tests were evaluated for proteolytic and lipolytic behavior at different temperatures. In this test, the strains were subcultured in milk agar (Plate Count Agar supplemented with 10% milk powder) for assessment of proteolytic activity, and in tributyrin agar (Plate Count Agar supplemented with 1% tributyrin) to evaluate the lipase activity (BEERENS; LUQUET, 1990). The plates were incubated at 7°C for 10 days, 10°C for 7 days, and 30°C for 48 hours, the colonies were considered positive when presented a precipitation halo around the colony, indicating the release of enzymes into the growth medium.

The strains of *B. cereus* were also inoculated in agar MYP and incubated at 7°C for 10 days and at 10°C for 7 days, aiming to evaluate the psychrotrophic behavior. The strains were considered positive when they get multiplied under these conditions.

Results and discussion

B. cereus was found in 15 of 85 samples (17.6%). Among these samples, four were pasteurized milks (4.7%), one milk cream (1.2%), one fresh cheese (1.2%), four butters (4.7%) and five cream cheeses (5.9%), according to Table 1. The results found in this experiment were similar to described by Vidal-Martins et al. (2005), which identified that 11.8% of 110 UHT milk samples were positive for B. cereus. However, this result was lower than found by Rezende-Lago et al. (2007) in Ribeirão Preto (Brazil), which evaluated 120 samples of raw milk, milk powder, UHT milk and pasteurized milk with the same techniques used in this study. In that study, B. cereus was isolated and identified from 58.3% of samples.

Zhou et al. (2010) verified *B. cereus* in 60% of the 40 ice cream evaluated samples, in China. In another study, also in China, 293 samples of dairy products purchased in the trade have been evaluated, and the authors isolated *B. cereus* in 52% of ice cream, 35% of

soft ice cream, 29% of milk powder, 17% of fermented milk and 2% of pasteurized milk and pasteurized flavored milk (WONG et al., 1988).

Table 1. Psychrotrophic behavior of *Bacillus cereus* strains isolated from refrigerated dairy products.

Products	Positive Samples	Psychrotrophic strains of *B. cereus*	
		10°C, 7 days	7°C, 10 days
Pasteurized milk	4 (4.7 %)	3	2
Milk cream	1 (1.2 %)	1	1
Fresh cheese	1 (1.2 %)	1	1
Butter	4 (4.7 %)	4	0
Cream cheese	5 (5.9 %)	3	0
Total	15 (17.6 %)	12 (80%)	4 (26.7%)

The incidence of *B. cereus* and contamination level in pasteurized milk may increase significantly during storage. Larsen and Jorgensen (1999) found low counts (<10 to 10^2 CFU mL^{-1}) in 2 of 27 milk pasteurized samples, however, after 8 days stored at 7°C, the counts were higher than 10^5 CFU mL^{-1}, and 24 of 27 samples were considered contaminated. This is an important fact to be considered, because the positivity and contamination level may be higher or lower depending on the point of shelf life at the sampling.

Twelve of the fifteen strains (80%) of *B. cereus* isolated from refrigerated dairy products get multiplied at 10°C for 7 days. Four of these strains (26.7%) also have grown up at 7°C. Three isolated strains have had no growth at refrigeration temperatures (Table 1).

The higher prevalence of *B. cereus* incubated at 10°C can be considered a problem for dairy products stored in marginal refrigeration temperatures, above the temperature indicated or recommended for the storage. Brazilian law regulates that pasteurized milk should never get to the trade places and be sold at temperatures above 7°C (BRASIL, 2011).

Giffel et al. (1997) concluded that all 106 strains of *B. cereus* isolated from dairy products were able to grow up at 10°C for 7 days; but only 56 (53%) had multiplied at 7°C for the same period. Garcia-Armesto and Sutherland (1997) evaluated 50 strains of *Bacillus spp* isolated from dairy products and identified 26 (52%) as psychrotrophic *B. cereus*, able to grow up at 6.5°C for 10 days. Vaisanen et al. (1991) have isolated strains of *B. cereus* from dairy products with a minimum temperature for growth below to 10°C, and alerted about the damage that these psychrotrophic strains can cause for refrigerated stored products.

Several *B. cereus* strains can produce lipases and proteases under refrigeration temperature. The evaluation of proteolytic and lipolytic activity of *B. cereus* strains isolated from dairy products is listed in Table 2.

Table 2. Proteolytic and lipolytic activities of *Bacillus cereus* strains isolated from refrigerated dairy products.

Activity	Positive strains of *B. cereus*		
	30°C, 48h	10°C, 7 days	7°C, 10 days
Proteolytic	15 (100%)	6 (40%)	0
Lipolytic	5 (33.3%)	1 (6.7%)	0

At 30°C for 48 hours, all strains of *B. cereus* showed proteolytic activity, and 5 strains (33%) also had lipolytic activity (Table 2). This data could be a negative statistic for products stored at regular room temperature, such as UHT milk, milk powder and milk cream. Nevertheless, in the present study it was evaluated only samples of products stored under refrigeration temperatures.

Five strains of *B. cereus* (33%) had both lipolytic and proteolytic activity. This result is lower than reported by Chen et al. (2004) that found 7 strains of *Bacillus spp* isolated from milk powder releasing both enzymes at 37°C.

At 10°C for 7 days, 6 strains (40%) had proteolytic activity; however no strain presented this behavior at 7°C for 10 days. The proteolytic action of *B. cereus* may produce the bitter taste and a sweet coagulation after 6 days of processing of dairy products (MEER et al., 1991).

Only one strain of *B. cereus* (6.7%) had lipolytic activity at 10°C, and none, at 7°C. Matta and Punj (1999) evaluated 100 samples of raw milk and found 48% of samples contaminated by psychrotrophic *Bacillus spp* with lipase activity at 4°C; 32.2% were identified as *B. cereus*.

Pasteurized milk samples sold in New York (USA) presented 32% of *Bacillus* spp. and 9% were *B. cereus*. The presence of free fatty acids was detected by a trained panel, which felt the rancid flavor and pointed it as a defect in the samples stored under refrigeration for 14 days (FROMM; BOOR, 2004).

The present study evidenced that the temperature of 7°C inhibits the production of proteases and lipases by *B. cereus*, being ideal for storing dairy products. Nevertheless, at 10°C, some strains have presented lipolytic and proteolytic activities, indicating that within a marginal temperature the quality of refrigerated dairy products might be undermined.

Conclusion

The presence of psychrotrophic, proteolytic and lipolytic strains of *B. cereus* represents a potential risk for dairy products stored under refrigeration temperatures, especially for products stored at temperatures above the recommended. The refrigeration under ideal condition inhibits the multiplication and production of enzymes by *B. cereus.*

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