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Centola Vidal, Ana Maria; Durival Rossi Junior, Oswaldo; Leite de Abreu, Irlan; Paes
Bürger, Karina; Vedovelli Cardoso, Marita; Siqueira Gonçalves, Ana Carolina; Marques
Rossi, Gabriel Augusto; Furlan D'Abreu, Léa
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Detection of *Bacillus cereus* isolated during ultra high temperature milk production flowchart through random amplified polymorphic DNA polymerase chain reaction

Detecção de *Bacillus cereus* isolado durante o fluxograma de produção do leite tratado por ultra alta temperatura através do DNA polimórfico amplificado ao acaso através da reação em cadeia pela polimerase

Ana Maria Centola Vidal^I Oswaldo Durival Rossi Junior^{II} Irlan Leite de Abreu^{III}
Karina Paes Bürger^{II} Marita Vedovelli Cardoso^{II} Ana Carolina Siqueira Gonçalves^{II}
Gabriel Augusto Marques Rossi^{II} Léa Furlan D'Abreu^{IV}

ABSTRACT

The present study focused on isolation *Bacillus cereus* during the UHT milk production and shelf life, to assess the enterotoxigenic production capacity of isolates and to evaluate the use of the RAPD-PCR technique to verify whether *Bacillus cereus* isolated at different phases of UHT milk processing belongs to the same strain. For this, six groups of milk samples composed of raw, pasteurized and UHT milk were collected from a processing plant. The results revealed that bacteria belonging to the *Bacillus cereus* group were isolated from 51.6%, 81.6% and from 13.8% of raw, pasteurized and UHT milk samples, respectively. About 50.0% of isolates from raw milk, 19.2% isolates from pasteurized milk and 70.7% isolates from UHT milk were capable of producing enterotoxins. It was confirmed the genetic similarity among *Bacillus cereus* isolates from raw, pasteurized and UHT milk, therefore demonstrating that the microorganism is able to withstand UHT treatment. These results should serve as a warning to health authorities, given that 13.8% of samples were not in accordance with standards established by the Department of Health for containing a potentially pathogen agent, therefore indicating that contamination of milk by sporulating bacteria should be avoided.

Key words: *Bacillus cereus*, enterotoxins, microbiological quality, pasteurized milk, raw milk, UHT milk.

RESUMO

Objetivou-se realizar o isolamento de bactérias do grupo do *Bacillus cereus* e verificação da semelhança genética entre elas, através da PCR-RAPD, durante a produção e vida de prateleira do leite UAT, bem como verificar sua capacidade enterotoxigênica. Para isso, foram colhidas em uma usina de beneficiamento seis grupos de amostras de leite compostos cada

um por amostras de leite cru, pasteurizado e UAT. Os resultados obtidos evidenciaram que bactérias do grupo do *Bacillus cereus* foram isoladas de 51,6% das amostras de leite cru, de 81,6% das de leite pasteurizado e de 13,8% das de leite UAT. Demonstraram-se capazes de produzir enterotoxinas 50,0% dos isolados de leite cru, 19,2% de leite pasteurizado e 70,7% de leite UAT. Também se pode constatar a semelhança genética entre as cepas de *Bacillus cereus* isoladas do leite cru, pasteurizado e UAT, evidenciando assim que o micro-organismo é capaz de resistir ao tratamento por UAT. Tais resultados devem servir de alerta às autoridades sanitárias, tendo em vista que 13,8% deles estavam em desacordo com o estabelecido pelo Ministério da Saúde, por serem capazes de veicular um agente potencialmente patogênico, demonstrando assim que a contaminação do leite por bactérias esporuladas deve ser motivo de preocupação.

Palavras-chave: *Bacillus cereus*, enterotoxinas, qualidade microbiológica, leite pasteurizado, leite cru, leite UHT.

INTRODUCTION

Milk is considered one of the noblest foods, given its peculiar composition rich in proteins, fat, carbohydrates, minerals, essential amino acids and vitamins, making it an essential component in the diet of all mammals, including humans. After milking, contaminating microorganisms from equipments and utensils, from environment and even from the employees responsible for obtaining and handling milk are

^IDepartamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos (FZEA), Universidade de São Paulo (USP), Av. Duque de Caxias Norte 225, 13635-900, Pirassununga, SP, Brasil. E-mail: anavidal@usp.br. Corresponding author.

^{II}Departamento de Medicina Veterinária Preventiva e Reprodução Animal, Faculdade de Ciências Agrárias e Veterinárias (FCAV), Universidade Estadual Paulista (UNESP), Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP, Brasil.

^{III}Centro Universitário de Rio Preto (UNIRP), São José do Rio Preto, SP, Brasil.

^{IV}Departamento de Zootecnia, FZEA, USP, Pirassununga, SP, Brasil.

the most important sources of contamination under the technological point of view, since it may cause undesirable alterations in the product, compromising the quality of milk and its derivatives and may even make it unsuitable for human consumption (SILVA et al., 2011).

Among the various microorganisms that can contaminate raw milk, bacteria belonging to the genera *Bacillus* and *Clostridium* should be highlighted (RAY, 2004). *Bacillus cereus* is important in the food industry due to its ability to produce toxins responsible for foodborne disease outbreaks (ARSLAN et al., 2014), production of extracellular enzymes that determine potential for food deterioration (MONTANHINI et al., 2013), and also production of heat-resistant spores that can withstand UHT treatment (BARTOSZEWICZ et al., 2008).

This microorganism is present in the milk submitted to UHT treatment may be due to some factors: bacterial spores resistant to heat treatment that were present in the raw milk, the improper packaging of milk after heat treatment allowing the entrance of microorganisms, recontamination after heat treatment (SALUSTIANO et al., 2009), whereas the spores of *Bacillus cereus* can form biofilm (PAGEDAR & SINGH, 2012).

At Brazil, milk samples were analyzed (30 of raw milk, 30 of pasteurized milk, 30 of UHT milk and 30 of powdered milk) and it was observed contamination by *Bacillus cereus* in 50%, 97%, 73% and 13%, respectively. Enterotoxin production by *Bacillus cereus* isolated was also observed in 64%, 31%, 33% and 80% of raw, pasteurized, powdered and UHT milk samples, respectively (REZENDE-LAGO, 2002). This bacterium with enterotoxigenic capacity was also described in dried milk products (REYES et al., 2007).

The genetic diversity and toxin production was evaluated in food samples for 30 years and it was noted that most isolates present in Brazilian foods have potential to cause foodborne diseases due to the presence of toxin-producing genes (CHAVES et al., 2011), but without a specific genetic profile (SANTOS et al., 2011).

In this context, the present study focused on isolation *Bacillus cereus* during the UHT milk production and shelf life, to assess the enterotoxigenic production capacity of isolates and to evaluate the use of the RAPD-PCR technique to verify whether *Bacillus cereus* isolated at different phases of UHT milk processing belongs to the same strain.

MATERIALS AND METHODS

The industry in which the work was carried is located in São Paulo State and has permanent sanitary-hygienic control from the Federal Inspection Service (SIF). The production of UHT milk is based on the time-temperature binomial of 150°C for 4 seconds and on the direct heating process using direct injection of heated steam to the milk.

Six groups of UHT milk samples, with 5 replicates each, collected at different points along the production process and shelf life were studied. The samples were collected in 500mL sterilized glass flasks, protected from heat and kept under aseptic conditions, being transported in insulated boxes containing ice.

All raw milk supplied to the milk industry was previously cooled in the farms and transported in isothermal tank trucks (4-7°C). Upon arrival to the industry, and after sample collection for laboratory analysis, sodium citrate was added to the milk. The silo had capacity to store 75,000L of milk for up to 24 hours at temperature of 4°C before being submitted to heat treatment. Raw milk samples were collected from taps located at the lower end of the silo, at the beginning and end of its filling. Another silo stored milk after rapid pasteurization on plates and homogenization at maximum temperature of 6°C for 24 hours. Pasteurized milk samples were collected at the beginning and end of its filling.

After thermal processing, the milk was packaged in 1L, hermetically sealed containers and stored at room temperature. After packaging, containers were collected as samples to be analyzed according to the standards established by Brazilian legislation. For this, they were transported protected from heat and, in the laboratory, they were incubated for 7 days at 35-37°C. Subsequently, standard plate count was performed for survey of aerobic or facultative heterotrophic microorganisms and viable mesophilic microorganisms. In addition, samples were analyzed after 30, 60, 90, 120 and 150 days of storage to monitor shelf-life.

All samples were investigated for presence of vegetative cells of bacteria belonging to the *Bacillus cereus* group and, if it was confirmed, if the production of enterotoxins was detected. In the first stage, 10ml of each sample (milk or water) was transferred to Erlenmeyer flasks containing 90ml of tryptone soy broth (TSB) supplemented with polymyxin B at a ratio of 20µg mL⁻¹ (STADHOUDERS, 1992). The set was incubated at 30°C for 24-30 hours and, after this period, selective plating was performed.

For the selective plating, an aliquot of 0.1mL of selective enrichment culture was inoculated in Petri dishes containing agar mannitol egg yolk polymyxin B (MYP) according to MOSSEL et al. (1967). The plates were incubated at 30°C for 18-40 hours and, at the end of this period, the colonies present were observed. Those with characteristics described by MOSSEL et al. (1967) and STADHOUDERS (1992) were considered suggestive of *Bacillus cereus*.

Colonies with attributes suggestive of belonging to the *Bacillus cereus* group were transferred to tubes containing tryptone soy agar (TSA), which were tilted, properly identified and incubated at 30°C for 24 hours. Subsequently, smears stained according to Gram's and Wirtz-Conklin methods were performed (BIER, 1975). If the presence of Gram positive rod-shaped cells with center terminal spores was detected, biochemical tests were performed to confirm the species as belonging to the *Bacillus cereus* group (APHA, 2001), according to methodology proposed by MACFADIN (1976).

Enterotoxin production by *Bacillus cereus* was determined. For this, the isolates were treated and centrifuged, and the supernatant was tested via passive latex agglutination using the BCET-RPLA kit (Product - TD950, OXOID, Basingstoke, Hampshire, England).

Bacillus cereus isolates obtained from milk samples were submitted to RAPD-PCR according to the method described by NILSSON et al. (1998). *Bacillus cereus* isolates were grown in brain heart infusion broth added with agar-agar (BHI) (overnight) and after that, aliquots of the cultures were transferred to 50ml of BHI broth and incubated for 4 hours, at 30°C, under constant stirring at 180rpm. After incubation, the cultures were centrifuged for 15 minutes at 8000rpm and 4°C according to protocol described by MARMUR (1961), with some modifications.

The samples containing genomic DNA of each isolate were quantified in spectrophotometer (Beckman Model DU® 640B), measuring the absorbance in contrast with distilled water at wavelengths of 260 and 280nm, and the 260/280 ratio was calculated. Then, the samples were diluted so that the concentration was adjusted to 500ng μL^{-1} . The samples were submitted to gel electrophoresis in 0.8% agarose in TBE buffer (89mM Tris, 89mM boric acid and 2.5mM EDTA, pH 8, 3) containing 0.05 $\mu\text{g mL}^{-1}$ of ethidium bromide with voltage of 70 volts for 1 hour. A DNA sample containing fragments of known size, multiple of 1kb, was applied (1kb DNA Ladder, GIBCO/BR). The banding patterns

were recorded using a Gel Documentation System (Bio-Rad Gel Doc 2000).

The RAPD-PCR reaction was performed according to the methodology described by NILSSON et al. (1998). PCR reactions were carried out on 20 μL volume plates, containing 18 μL of Mix and 2 μL of DNA to be amplified at concentration of 15ng. Initially, Mix containing 2 μL of buffer solution (10%), 1 μL of MgCl_2 , 0.5 μL of dNTPs, 0.4 μL of taq, 12.1 μL of H_2O and 1 μL of each primer (5'CCGAGTCCA 3' and 5'CCGGCGGCG 3') was prepared. For samples processing, the amplification conditions were as follows: step 1: 94°C (3min); step 2: 94°C (45sec); step 3: 30°C (2min); step 4: 72°C (1min); step 5: 4 cycles from step 2, step 6: 94°C (45sec); step 7: 36°C (1min); step 8: 72°C (2min) step 9: 26 cycles from step 6; step 10: 75°C (10min) and step 11: 4°C to keep samples cooled until ready to be removed. The reactions were performed in a thermal cycler model PTC-100 (MJ Research).

The amplified samples were analyzed in 1.5% agarose gel. DNA sample containing fragments of known size, multiple of 1kb (1kb DNA Ladder, GIBCO/BR), was applied. Electrophoresis were performed in horizontal tank model HORIZON 11-14, using TBE buffer, stained with ethidium bromide and processed at 65 volts for 2 hours. The fragments amplified by PCR and separated by electrophoresis were visualized by UV light incidence and recorded using a Gel Documentation System (Bio-Rad Gel Doc 2000).

RESULTS

Table 1 shows the results of vegetative cells of bacteria belonging to the *Bacillus cereus* group identification in raw, pasteurized and UHT milk samples from six collections performed during UHT milk production flowchart and shelf life. This table evidences that all pasteurized and raw milk samples were positive for the presence of bacteria from the *Bacillus cereus* group, pasteurized milk samples showing the highest count (81.6%).

The Ministry of Health, through RDC No. 12 (BRAZIL, 2001), establishes that UHT milk, after incubation for 7 days at 35-37°C, should not present pathogenic microorganisms under normal storage conditions. Although the samples in this study have not been incubated, the presence of a pathogenic microorganism belonging to the *Bacillus cereus* group was observed through entire UHT milk production line and shelf life, as shown in table 1.

Table 1 - Results of vegetative cells of bacteria belonging to the *Bacillus cereus* group found in raw, pasteurized and UHT milk samples in the six groups of samples (A, B, C, D, E, F) collected during UHT milk production flowchart and shelf-life.

Milk	Number of samples	-----No. of positive samples for the presence of <i>Bacillus cereus</i> -----						Total (%)
		A	B	C	D	E	F	
Raw milk	60	7	2	7	5	6	4	31 (51.6)
Pasteurized milk	60	8	4	7	10	10	10	49 (81.6)
0 days	30	0	1	4	0	2	4	11 (36.6)
30 days	30	3	0	0	0	2	0	5 (16.6)
UHT milk	60	0	0	1	1	0	0	2 (6.6)
90 days	30	0	1	0	1	0	0	2 (6.6)
120 days	30	1	1	0	2	0	0	4 (13.3)
150 days	30	0	0	0	1	0	0	1 (3.3)

GRIFFITHS (1995) found that psychrotrophic *Bacillus* spp isolates were present in over 70% of pasteurized milk samples, and of these, over 75% contained *Bacillus cereus*, resembling the results of the present study. Also, REZENDE et al. (2000) assayed 120 UHT milk samples for the presence of *Bacillus cereus* and observed that 34.2% were positive. These results were higher than those found in this study, which analyzed 180 UHT milk samples, and just 13.8% (25) were positive.

Bacillus cereus isolates from milk were submitted to latex agglutination test for the detection of enterotoxins using the BCET-RPLA kit. Results in table 2 show that 29 (42.6%) of the 68 isolates tested were positive for the production of diarrhea toxin and the highest number of positive samples were isolated from UHT milk. Among the 39 negative isolates, those from pasteurized milk accounted for the greatest number.

Regarding the enterotoxin production, REZENDE-LAGO (2002) isolated *Bacillus cereus* from raw, pasteurized and UHT milk samples and found that of the 11 isolates from raw milk, seven (63.6%) were positive for enterotoxin production; in the pasteurized milk, of the 13 isolates, four (30.8%) were positive and in the UHT milk, of the 10 isolates, eight (80.0%) were positive. These results are similar

to those found in the present work, which revealed that 50.0% of the isolates from raw milk, 19.2% of isolates from pasteurized milk and 70.7% of the isolates from UHT milk were positive for enterotoxin production.

RAPD-PCR was subsequently performed using DNA (genetic material) extracted from *Bacillus cereus* strains isolated at three points in the milk processing line (raw, pasteurized and UHT). Thus, it could be demonstrated whether these microorganisms belonged to the same strain in all processing phases, indicating resistance to heat treatment, or if they were originated from other sources of contamination along the flowchart production.

The dendrogram (Figure 1) demonstrates that there was clustering by genetic similarity and these were divided into five groups (A, B, C, D and E). Group A showed the largest number of individuals isolated from raw, pasteurized and UHT milk from the same collections points (D and E); and of the 25 isolates grouped, 88% were similar. Group D also demonstrated genetically similar individuals that were isolated from the three types of milk from collections C and F, since of the 20 isolates clustered, 75% showed similarity.

Isolates from samples of the three types of milk of collection A were clustered into group B and group E, showing 46% and 60% of similarity,

Table 2 - Results of bacterial strains of the *Bacillus cereus* group isolated from raw, pasteurized and UHT milk samples, which were submitted to latex agglutination test for detection of enterotoxin using the BCET-RPLA kit (OXOID).

Milk	No. of samples	No. of strains tested	-----Enterotoxigenic capacity-----	
			Positive	Negative
Raw milk	60	14	7 (50.0%)	7 (50.0%)
Pasteurized milk	60	26	5 (19.2%)	21 (80.8%)
UHT milk	180	28	17 (70.7%)	11 (29.3%)
Total	300	68	29 (42.6%)	39 (57.4%)

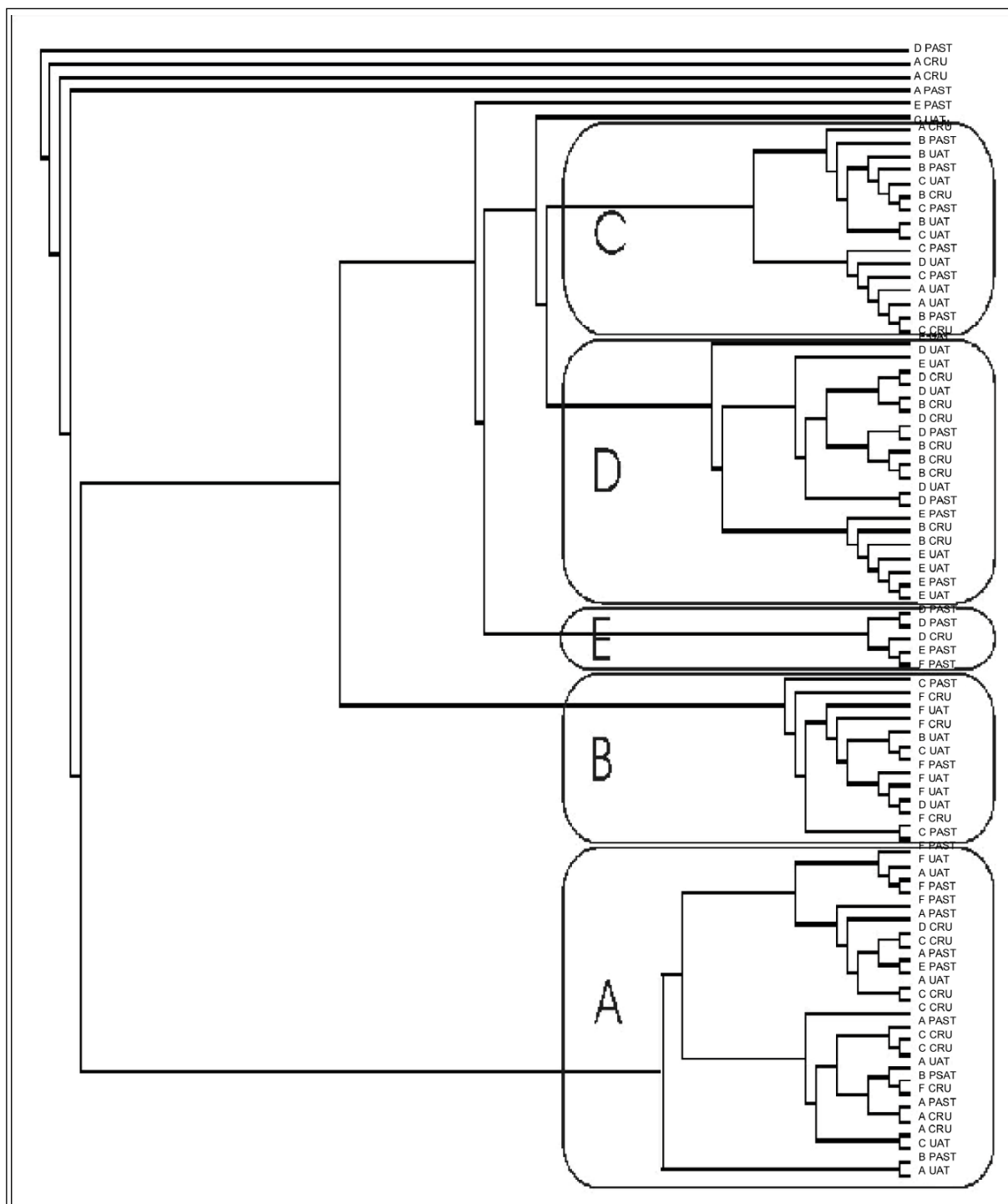


Figure 1 - Dendrogram showing the genetic relatedness between *Bacillus cereus* strains isolated during UHT milk production flowchart and shelf life based on RAPD shared in five groups (A, B, C, D, E). The tree was constructed by performing “bootstrap” by PAUP 4.0. and using the PHYLIP 3572 software to generate the dendrogram.

samples, respectively, were grouped as individuals isolated from pasteurized and UHT milk of collections B and C. This also occurred in group D, showing individuals of collection A isolated from pasteurized

and UHT milk samples and individuals of collection D, isolated from raw and UHT milk samples.

SVENSSON et al. (1999) isolated *Bacillus cereus* from raw and pasteurized milk, and as in the present study, found several microorganism strains and most isolates from raw milk was genetically similar to those isolated from pasteurized milk. Although, ENEROTH et al. (2000) found no genetic similarity between psychrotrophic *Bacillus cereus* isolated from raw milk and after pasteurization by RAPD-PCR. The authors attributed this to the possibility of recontamination during processing or to the presence of biofilm in the pipeline.

In another study, SALUSTIANO et al. (2009) also isolated *Bacillus cereus* in a dairy industry in Brazil. The bacteria were detected in 12 isolates from pasteurized milk and in 30 isolates from the surfaces of post-pasteurization equipment. The presence of seven ribotypes was demonstrated, but most belonging to the same microorganism. They were found on four surfaces and in milk, indicating post-processing contamination.

Presence of *Bacillus cereus* in UHT milk is related to improper cleaning of the equipment, inadequate pasteurization temperature or post-processing contamination (BAHOUT, 2000); however, as can be observed in the present work, after RAPD-PCR, microorganisms isolated from UHT milk had a genetic profile similar to those isolated from raw and pasteurized milk, thus demonstrating that the microorganism survives to the double heat treatment to which milk is submitted.

Through observation of data present in figure 1, it is concluded that 82% of the 79 *Bacillus cereus* isolates during the stages of UHT milk production process were genetically similar, showing that the microorganisms present in the raw material were able to survive to two subsequent heat treatments, since they were isolated from the final product, also with ability to produce toxins (Table 2). This acts as a warning for health authorities, since it shows the necessity to improve the quality of the raw material used to produce UHT milk.

CONCLUSION

The results should serve as a warning for health authorities, since the presence of a pathogenic microorganism capable of producing toxin throughout the UHT milk production process and shelf life was detected. Furthermore, genetic similarity was observed demonstrating that this microorganism

was originated in the raw milk, thus demonstrating the capacity to withstand UHT treatment steps and the necessity for more stringent hygienic-sanitary measures for obtaining the product.

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