ISABELLA MARIA FERNANDES BOTELHO MOREIRA

MICROBIOLOGICAL QUALITY OF BRAZILIAN UHT MILK AND GENETIC BIODIVERSITY OF SPORE-FORMING BACTERIA

Dissertation submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of Magister Scientiae.

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“ The difference between winning and losing is most often not quitting. If you can dream it, you can do it, always remember that this whole thing was started with a dream! ”

Walt Disney

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RESUMO

MOREIRA, Isabella Maria Fernandes Botelho, M.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Qualidade Microbiológica de Leite UHT Brasileiro e Biodiversidade Genética de Bactérias Formadoras de Esporos.** Orientador: Antônio Fernandes de Carvalho. Coorientadora: Rosângela de Freitas.

O leite é considerado um alimento rico em nutrientes, o que faz com que o mesmo seja o ambiente ideal para o desenvolvimento de uma série de micro-organismos. Entre os micro-organismos contaminantes do leite, aqueles capazes de formar esporos termorresistentes são muito importantes do ponto de vista microbiológico, principalmente, para laticínios que utilizam a tecnologia UHT. O gênero Bacillus é o mais associado com a qualidade do leite, e entre as espécies de importância na indústria de laticínios, podemos destacar Bacillus sporothermodurans. Essa bactéria é classificada como mesófila aeróbia e apresenta capacidade de produzir esporos resistentes ao tratamento UHT. Durante o armazenamento, estes esporos podem germinar, com subsequente multiplicação até contagens próximas de 10^5 UFC·mL^{-1}. O objetivo deste estudo foi determinar a qualidade microbiológica de amostras de leite UHT produzidas no Brasil com foco na biodiversidade de micro-organismos esporulados termorresistentes. Este trabalho teve início com a avaliação de amostras de leite UHT doadas por sete empresas distintas (A, B, C, D, E, F e G), de três regiões do país (Sul, Sudeste e Centro-oeste) ao longo de seis meses. Para tal, foram realizadas as etapas de incubação e contagem total em placas de acordo com a Portaria do Ministério da Agricultura, Pecuária e Abastecimento. De a Portaria nº 370 de 1997, que estabelece que a contagem de mesófilos aeróbios não pode exceder ao limite de 100 UFC·mL^{-1}, 50,5% das amostras avaliadas encontraram-se fora dos padrões. A cada uma das amostras avaliadas foram aplicados dois tratamentos térmicos (T1: 80 ºC/10 min e T2: 100 ºC/30 min) para a inativação das células vegetativas e avaliação da presença de esporulados. Cerca de cinco colônias foram isoladas de cada amostra em estudo e submetidas à caracterização fenotípica para identificação da presença de B. sporothermodurans. Para isso as análises foram baseadas na Instrução Normativa n° 62 de 2003 do Ministério da Agricultura, Pecuária e Abastecimento na qual cada isolado purificado foi submetido a uma série de testes bioquímicos (coloração de Gram, catalase, oxidase, crescimento em

anaerobiose, fermentação da glicose, hidrólise da esculina, redução do nitrato e prova da urease). De acordo com os resultados dos testes, nenhum dos isolados obtidos foi considerado B. sporothermodurans. Posteriormente, foi realizada a análise de Rep-PCR que permitiu agrupar os isolados em clusters. Após escolher, aleatoriamente, os representantes de cada grupo formado, considerando uma similaridade de 90%, estes foram enviados para o sequenciamento do rDNA 16S e pode-se observar que o gênero Bacillus, foi encontrado em abundância correspondendo a 100% dos isolados. Dentro do gênero Bacillus foram identificadas seis espécies: Bacillus amyloliquefaciens, Bacillus proteolyticus, Bacillus cereus, Bacillus subtilis, Bacillus licheniformis e Bacillus velezensis. A identificação da presença destas bactérias esporuladas nas amostras de leite UHT indica a importância da qualidade da matéria-prima, da implementanção dos programas de autocontrole, e da conscientização de todos os elos da cadeia produtiva, afim de obter um produto com qualidade.

ABSTRACT

MOREIRA, Isabella Maria Fernandes Botelho, M.Sc., Universidade Federal de Viçosa, February, 2019. **Microbiological Quality of Brazilian UHT Milk and Genetic Biodiversity of Spore-Forming Bacteria.** Advisor: Antônio Fernandes de Carvalho. Co-advisor: Rosângela de Freitas.

Milk is considered a food rich in nutrients, which makes it the ideal environment for the development of a series of microorganisms. Among the milk contaminants microorganisms, which are able to form heat-resistant spores are very important, especially for dairy companies that use UHT technology. The genus Bacillus is the most associated with milk quality, and between the species of importance in the dairy industry, we can highlight Bacillus sporothermodurans. This bacterium is an aerobic mesophyll able to produce spores resistant to UHT treatment, and throughout the storage can germinate and multiply until counts close to 10^5 CFU·mL^{-1}. The objective of this study was to determine the microbiological quality of UHT milk commercialized in Brazil with a focus on the biodiversity of sporulated thermoresistant microorganisms. This work began with the evaluation of samples of UHT milk donated by seven different companies (A, B, C, D, E, F and G), of three regions of the country (South, Southeast and Midwest) over six months. For that the steps of incubation and total plate count were performed in accordance with the Ordinance nº 370 of 1997 of Ministério da Agricultura, Pecuária e Abastecimento. According to this Ordinance, which establishes that the count of aerobic mesophylls can not exceed the limit of 100 CFU·mL^{-1}, 50,5% of the samples evaluated were out of standards. After the seven days of incubation, two heat treatments (T1: 80 ºC / 10 min and T2: 100 ºC / 30 min) were applied to the inactivation of the vegetative cells and evaluation of the presence of sporulates. About five colonies were isolated from each sample and submitted to phenotypic characterization to identify the presence of B. sporothermodurans. For this, the analyzes were based on Normative Instruction n° 62 of 2003 of Ministério da Agricultura, Pecuária e Abastecimento in which each purified isolate was submitted to a series of biochemical tests (Gram staining, catalase, oxidase, anaerobic growth, glucose fermentation, esculin hydrolysis, nitrate reduction and urease test). According to the test results, none of the isolates obtained was considered B. sporothermodurans. Subsequently, the Rep-PCR analysis was

carried out, which allowed grouping the isolates into clusters. After choosing, randomly, the representatives of each group, considering a similarity of 90%, these were sent to the 16S rDNA sequencing and it can be observed that the genus Bacillus was found in abundance corresponding to 100% of the isolates. Within the genus Bacillus six species were identified: Bacillus amyloliquefaciens, Bacillus proteolyticus, Bacillus cereus, Bacillus subtilis, Bacillus licheniformis and Bacillus velezensis. The identification of the presence of these sporulated bacteria in UHT milk samples indicates the importance of quality maintenance of raw material, the implementation of self-control programs, and the awareness of all links in the production chain in order to obtain a quality product.

GENERAL INTRODUCTION

Considering the availability of formal fluid milk in 2017, about 7,026 billion liters (27.89%) were directed to the segment of Ultra High Temperature milk, which is only lower than that used for cheese production (33.37%). UHT milk consumption in Brazil has increased significantly and currently accounts for about 85% of the country's fluid milk consumption (ABLV, 2018). This fact can be explained by the facility of storing the product, the long shelf life, the practicality of the packaging and the fact that the cold chain is dispensed during the internal logistics and distribution of the product.

In the 90s, some bacteria capable of producing highly heat resistant spores were isolated from this product (PETTERSSON et al., 1996). Due to their surface characteristics, some spores have greater easily of adhesion than the vegetative cells, which increases the possibility of biofilm formation in the pipes along the processing lines, a condition that favors sporulation. In addition, these spores are able to survive heat treatment at high temperatures and during product storage can germinate. After germination, the bacteria can multiply and produce proteolytic and lipolytic enzymes that cause various technological problems such as thermal instability, off-flavor and sweet coagulation (MOSTERT et al., 1979; WESTHOFF et al., 1981; FOSCHINO et al., 1990; PETTERSSON et al., 1996; COSTA et al., 2016).The presence of these spores in Brazilian UHT milk results in an increase in the total count of viable aerobic mesophiles when they germinate. In this way, most of the time the count exceeds the limits of the current legislation (BRASIL, 1997).

Among these sporulated bacteria isolated in UHT milk, the genus Bacillus is of great importance, especially B. sporothermodurans. Because it is an isolated bacterium relatively recently, there is little information in the literature, and some of them are still divergent about how this bacterium behaves in different situations, as well as its physiological and biochemical characteristics, its thermal resistance, levels of contamination, isolation techniques, deterioration mechanisms of products and their ecological characteristics.

The objective of this work was to evaluate the microbiological quality of UHT milk samples of different problem batches from different Brazilian regions, to determine

the presence of B. sporothermodurans, as well as, to determine the biodiversity of the isolates from molecular techniques.

GOALS

General Objective

To determine the microbiological quality of Brazilian UHT milk samples with a focus on the biodiversity of thermoresistant sporulated microorganisms.

Specific Objectives

- To evaluate the microbiological quality of UHT milk samples produced in different regions of Brazil;
- To determine the presence of B. sporothermodurans according to the parameters stipulated by the legislation through phenotypic techniques;
- To determine the biodiversity and identify the isolates at the genus and species level by molecular techniques.

CHAPTER 1: PRESENCE OF SPORE-FORMING BACTERIA IN UHT MILK: A REVIEW

PRESENCE OF SPORE-FORMING BACTERIA IN UHT MILK: A REVIEW

1. INTRODUCTION

According to the Ministério da Agricultura, Pecuária e Abastecimento (MAPA), Ultra High Temperature (UHT) milk is the homogenized milk heat-treated at a temperature between 130 ºC and 150 ºC for a period of 2 to 4 seconds, at heat continuous flow process, immediately cooled to below 32 ºC and packed under aseptic conditions in sterile and hermetically sealed packages (BRASIL, 1997). The effectiveness of this treatment is defined by the number of decimal reductions in the bacterial spore count achieved by the process, being influenced not only by the time/temperature binomial, but also by the initial number of microorganisms present in the raw material and by the type heat treatment (direct/indirect) (MEMBRÉ et al., 2011).

As in any production process, to obtain a quality UHT milk, in addition to the need to apply quality tools along the entire production chain such as Good Manufacturing Practices (GMP) and Hazard Analysis and Critical Points of Control (HACCP), the quality of the raw material is a fundamental prerequisite. Thus, raw milk must meet at least the quality requirements recommended by the current legislation (SOUSA et al., 2006; COSTA et al., 2016).

In addition to the low microbiological quality of the raw material, the presence of contaminating microorganisms in UHT milk may be associated with failures in the production line, especially during packaging; flaws in piping sanitation procedures, which favors microbial adhesion and biofilm formation; as well as shortcomings in packaging asepsis procedures (WESTHOFF, 1981; WESTHOFF et al., 1981; PEREIRA et al., 2013).

2. MICROBIOTA OF RAW MILK

Milk is considered a sterile product when it is synthesized and secreted into the mammary glands of healthy cows, but bacteria inside the udder, such as, Micrococcus spp., Corynebacterium spp. and Streptococcus spp., contaminate raw milk at the time of milking. In addition, contamination of this raw material can occur

through the milking of sick animals; inadequate management of animals during milking; poor hygiene of the external surface of the udder, ceilings and hands of milkers, in the case of manual milking, or poor sanitation of the nets and pipes, in the case of mechanical milking; milking performed in environments with inappropriate hygienic-sanitary conditions; the use of poorly sanitized utensils and tanks; as well as from several environmental sources through microorganisms present in the soil and in the pastures that are capable of contaminating and multiplying in the milk (BRITO et al., 2000; SANTOS et al., 2001; BUSATTA et al., 2005; JAY, 2005; MENEZES et al., 2014; COSTA et al., 2016).

When the animal is diseased the main microorganisms that are transferred to the milk during milking are Mycobacterium bovis, Coxiella burnetii and Brucella abortus (ORDOÑEZ, 2005). After milking if the raw milk is stored for many days under refrigeration, there may be development of bacteria of the following genera: Enterococcus, Lactococcus, Streptococcus, Leuconostoc, Lactobacillus, Microbacterium, Propionobacterium, Micrococcus, Proteus, Pseudomonas, Bacillus and Listeria, as well as some representatives of the coliform group (Escherichia, Edwardsiella, Citrobacter, Salmonella, Shigella, Klebsiella, Enterobacter, Serratia, Proteus and Yersinia) (LAVEN et al., 2003; JAY, 2005; TEBALDI et al., 2008; MENEZES et al., 2014).

Normative Instruction n° 62 of 2011 is the law that presents the microbiological and physical chemical standards for raw milk produced in Brazil. In many cases, Brazilian raw milk does not meet the standards established by this legislation, presenting high counts of aerobic mesophilic and psychrotrophic (CERQUEIRA et al., 1994; BELOTI et al., 1997; FRANCO et al., 2000; BRASIL, 2011; CATTANI et al., 2013). When evaluating samples of raw milk obtained through mechanical milking in the state of Goiás, Brasil et al. (2012) obtained an average of 9.1×10^5 $CFU\cdot mL^{-1}$ for counts of aerobic mesophilic, value that is above the maximum allowed by the legislation. In order to determine the quality of raw milk samples from the region of Ivaiporã in Paraná, Ribeiro Júnior et al. (2013) evaluated 98 samples of which 17.2% were above the limits permitted by legislation, with a mean aerobic mesophil count of 3.6×10^6 $CFU\cdot mL^{-1}$.

After milking, milk is in the temperature range that favors the development of contaminating mesophilic microorganisms (25 – 35 ºC) and if it is not immediately cooled to temperatures between 4 and 7 ºC, these microorganisms will multiply at high rates leading to milk biodeterioration. In contrast, the storage of raw milk under refrigeration conditions for a long time favors the multiplication of psychrotrophic microorganisms (JAY, 2005; ARCURI, 2006; MENEZES et al., 2014).

Psychrotrophic microorganisms and bacterial spores comprise groups that are associated with most of the microbiological problems confront by dairy companies. The psychrotrophs, due to their ability to multiply in refrigeration temperature and the produce of proteolytic and lipolytic thermoresistant enzymes, cause several technological and sensorial problems in dairy products. The production of these enzymes is dependent on the initial charge of microorganisms, nutrient availability, growth phase and time in which the milk is kept under refrigeration (SANTANA et al., 2001; NÖRNBERG et al., 2009; ZENI et al., 2013).

Bacterial spores are also associated with problems in the dairy industry due to the possibility of germination and multiplication during storage of the finished product, as well as the ease of forming biofilms along the processing line. Their origins may be distinct, but generally they come from the environment of rearing animals and can be transferred from the udder to the milk during milking (VISSERS et al., 2009).

It is important to note that even if milking is carried out following the necessary hygiene conditions, it is practically impossible to obtain a milk free of microorganisms. Thus, it is important to keep the raw milk at an appropriate storage temperature and the time must be minimized in order to prevent a high multiplication of contaminating microorganisms (MENEZES, et al., 2014).

3. IMPACTS OF UHT PROCESSING IN MILK MICROBIOTA

Upon receipt in the dairy industry, raw milk undergoes a series of operations corresponding to the technology to which it will be destined. Many of these operations aim to increase the shelf life of the final product by eliminating contaminating microorganisms from the milk.

Considering that the temperature of the raw milk can reach up to 10 ºC during the transportation from the farm to the industry (BRASIL, 2011), this raw material must be cooled immediately at 4 ºC at the reception of the dairy company and maintained at this temperature until the processing, to avoid the multiplication of contaminating microorganisms (MUCIDAS, 2010; BRASIL, 2011). It is important to note that milk should not be stored for a long time under refrigeration in order to avoid the development of psychrotrophic microorganisms. The ideal is to start the processing immediately after receiving the raw milk by the industries (JAY, 2005; MENEZES et al., 2014).

Upon receipt, the raw milk is subjected to the centrifugation, which is the first step in UHT processing and has as principle to standardize the fat content and remove dirt by density difference. Through centrifugation it is possible to separate mechanically, by a centrifugal force, dirt solid, somatic cells and microorganisms (WALSTRA et al., 1999; KROLOW et al., 2006). There is also an alternative centrifugation process, optimized to achieve high speeds of rotation, called ultracentrifugation, which, according to Schuck (2014), allows a reduction of up to 90% in spore counts, making possible the elimination of Bacillus sp. and Clostridium sp. spores which are not inactivated by conventional heat treatments.

After centrifugation, the standardization step is carried out, combining the skimmed milk with the cream in order to obtain specific proportions, ensuring a uniformity of the final product in relation to the fat content (whole UHT milk - min 3%, semi UHT milk or partly skimmed - from 0.6 to 2.9%, and skimmed UHT milk - max 0.5%) (BRASIL, 1997; FEAM, 2011). At this stage there are no significant effects on the microbiota present in milk.

The next step is pasteurization, which consists in the application of a milk heat treatment to eliminate pathogenic microorganisms and most deteriorators, except spores (VARNAM et al., 1995; FRANCO et al., 2008; LEWIS et al., 2009; TRONCO, 2010). There are two types of pasteurization: Rapid Pasteurization or HTST - High Temperature Short Time (71.7 ºC / 15 s) and Pasteurization Slow or LTLT - Low Temperature Long Time (62.8 ºC / 30 min), both aim to inactivate the microorganisms Mycobacterium tuberculosis and Coxiella burnetii, the non

sporulated pathogen more resistant to the heat treatment present in milk (JONG, 2008).

Subsequently, homogenization is performed, which aims to mechanically reduce the size of the milk fat globules, avoiding during storage that these globules coalesce and accumulate on the surface of the package. In addition, homogenization provides a product of better viscosity and texture. This operation is carried out in a homogenizer and in case of unsatisfactory conditions of hygiene and sanitization of the equipment, an increase in the microbial count of the milk due to the biofilm detachment may occur (MUCIDAS, 2010; FEAM, 2011; SCHUCK, 2014).

The next step is ultrapasteurization, or UHT treatment. This heat treatment is able to eliminate all vegetative cells of microorganisms present in the milk, as well as a large number of spores, except for thermoresistant spores, as is the case of spores of Bacillus sporothermodurans (FRANCO et al., 2008; MUCIDAS, 2010; TRONCO, 2010; FEAM, 2011, MENEZES et al., 2014). Despite being considered an intense heat treatment, proteases and lipases can maintain their activity after a treatment of 140 ºC/5 s and in this way continue to act in the milk throughout its storage. Thus, in order to maintain the quality of the final product, it is necessary to minimize the contamination of the raw material and avoid its storage for a prolonged period, since this condition favors the production of these enzymes by psychrotrophic microorganisms (WALSTRA et al., 1999; ROWE et al., 2011).

To avoid contamination of the milk that has undergone UHT treatment, the packaging must be made in a completely aseptic environment, in sterile containers and with hermetic closure. Thus, the product does not need to be kept refrigerated during its storage (VARNAM et al., 1995; MUCIDAS, 2010; FEAM, 2011; MENEZES et al., 2014).

4. SPORULATED BACTERIA IN UHT MILK

The sources of milk contamination by spores are several, and we can highlight as the main the soil, the feces of the animals and the equipment of feeding and milking. It is extremely important to identify these possible sources in the farm environment, since

contamination of raw milk by spores directly reflects the quality and food safety of the final product (LANDSCHOOT, et al., 2011).

The main genera of microorganisms capable of producing spores include Bacillus, Clostridium, Sporosarcina, Sporolactobacillus, Thermoactinomyces e Paenibacillus (COSTA et al., 2016). The genus Bacillus and Paenibacillus are the most associated with milk quality (HUCK et al., 2007; IVY et al., 2012 ; COSTA et al., 2016). The genus Bacillus was the first group of sporulated gram-positive found in raw milk and pasteurized milk, some species of this genus have already been identified in several situations in dairy products such as B. cereus (WESTHOFF et al., 1981; CATTANI et al., 2013; AOUADHI et al. 2014), B. coagulans (WESTHOFF et al., 1981), B. licheniformis (WESTHOFF et al., 1981; AOUADHI et al., 2014; PINTO et al., 2017), B. pumilus (WESTHOFF et al., 1981; PINTO et al., 2017), B. sphaericus (WESTHOFF et al., 1981; AOUADHI et al., 2014), B. sporothermodurans (ZACARCHENCO, et al., 2000; SCHELDEMAN et al., 2002; BUSATTA et al., 2005; CATTANI et al., 2013; AOUADHI et al., 2014), Geobacillus stearothermophilus (WESTHOFF et al., 1981) and B. subtilis (WESTHOFF et al., 1981; PINTO et al., 2017).

There are also some species of the B. subtilis group that normally contaminate dairy products. B. subtilis, B. mojavensis, B. sonorensis, B. vallismortis, B. amyloliquefaciens, B. atrophaeus, B. licheniformis, B. tequilensis and B. velezensis consist of members of the Bacillus subtilis group. The most commonly found in dairy products are: B. subtilis, B. licheniformis and B. pumilus. Being present in most of the farms environment, B. licheniformis is the most found in raw milk (PHILLIPS et al., 1986; JEYARAM et al., 2011).

Characteristics of the major spore-forming gram-positive bacteria isolated from dairy products will be described in the following topics.

4.1. B. amyloliquefaciens

B. amyloliquefaciens is a bacterium normally isolated from soil. Its optimum temperature range is between 30 and 40 ºC, it is able to grow in concentrations of up to 10% (w/v) of NaCl, produces catalase and oxidase, presents a positive reaction to

the Voges-Proskauer test, reduces nitrate to nitrite, hydrolyses esculin, and produces acid through the fermentation of glucose (PRIEST et al., 1987; WANG et al., 2008).

B. amyloliquefaciens is widely used industrially for the production of proteases, thermostable amylases and amino acids. These products have many applications which include their use as food additives (PRIEST et al., 1987; MIKKOLA et al., 2004; WANG et a., 2008).

McKillip et al. (2016) identified isolates of these bacteria in organic UHT milk and proved that it is a biofilm producer and virulence genes were present in isolates that was isolated.

4.2. B. cereus

B. cereus has a natural habitat on the soil, which allows it to easily contaminate several foods, such as raw milk (SCHOKEN-ITURRINO et al., 1996; CHRISTIANSSON et al., 1998; ROSSLAND et al., 2003; BARTOSZEWICZ et al., 2008; WATANUKI et al., 2008). Its optimal temperature range is between 27 and 35 ºC, but some strains can multiply between 3 and 75 ºC. This microorganism is classified as thermoduric, in addition some strains are psychrotrophic as they are capable of multiplying in cooling temperatures (BRADSHAW et al., 1975; KRAMER et a., 1989; DROBNIEWSKI, 1993; DUFRENNE et a., 1995; GARCIA-ARMESTO et al., 1997; MATTA et al., 1999; CRONIN et al., 2008; MONTANHINI et al., 2012).

In addition to being a sporulated able to withstand the conventional heat treatments used in the dairy industries, B. cereus can cause sensorial changes in milk, because it produces proteolytic and lipolytic enzymes, which makes it a major concern in this type of industry (WONG et al., 1988; LARSEN et al., 1999; CHEN et al., 2004; BARTOSZEWICZ et al., 2008; MONTANHINI et al., 2012).

This bacterium has already been isolated from several dairy products such as: raw milk, pasteurized milk, UHT milk, milk powder, ice cream and fermented milk (WONG et al., 1988; CHRISTIANSSON et al., 1998; VIDAL-MARTINS et al., 2005; REZENDE-LAGO et al., 2007, BARTOSZEWICZ et al., 2008; ZHOU et al., 2008; ZHOU et al., 2010). This is a matter of concern, once the presence of B. cereus

in food is associated with two types of food-borne diseases: the emetic syndrome and the diarrheal syndrome. The first is caused by ingestion of the food with the preformed toxin, and the second by the enterotoxin produced in the host intestine (GRANUM et al., 1997; EHLING-SCHULZ et al., 2004; RAJKOVIC et al., 2008).

4.3. B. coagulans

This bacterium was first identified as Lactobacillus sporogenes by Horowitz-Wlassowa and Nowotelnow in 1933, after being isolated from spoiled milk. Subsequently, it was called B. coagulans (KRISTJANSSON, 1991; KARRI et al., 2016). It consists of a facultative, non-pathogenic and lactic acid-producing anaerobic bacterium. Its optimum range of temperature is between 35 and 50 ºC and pH between 5.5 and 6.5 (A¸SAN ÖZÜSA˘GLAM, 2010; KARRI et al., 2016; KONURAY, et al., 2018).

Due to its ability to produce acid, its presence is associated with deterioration in dairy products, fruits and vegetables. In addition, it exhibits proteolytic activity (DE CLERK et al., 2004; A¸SAN ÖZÜSA˘GLAM, 2010; KONURAY et al., 2018).

B. coagulans has the characteristics of a probiotic microorganism and has been considered safe by the US Food and Drug Administration (FDA) and the European Union Food Safety Authority (EFSA), and is on the Generally Recognized As Safe (GRAS) and Qualified Presumption of Safety (QPS) list (EFSA, 2013).

4.4. B. licheniformis

B. licheniformis is a bacterium widely distributed in nature, found mainly in soil and associated with plants. It is a microorganism of great industrial interest because it is capable of producing proteases on a large scale (SNEATH et al., 1986; VEITH et al., 2004).

Some members of B. licheniformis species capable of causing food poisoning have also been shown to be capable of producing substances toxic to mammalian cells, including those already associated with bovine abortion (AGERHOLM et al., 1995; SALKINOJA-SALONEN et al., 1999; MIKKOLA et al., 2000; SUOMINEN et al., 2001; MIKKOLA et al., 2004). Although commonly found in sick animals, B.

licheniformis has also been isolated from the udder of a clinically healthy cow recovered from mastitis (SALKINOJA-SALONEN et al., 1999). This fact makes the udder of animals that have already been affected by such pathology, a source of contamination of the raw milk by this bacterium.

4.5. Bacillus proteolyticus

Through a phylogenetic analysis Liu et al. (2017) suggested the addition of nine Bacillus species to the Bacillus cereus group. Among these species is B. proteolyticus which consists of a anaerobic facultative bacterium, without motility. It grows in the temperature range between 10 and 39 ºC and pH between 5 and 10. It presents positive reaction for the test of Voges-Proskauer and negative for the production of acid through the fermentation glucose, hydrolyses starch, casein and skim milk.

4.6. B. pumilus

B. pumilus is a aerobic bacterium, and like other members of the genus Bacillus, is found mainly in soil (PRIEST, 1993).

Some strains of B. pumilus are capable of producing chitinases that have antifungal activity against Rhizoctonia solani, Verticillium sp., Nigrospora sp., Stemphyllium botryosum and Bipolaris sp.(GHASEMI et al., 2010). In addition, they are capable of producing a proteolytic enzyme capable of coagulating soybean protein, an essential ingredient for the production of tofuyo (AOYAMA, et al., 2000).

As well as some members of the species B. licheniformis, representatives of the B. pumilus species are also capable of producing toxins harmful to mammalian cells, which makes them a problem for producers and companies that use milk as raw material (SALKINOJA-SALONEN et al., 1999; MIKKOLA et al., 2000; SUOMINEN et al., 2001; MIKKOLA et al., 2004).

4.7. B. sphaericus

It is an aerobic mesophile, producer of thermoresistant terminal spores. Due to the absence of some metabolic pathways, it is not able to use sugars as metabolites. It

presents a positive reaction in the catalase and oxidase test, and reduces nitrate to nitrite (NEIDE, 1904; ISAACSON, et al., 1976; CHARLES et al., 1996).

It is a heterogeneous species with toxic and non-toxic strains. Strains capable of producing toxins are usually used to control mosquitoes such as Culex, Anopheles, Aedes and Culiseta incidens. They present high toxicity to the larvae of these mosquitoes, becoming a form of alternative control to the use of pesticides (KELLEN et al., 1965; CHARLES et al., 1996; SURUADY et al., 2016). There are reports that some strains of B. sphaericus can be considered pathogenic, including cases where it was able to produce a massive pseudotumor in the patient's lung (ISAACSON, et al., 1976). According to WESTHOFF et al. (1981) and AOUADHI et al. (2014) this bacterium has already been identified in raw milk and dairy products.

4.8. B. sporothermodurans

Among the Bacillus species of importance in the dairy industry, we can highlight the B. sporothermodurans. This bacterium is an aerobic mesophyll able to produce spores resistant to UHT treatment, and that over the storage of this product can germinate and multiply until counts close to 10^5 $CFU \cdot mL^{-1}$ (HAMMER et al., 1995; PETTERSSON et al., 1996; ZACARCHENCO et al., 2000; SCHELDEMAN et al., 2002).

B. sporothermodurans is a strict aerobic bacterium, presents in the form of long and filamentous rods with irregular gram staining and present motility by means of perimeter flagella. The spores are ellipsoidal in shape, being located in terminal position. The colonies are small (1 – 2 mm in diameter), circular, flat and almost white to beige. They are able to form colonies at temperatures between 20 and 55 ºC, being the best around 37 ºC. They growing in pH between 5 and 9 and tolerate up to 5% (w/v) of NaCl (BUSATTA et al., 2005; HEYNDRICKX et al., 2012). They are oxidase and catalase positive, do not reduce nitrate to nitrite, present a negative reaction in Voges-Proskauer reactions, hydrolyze esculin, do not hydrolyze urea and do not ferment glucose (PETTERSSON et al., 1996; BRASIL, 2003).

Although B. sporothermodurans is able to reach high count after germination, it is not considered pathogenic and there is no description in the literature of its ability to deteriorate milk; in addition, it does not alter pH, stability and sensory quality (PETTERSSON et al., 1996; KLIJN et al., 1997).

This microorganism was originally termed as a highly heat-resistant spore former (HHRS or HRS) and was initially isolated from UHT milk in Austria and Italy in 1985 and Germany in 1990 (HAMMER et al., 1995; PETTERSSON et al., 1996; SCHELDEMAN et al., 2006). The name B. sporothermodurans was proposed by Pettersson (1996) after finding that the bacteria capable of forming these highly heat-resistant spores was a homogeneous group belonging to the genus Bacillus.

The spores formed by B. sporothermodurans have a $D_{140\ ^oC}$ value between 3.4 and 7.9 seconds, which makes them highly resistant to the heat treatments usually used in food preservation. Studies on spore survival have shown that complete inactivation of B. sporothermodurans spore occurs only after the application of a heat treatment of 130 ºC for 8 minutes, which is considered to be a totally non-viable treatment for affecting milk constituents (HUEMER et al., 1998; TABIT et al., 2010; CATTANI, et al., 2013).

Currently, B. sporothermodurans has been isolated in several countries and in different dairy products such as UHT milk, cream, chocolate milk, evaporated milk, reconstituted milk and raw milk (HERMAN et al., 2000; KMIHA, et al., 2017).

B. sporothermodurans is very close phylogenetically to other species of Bacillus, which makes it difficult to identify them. Some examples are: B. oleronius, B. lentus, Bacillus firmus, Bacillus benzoevorans and B. acidicola (SCHELDEMAN et al., 2006; CATTANI et al., 2013). The latter was responsible for false-positive results in the identification of B. sporothermodurans through the PCR technique developed by Scheldeman (2002).

4.9. G. stearothermophilus

G. stearothermophilus was the first, and for long, the only species of the B. thermophilus group with the name validated (DONK, 1920). Subsequently, after the

1980s, through phylogenetic and molecular studies, it was possible to determine the heterogeneity of the group (WALKER et al., 1971; LOGAN et al., 1984).

It is an aerobic bacterium with varying gram staining, most of the strains grow at temperatures between 30 and 75 ºC, being the optimum at 55 ºC, and pH between 6 and 9. It is capable of hydrolysing esculin, gelatin , casein and starch, and withstand up to 1% (w/v) of NaCl (DONK, 1920; NAZINA et al., 2001; COOREVITS, et al., 2012). G. stearothermophilus is very important for the food industries, especially for the dairy companies, since it is resistant to heat treatment of pasteurization of these products, besides producing thermoresistant enzymes (FURUKAWAA et al., 2003).

4.10. B. subtilis

B. subtilis is a optional aerobic, non-pathogenic bacterium, producer of acetic acid and thermoresistant spores (MAZZA, 1994). This bacterium, like the great majority of the species of the genus Bacillus, has as natural habitat the soil. However, they can be associated with plants and multiply even in the internal tissues of them (ONGENA et al., 2005; CAMPOS SILVA et al., 2008).

Due to its ability to sporulate, strains of B. subtilis have been used as probiotics for ruminants because the spores formed survive rumen transit and after germination reestablish the normal microbial flora of animals, especially after prolonged use of antibiotics. In addition, B. subtilis can be used as immunomodulators as they are capable of stimulating the immune response of the animals (BELIAVSKAIA, 2001; CONCEIÇÃO et al., 2002; COPPOLA et al., 2004).

Recent studies have revealed that B. subtilis isolates are capable of producing a variety of enzymes (proteolytic, aminolytic, lipolytic and fibrolytic), a number of metabolites and volatile substances with antifungal activity, and bacteriocins (SCHALLMEY et al., 2004; KAI et al., 2007; CHEN et al., 2008). Together with B. licheniformis and B. cereus are the most predominant mesophilic aerobic spore-formes in raw milk (WESTHOFF et al., 1981).

5. FINAL CONSIDERATIONS

According to Ministério da Saúde data, between 2000 and 2017, 12,503 food-borne outbreaks were reported in Brazil, with 236,403 sick people and 182 dead. Of these, it was not possible to determine the food involved in the outbreak in 46.82% of the cases. Mixed foods were the most involved in outbreaks with a percentage of 11.77%, followed by multiple foods, eggs and egg products, water, inconclusive, and milk and dairy products with 3.21%, in seventh place. Among the etiological agents involved, B. cereus appeared in the fifth position as responsible for 5% of the outbreak cases in question (FERREIRA et al., 2017).

Between 1960 and 1968 in Hungary, of the total outbreaks involving B. cereus, 9.6% had milk and cocoa as vehicle (GOEPFERT et al., 1972). Holmes et al. (1981) reported cases of food-borne toxinfections caused by the presence of B. cereus in milk powder that was used in the manufacture of cheese and pasta. In 1972, in Romania, there was a food-borne outbreak with 221 children, involving milk contaminated with B. cereus. (CHRISTIANSSON, 1992).

In this way, it is important to apply quality tools throughout the productive chain, mainly dairy products, in order to avoid the presence of these bacteria as contaminants of these products as much as possible and, consequently, to preserve the health of the consumer and maintain the characteristics of the product.

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CHAPTER 2: MICROBIOLOGICAL QUALITY OF UHT MILK AND GENETIC BIODIVERSITY OF SPORE-FORMING BACTERIA

MICROBIOLOGICAL QUALITY OF UHT MILK AND GENETIC BIODIVERSITY OF SPORE-FORMING BACTERIA ISOLATES OF UHT MILK FROM DIFFERENT REGIONS IN BRAZIL

ABSTRACT

The sporulated bacteria studied were isolated from UHT milk samples from seven different companies located in three regions of Brazil. The objective of the present study was to evaluate the microbiological quality of UHT milk produced by these companies according to Portaria n° 370 of 1997, to identify the microbial biodiversity of the isolated strains and to verify the presence of Bacillus sporothermodurans. Firstly, each of the UHT milk samples were incubated at 37 ºC for seven days followed by standard plate count for the determination of viable aerobic mesophiles. Afterwards, the samples were submitted to heat treatments to determine the presence of spore-forming bacteria. About five strains of each UHT milk sample were selected and subsequently subjected to isolation and biochemical tests (Gram staining, catalase, oxidase, glucose fermentation, esculin hydrolysis, nitrate reduction and urease test). According to these tests, none of the isolates presented typical characteristics of B. sporothermodurans. In sequence, the isolates, that presented rod-shaped in gram staining, were submitted to molecular analyzes of Rep-PCR and 16S rDNA sequencing to identify the microbial biodiversity existing between them. There was a great diversity, since several clusters were formed and among them about 44 % were formed by a single isolate. In conclusion, with this study it was possible to evaluate, in general terms, the quality of Brazilian UHT milk produced in three different regions. In addition, it was also possible to determine the biodiversity of the spore-forming bacteria found in the samples, thus opening a range of possible research aspects about the effects of the presence of these microorganisms on milk quality, as well as, the effects they may have on the consumer health.

1. INTRODUCTION

Ultrapasteurization or UHT treatment consists of a heat treatment in continuous flow (130 – 150 ºC / 2 – 4 s) followed by cooling at 32 ºC. This heat treatment is able to eliminate all vegetative cells of microorganisms present in milk, as well as, a large number of spores, except for thermoresistant spores, as is the case of spores of B. sporothermodurans (BRASIL, 1997; FRANCO et al., 2008; MUCIDAS, 2010; TRONCO, 2010; FEAM, 2011; MENEZES et al., 2014).

The thermoresistant bacterial spores are able to survive the heat treatment at high temperatures and there is a great possibility that they germinate along the storage and distribution of the product. In addition, after germinating, the bacteria can multiply, reach high counts and produce enzymes (proteolytic and lipolytic) that reduce the quality of the product (HAMMER et al., 1995; PETTERSSON et al., 1996; COSTA et al., 2016).

In Brazil, the Ordinance n° 370 of 1997 establishes that UHT milk must not contain microorganisms capable of multiplying under normal conditions of storage and distribution, and after incubation of the closed package at 35 – 37 ºC for seven days, the count of aerobic mesophiles can not exceed the limit of 100 $CFU \cdot mL^{-1}$. The problem regarding the contamination of UHT milk by spore-forming bacteria is in the increase in the total count of viable aerobic mesophilic microorganisms, exceeding the limits determined by this legislation (BRASIL, 1997; ZACARCHENCO et al., 2000).

In Chapter III of Normative Instruction n° 62 of 2003 (Microbiological Analyzes for Dairy Products), which describes the count of viable aerobic mesophilic microorganisms capable of causing change in UHT liquid dairy products, it is clear in item 5.5 that the differentiation of B. sporothermodurans is necessary since this species should not be accounted in the calculation of viable aerobic mesophiles. However, the Laboratórios Nacionais Agropecuários (LANAGRO) of the Ministério da Agricultura, Pecuária e Abastecimento (MAPA) started to use ISO 4833 of 2013 (specifies a horizontal method for enumeration of microorganisms that are able to grow and form colonies in a solid medium after aerobic incubation at 30 °C) as the

official method and as a legal reference to Ordinance n° 370 of 1997 for the analysis of UHT milk, which does not differentiate the presence of B. sporothermodurans of the other aerobic mesophilic microorganisms (BRASIL, 1997; BRASIL, 2003). The consequence of this, is that the companies that presented samples of UHT milk with high counts of B. sporothermodurans happened to be outside the standards determined by the legislation for standard counting in plates of viable aerobic mesophiles.

Based on this, the objective of this study was to evaluate the microbiological quality of UHT milk from some Brazilian regions, to determine the presence of B. sporothermodurans, through techniques determined by the legislation and, finally, to determine the genetic biodiversity among the isolates.

2. MATERIAL AND METHODS

2.1. UHT milk samples

For this study, seven Brazilian companies producing UHT milk from three different regions of the country (South, Southeast and Central-West) sent to INOVALEITE - Laboratory of Milk and Derivatives, Department of Food Technology, Federal University of Viçosa - UHT milk samples of different problem batches over six months. A total of 184 samples were evaluated. Of the three regions that were studied, the Southeast region was the one with the most numbers of UHT milk samples and companies evaluated, were 154 samples from five companies from two different states. From the Central-West and South regions, only one company was evaluated, with a total of six and 24 samples, respectively, as shown in Table 1.

Table 1. Dairy companies, regions of origin and total number of samples used in this study.

Dairy Companies	Region	Total of samples evaluated
A	Southeast	18
B		42
C		40
D		40
E		14
F	South	6
G	Central-West	24
Total	**-**	**184**

2.2. Microbiological quality of UHT milk samples and determination of the presence of sporulates

UHT milk samples were incubated for a period of seven days at 36 ± 1 ºC. Subsequently, a visual check was made on the occurrence of changes in product characteristics (coagulation, flocculation, desorption, non-characteristic odor or other). When evident change occurred, no further analysis was performed with the sample in question, and the result was reported as "altered product after incubation at 36 ± 1 ºC for 7 days".

Samples that did not show alteration were diluted in tubes containing 9 mL of 0.1% (w/v) peptone saline solution until dilution 10^{-2}. The dilutions were pour plated on brain-heart agar (BHI) (Kasvi, Curitiba, Paraná, Brazil) and nutrient agar free of yeast extract (Kasvi), in duplicate. The plates were incubated at 30 ± 1 ºC for 72 hours (Figure 1). After incubation, the colonies formed on the plates were counted and the results expressed in $CFU \cdot mL^{-1}$.

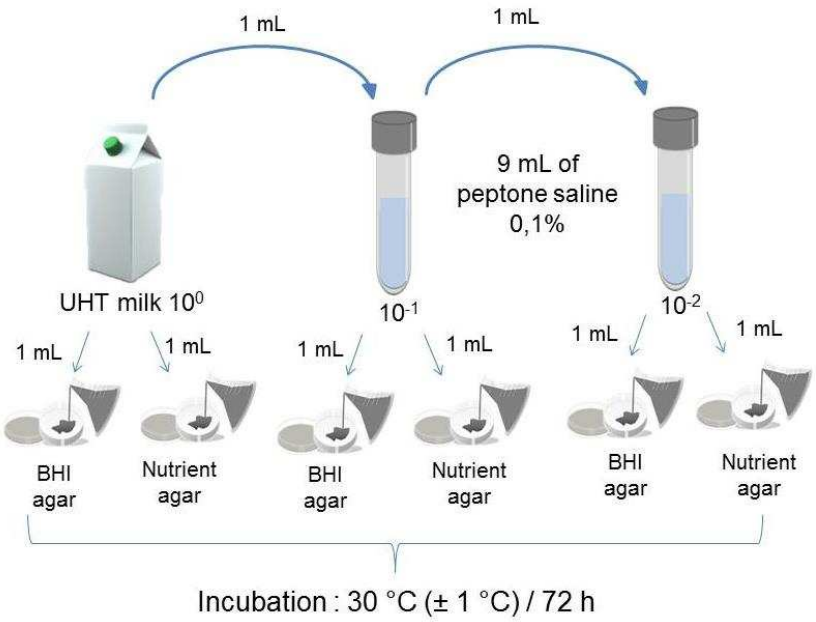


Figure 1. Scheme of the dilution and plating stages of UHT milk samples.

To determine the presence of spore-forming bacteria, two aliquots of approximately 10 mL of each UHT milk sample were collected in previously sterile test tubes. Each of the aliquots was subjected to a different heat treatment (T1: 80 ºC / 10 min and

T2: 100 ºC / 30 min), in order to eliminate all vegetative forms of microorganisms present and to allow the detection of spores (Figure 2).

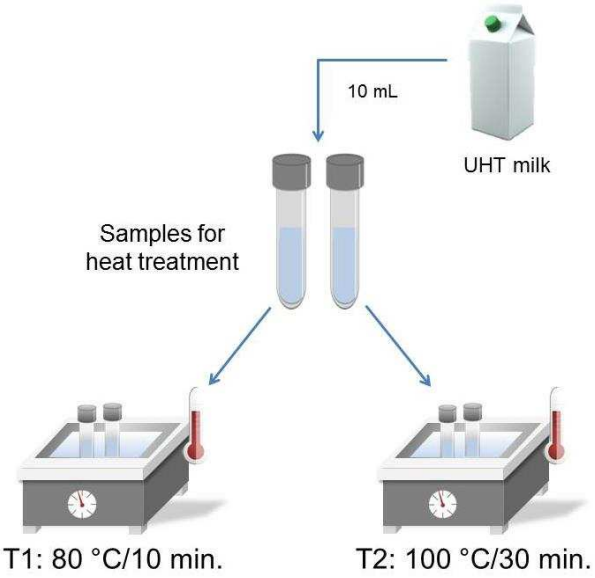


Figure 2. Scheme of the thermal treatments applied in UHT milk samples.

After the heat treatment, the aliquots were submitted to dilution (up to 10^{-2}) in 0.1% (w/v) peptone saline solution and pour plated, in duplicate, in the BHI agar (Kasvi) and nutrient agar free of yeast extract (Kasvi), followed by incubation at 30 ± 1 ºC for 72 hours.

After the incubation period, the colonies formed on the plates here counted and the results expressed in CFU·mL^{-1}. After incubation, 10% of the colonies were randomly selected, purified by composed streaks in PCA (Plate Count Agar) plates (Kasvi, Curitiba, Paraná, Brazil) and stored at − 80 °C in isolation BHI broth media (Kasvi) supplemented with 30% (v/v) glycerol. Prior to phenotypic and molecular analysis, the isolates were transferred in BHI broth and incubated at 37 °C for 24 h.

2.3. Phenotypic analyzes of isolates

The phenotypic characterization of the 335 bacteria isolated from the UHT milk samples was performed according to the methodology described by Normative Instruction n° 62 of 2003.

Each isolate was submitted to a series of biochemical tests regarding to the phenotypic characterization to identify the presence of B. sporothermodurans. The

tests performed were: Gram staining, catalase, oxidase, anaerobic growth, glucose fermentation, esculin hydrolysis, nitrate reduction and urease test. The tests were carried out according to the recommendation of the same legislation.

2.4. Biodiversity by molecular analysis

2.4.1. DNA extraction

The pellet of the activated culture of isolated strains was obtained by centrifugation at 10.000 x g for 10 min. The DNA of the bacteria was isolated by DNA Purification Wizard® Genomic kit (Promega Corp., Madison, WI, USA). The quality and the concentration of the extracted DNA was measured by the equipment NanoDrop™ Lite Spectrophotometer (Thermo Scientific, Massachusetts, EUA) and the concentration was standardized to 100 ng/μL**.**

2.4.2. Characterization of isolated strains by Rep-PCR

Rep-PCR analysis was performed according to the protocol described by Dal Bello et al. (2010) using a single universal primer $(GTG)_5$ (5'-GTGGTGGTGGTGGTG-3'). PCR reactions contained 12.5 μL of Go Taq Green Master Mix 2x (Promega), 0.5 μL of the primer (100 mol/L), 100 ng DNA and ultra-pure water (Promega) to a final volume of 25 μL. PCR amplification was carried out in a thermal cycler and the cycle used was 95 ºC for 5 min as initial step, 95 ºC for 30 s, annealing at 40 ºC for 30 s and 65 ºC for 8 min for the next 30 cycles, 65 ºC for 16 min concluded the amplification.

The PCR products were electrophoresed on 2% (w/v) agarose gel for 2 h at a constant voltage of 75 V in 0.5x TBE buffer. The gels were stained using GelRed (Biotium Inc.) and developed using an LPIX transilluminator (Loccus Biotechnology, SP, Brazil). The bands profile was analyzed using BioNumerics software 6.6 (Applied Maths). The similarities between the profiles were calculated using the Pearson correlation. Dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic (UPGMA).

2.4.3. 16S rDNA sequencing

A representative of each group generated by Rep-PCR analysis was randomly selected and subjected to 16S rDNA gene amplification using the 8f (5 "-CACGGATCCAGACTTTGATYMTGGCTCAG-3") and 1512r (5 "-GTGAAGCTTACGGYTAGCTTGTTACGACTT-3") primers (FELSKE et al., 1997), which give rise to fragments of approximately 1500 bp. The amplification reaction was done using 12.5 μL of Go Taq Green Master Mix 2x (Promega), 1.0 μL of each primer (100 mol/L), 100 ng DNA and ultra-pure water (Promega) to a final volume of 25 μL. PCR amplification was carried out in a thermal cycler and the cycle used was 95 ºC for 5 min as initial step, 94 ºC for 20 s, annealing at 54 ºC for 20 s and 68 ºC for 2 min for the next 35 cycles, 72 ºC for 7 min concluded the amplification. The fragments obtained were sent to Macrogen Inc. in South Korea for sequencing the amplified gene, and the sequences obtained were aligned with 16S rRNA gene sequences present in the GenBank database (http://www.ncbi .nlm.nih.gov/genbank/) from the National Center for Biotechnology Information (NCBI) using BLAST software (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST).

3. RESULTS AND DISCUSSION

3.1. Microbiological quality of UHT milk samples and determination of the presence of sporulates

After incubation of the UHT milk samples for seven days at 36 ± 1 ºC, only one sample, from company B, presented alteration (gelation) and was not considered in the other stages of the study. The gelation of UHT milk consists of the change in the physical state of the product - from fluid to gel. This modification can occur due to a series of distinct factors, such as the action of proteolytic enzymes produced by psychrotrophic bacteria (SANTANA et al., 2001; NÖRNBERG et al., 2009; ZENI et al., 2013). The presence of these microorganisms is closely related to the microbiological quality of raw milk, because they produce proteolytic and lipolytic

thermoresistant enzymes that lead to a series of technological problems (DATTA et al., 2001). Kappa-casein is easily degraded by this proteases because it is located on the micelle surface. When it is hydrolyzed, a destabilization of the casein micelle can lead to milk coagulation (HANTSIS-ZACHAROV et al., 2007; ANGELO et al., 2014). The time required for gel formation over UHT milk storage is dependent on the extent of contamination of raw milk, according to a study by Law et al. (1977) with Pseudomonas fluorescens.

The most of the psychrotrophs belong to the genera Pseudomonas, Achromobacter, Aeromonas, Alcaligenes, Micrococcus, Bacillus, Lactobacillus or Arthrobacter. The genus Pseudomonas is the main one among them, being Pseudomonas fluorescens the predominant species (COUSIN, 1982; HANTSIS-ZACHAROV et al., 2007; STULOVA et al, 2010; ÂNGELO et al., 2014).

Regarding the microbiological quality, approximately 51% of the analyzed UHT milk samples presented viable aerobic mesophiles counts above 100 $CFU \cdot mL^{-1}$, which makes these samples in disagreement with the current legislation (Table 2). Similar results were found in other studies on Brazilian UHT milk quality. Coelho et al. (2000) evaluated 80 samples of UHT milk produced in Belo Horizonte (Minas Gerais) and 41.2% of the samples presented a viable aerobic mesophil count above 100 $CFU \cdot mL^{-1}$, varying from 1.3×10^4 to 1.4×10^5 $CFU \cdot mL^{-1}$. In a study conducted by Souza et al. (2014) with UHT milk samples from the state of Minas Gerais, 35% of the evaluated companies presented viable aerobic mesophil counts above the standards established by the legislation. Bersot et al. (2010) evaluated 150 samples of UHT milk produced in the state of Paraná, of which 24% were in disagreement with the legislation, presenting counts above 100 $CFU \cdot mL^{-1}$.

The table 2 shows the unit and percentage values of the quantity of UHT milk samples with counts of viable aerobic mesophiles above 100 $CFU \cdot mL^{-1}$, and thus are out of legal standards, and the amount of samples that after the heat treatments presented counts of spore-forming bacteria.

Table 2. Data about the plate counts of the UHT milk samples evaluated.

Dairy Companies	Total of nonstandard samples (> 100 CFU·mL^{-1})		Total of samples contaminated by sporulates	
	n° samples	%	n° samples	%
A	17	94.4	17	94.4
B	8	19.0	16	38.1
C	16	40.0	15	37.5
D	39	97.5	39	97.5
E	1	7.1	4	28.6
F	6	100.0	6	100.0
G	6	25.0	8	33.3
Total	**93**	**50.5**	**105**	**57.1**

The dairy company "F" was the one that presented the worst results, 100% of the samples evaluated were outside the standards established by the legislation. Next, we have the companies "D" and "A" with 97.5% and 94.4% of the samples above the limits. The other companies varied between 7.1% and 40% (Table 2). The reasons for the presence of these microorganisms in UHT milk are diverse and may range from the low quality of raw milk, due to unsatisfactory hygiene conditions at the time of milking, until the recontamination of milk after heat treatment, due to the existence of biofilms along processing line (PEREIRA et al., 2013; MENEZES et al., 2014).

Of the 184 samples evaluated, 57.1% had presented counts of microorganisms after the application of thermal treatments. Pinto et al. (2017) also evaluated samples of Brazilian UHT milk for the presence of spore-forming bacteria, and of the 20 milk brands evaluated, 45% had sporulated bacteria counts. In addition, 18.7% of the brands had sporulated counts greater than 100 CFU·mL^{-1}.

These results reaffirm the importance of the use of quality raw material and the lowest level of contamination possible, since some sporulated microorganisms besides producing thermoresistant enzymes and forming biofilms are considered pathogenic and represent a great risk to the health of the consumer, as is the case of Bacillus cereus (MONTANHINI et al., 2013).

After enumeration of microorganisms in the UHT milk samples, 335 isolates were selected, of which 231 (68.9%) were isolated from heat-treated samples, therefore they were spore-forming bacteria (Table 3).

Table 3. Amount of isolates and sporulates that were isolated from UHT milk samples.

Dairy Companies	Total isolates	Total Sporulates	% sporulates
A	55	41	74.5
B	26	22	84.6
C	48	33	68.8
D	160	98	61.3
E	3	3	100.0
F	24	17	70.8
G	21	17	81.0
Total	**335**	**231**	**68.9**

It is important that companies apply all the necessary tools for quality management and increasingly encourage their suppliers of raw material to always seek to produce high quality milk, since it is only in this way that it will be possible to minimize the problems associated with microbiological contamination in these products and ensure the consumer a high quality product.

3.2. Phenotypic analyzes of isolates

According to the Normative Instruction n° 62 of 2003, colonies of B. sporothermodurans present gram-positive rod morphologies, catalase and oxidase positive reactions, are not able to grow in anaerobiosis, hydrolyze esculin, do not ferment glucose, do not reduce nitrate and do not produce urease. Therefore, to be considered a strain of B. sporothermodurans, the isolates must own all characteristics. Table 4 shows the number of isolates that presented such characteristics in each of the biochemical tests.

Table 4. Isolates that showed characteristics of B. sporothermodurans in the biochemical tests.

Biochemical tests	Total isolates
Gram staining	114
Catalase	332
Oxidase	192
Anaerobic growth	5
Glucose fermentation	42
Esculin hydrolysis	229
Nitrate reduction	5
Urease test	111

Although several isolates presented results consistent with those expected for B. sporothermodurans strains in some of the biochemical tests, none of them had all the necessary characteristics for such identification. Thus, according to Normative Instruction n° 62 of 2003, of the 335 isolates none can be identified as B. sporothermodurans, therefore, none were excluded from the count of viable aerobic mesophiles. Similar result was found by Pinto et al. (2017) that evaluated 91 samples of Brazilian UHT milk, and of the 46 isolates obtained, none were identified as B. sporothermodurans. Rezer (2010) evaluated UHT milk samples commercialized in Rio Grande do Sul (Brazil) and also did not find strains of B. sporothermodurans. In contrast some researchers found this bacterium in Brazilian UHT milk samples. Zacarchenco et al. (2000) evaluated 100 samples of UHT milk from 6 Brazilian states and identified 24 isolates of B. sporothermodurans. Busatta et al. (2005) and Pereira et al. (2013) also identified the presence of this bacterium, respectively, in 54.5% and 60% of the brands evaluated. In a study on the presence of thermoresistant spore-forming bacteria in UHT milk from Thailand, Kmiha et al. (2017) evaluated 41 samples that were taken at different stages during the UHT milk manufacturing. The presence of B. sporothermodurans was identified only in raw milk samples.

Although simple, the biochemical tests are very laborious and require a lot of time and material to perform. In addition, the results are obtained, on average, after 72 h. Given the routine and flow of products from a dairy company, the identification of B. sporothermodurans strains through these techniques becomes completely infeasible.

Even though a negative result was obtained regarding the presence of B. sporothermodurans, the isolates that presented bacilli morphology were directed to the molecular analyzes to confirm the results of the biochemical tests and to identify which bacteria were present in UHT milk samples. This choice was made through the decision to keep the focus of the studies on the possible representatives of the genus Bacillus, due to the ability of some species to form thermoresistant spores. The other isolates (gram-positive coccus) were stored in the culture bank for further studies. In studies about the quality of UHT milk samples, Pereira et al. (2013), Bersot et al. (2010) and Coelho et al. (2001) also found gram-positive coccus in the morphological identification of the isolates through gram staining.

3.3. Biodiversity by molecular analysis

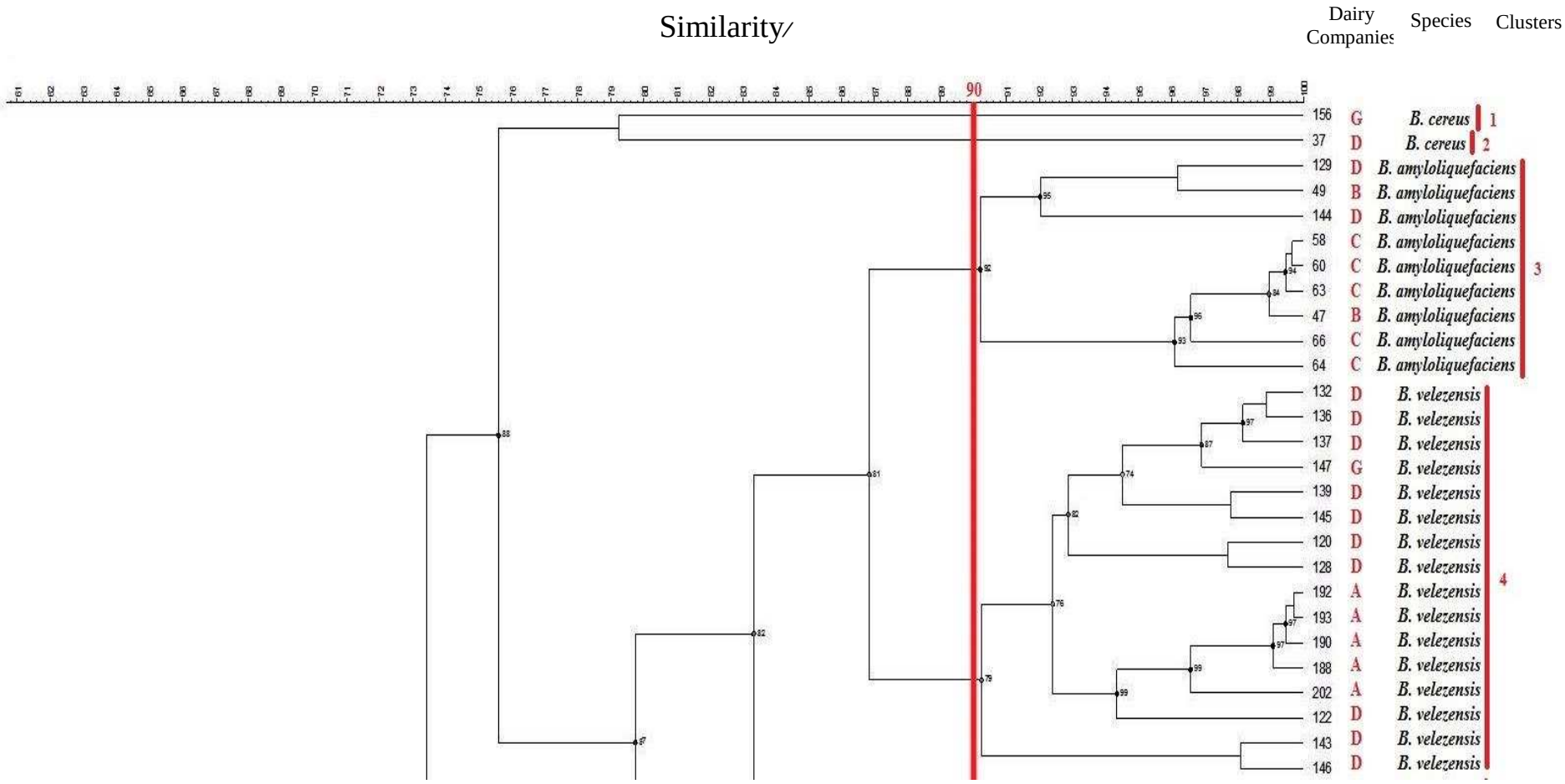
The Rep-PCR method has been shown to be a good tool for the study of the bacterial genome, microbial ecology, environmental microbiology, molecular diagnosis and medical microbiology (DAL BELLO et al., 2010; PERIN et al., 2012).

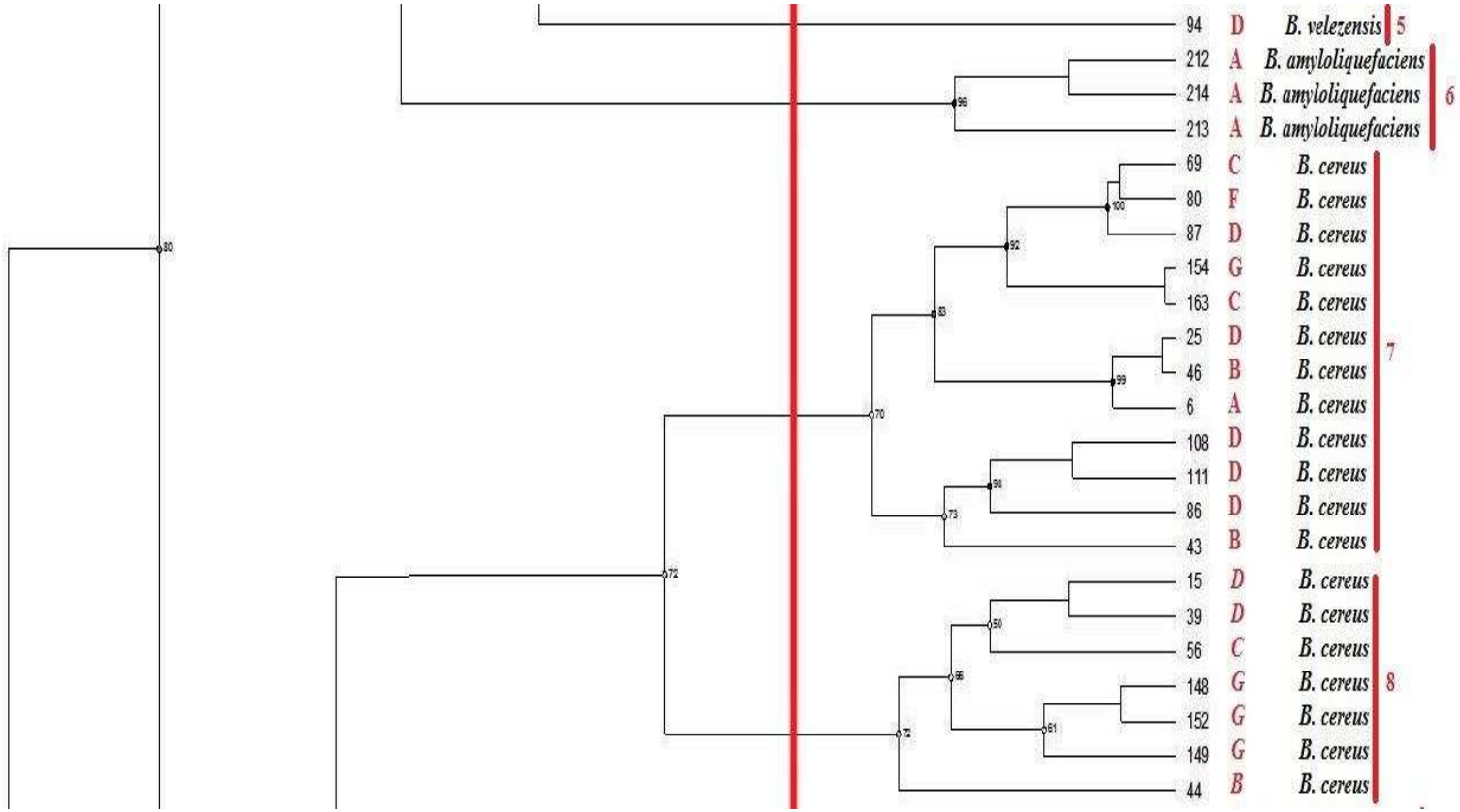
Considering a level of 90% similarity, the dendrogram generated 25 clusters (Figure 3). Rep-PCR analysis revealed a very varied band profile indicating a high diversity among the isolates, and even though the similarity between some isolates was very high, there was no case where it was 100%. Of the 25 clusters formed, 11 were formed by single strains; 11 clusters from 2 to 9 isolates and 3 ranging from 12 to 17 isolates.

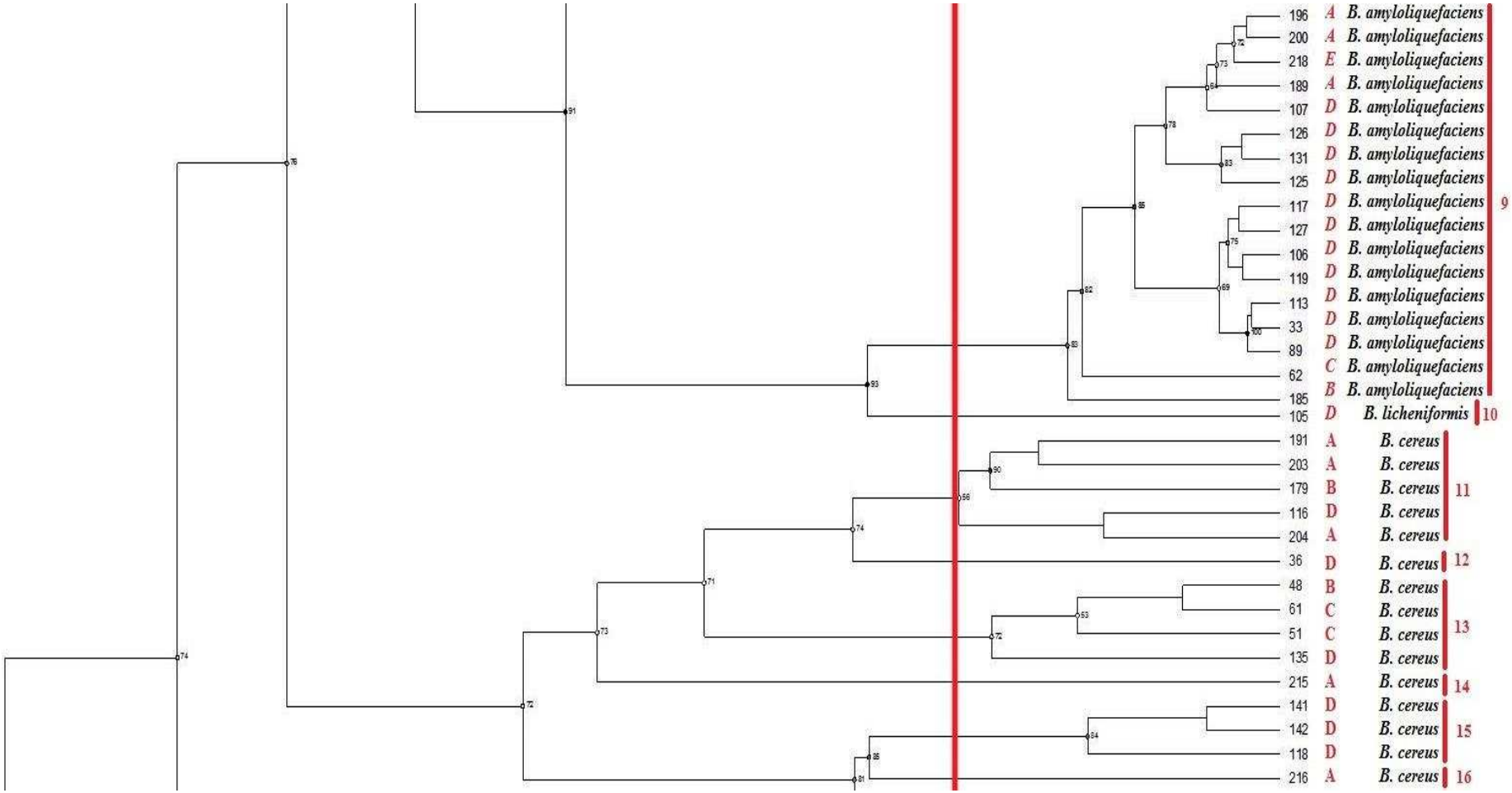
Considering the formation of clusters, among isolates from a single company, it was possible to perceive that, even within a single production line, there was a high diversity. Between the isolates of each of the companies (from "A" to "G"), 9, 8, 6, 18, 1, 1 and 6 clusters were, respectively, formed. With the exception of the companies "E" and "F", which formed the custer with only one isolate, the others would require a level between 50 and 70% of similarity for all the isolates to be grouped in a single cluster. Regarding the geographic region, of the 25 groups obtained, 18 were composed only of isolates from the Southeast, 1 of isolates from the central-west, 5 of isolates from the Southeast and Center-West regions, 1 of

isolates from the three regions, and none group was formed, exclusively, by isolates from the Southern region (Figure 3).

Within each of the groups formed in Rep-PCR analysis were randomly selected representatives for identification through the 16S rDNA sequencing. The genus found, in 100% of cases, was Bacillus. Within the genus Bacillus, our focus of the study, six species were identified: B. amyloliquefaciens, B. proteolyticus, B. cereus, B. subtilis, B. licheniformis and B. velezensis. The presence of Bacillus spp. in UHT milk can be attributed to a raw material with a high spore count, insufficient heat treatment, or post process contamination due to the presence of biofilms in the pipes (CHRISTIANSSON et al., 1998; SVENSSON et al., 2004; VIDAL-MARTINS et al., 2005; REZENDE-LAGO et al., 2007).


Similarity
Dairy Companies
Species
Clusters
90
156 G B. cereus 1
37 D B. cereus 2
129 D B. amyloliquefaciens
49 B B. amyloliquefaciens
144 D B. amyloliquefaciens
58 C B. amyloliquefaciens
60 C B. amyloliquefaciens
63 C B. amyloliquefaciens
47 B B. amyloliquefaciens
66 C B. amyloliquefaciens
64 C B. amyloliquefaciens
3
132 D B. velezensis
136 D B. velezensis
137 D B. velezensis
147 G B. velezensis
139 D B. velezensis
145 D B. velezensis
120 D B. velezensis
128 D B. velezensis
192 A B. velezensis
193 A B. velezensis
190 A B. velezensis
188 A B. velezensis
202 A B. velezensis
122 D B. velezensis
143 D B. velezensis
146 D B. velezensis
4


94 D B. velezensis 5
212 A B. amyloliquefaciens
214 A B. amyloliquefaciens 6
213 A B. amyloliquefaciens
69 C B. cereus
80 F B. cereus
87 D B. cereus
154 G B. cereus
163 C B. cereus
25 D B. cereus 7
46 B B. cereus
6 A B. cereus
108 D B. cereus
111 D B. cereus
86 D B. cereus
43 B B. cereus
15 D B. cereus
39 D B. cereus
56 C B. cereus
148 G B. cereus 8
152 G B. cereus
149 G B. cereus
44 B B. cereus


196 A B. amyloliquefaciens
200 A B. amyloliquefaciens
218 E B. amyloliquefaciens
189 A B. amyloliquefaciens
107 D B. amyloliquefaciens
126 D B. amyloliquefaciens
131 D B. amyloliquefaciens
125 D B. amyloliquefaciens
117 D B. amyloliquefaciens 9
127 D B. amyloliquefaciens
106 D B. amyloliquefaciens
119 D B. amyloliquefaciens
113 D B. amyloliquefaciens
33 D B. amyloliquefaciens
89 D B. amyloliquefaciens
62 C B. amyloliquefaciens
185 B B. amyloliquefaciens
105 D B. licheniformis 10
191 A B. cereus
203 A B. cereus
179 B B. cereus 11
116 D B. cereus
204 A B. cereus
36 D B. cereus 12
48 B B. cereus
61 C B. cereus
51 C B. cereus 13
135 D B. cereus
215 A B. cereus 14
141 D B. cereus
142 D B. cereus 15
118 D B. cereus
216 A B. cereus 16

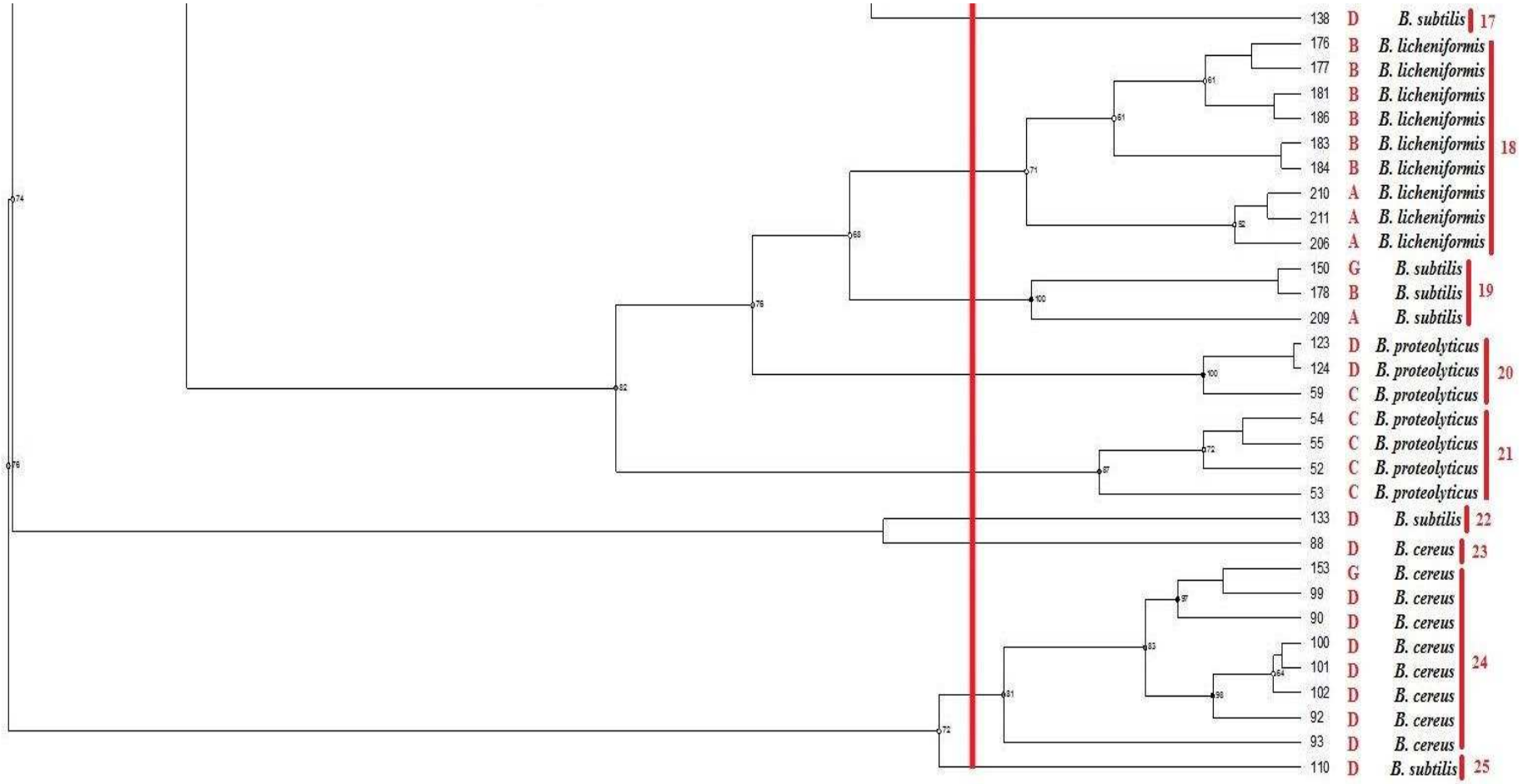


Figure 3. Dendrogram based on UPGMA clustering (Pearson coefficient) of Rep-PCR profiles of strains isolated from UHT milk samples.

Of all the species of Bacillus found, B. cereus was the most abundant representing 39.5% of the isolated strains (Table 5). This result was superior to those found in other studies on the microbiological quality of Brazilian UHT milk, which can be explained by the fact that the samples evaluated in this study were from problematic lots. Vidal-Martins et al. (2005) evaluated 110 samples of UHT milk in the city of São José do Rio Preto (SP), of which 11.8% presented B. cereus strains, Montanhini et al. (2011) also evaluated 110 samples of UHT milk and 16.4% of them contained B. cereus strains. Rezende-Lago et al. (2007) in a study in the city of Jaboticabal (SP) evaluated 30 UHT milk samples from which 13.3% presented B. cereus strains. Rezende et al. (2015) evaluated 120 samples of UHT milk and 7.5% were positive for the presence of this bacterium. The presence of B. cereus in food is very important from the point of view of food safety, since it is responsible for two food-borne diseases: the emetic and the diarrheal syndromes. The emetic syndrome is associated with the ingestion of a toxin previously produced in the food, whereas the diarrhea syndrome is the ingestion of the microorganism that in the intestine of the host produces an enterotoxin (DROBNIEWSKI, 1993; SENESI et al., 2010; MAZIERO et al., 2011). The emetic toxin is resistant to heat and pH (GRANUM et al., 1997; MAZIERO et al., 2011).

Table 5. Data on identified Bacillus species.

Species of Bacillus	n° of clusters	n° of strains	%	Geographic region
B. amyloliquefaciens	3	29	25.4	Southeast
B. cereus	12	45	39.5	Southeast, South and Central-West
B. licheniformis	2	10	8.8	Southeast
B. proteolyticus	2	7	6.1	Southeast
B. subtilis	4	6	5.3	Southeast and Central-West
B. velezensis	2	17	14.9	Southeast and Central-West
Total	**25**	**114**	**100.0**	**-**

B. cereus was the only isolated species of samples from all regions (Southeast, South and Central-West). In addition, it was the species that formed the most simple groups, of the 11 formed six (54.5%) were identified as B. cereus, which indicates

that although this bacterium is present in several distinct environments, the biodiversity existing among the strains of this same species is very large.

A new species belonging to the B. cereus group was identified by Liu et al. (2017) as B. proteolyticus. This bacterium is capable of producing proteinases that hydrolyze starch and casein. About 6% of the strains of this study were identified as B. proteolyticus, and together with the B. cereus strains represent 45.6% of the isolates, which is alarming because associated with the toxigenic potential of B. cereus such bacteria have a high potential for deterioration. This bacterium was isolated from UHT milk samples from only two dairy companies in the Southeast.

Six isolates (5.3%) of samples from the Southeast and Central-West region were identified as B. subtilis. This bacterium are associated with deterioration of various foods, which mainly include dairy products such as raw, pasteurized and UHT milk (HEYNDRICKX et al., 2002). Pinto et al. (2017) in a study on the microbiological quality of Brazilian UHT milk, isolated 46 strains of Bacillus sp., of which 68% were identified as being of B. subtilis, result much higher than the found in the present study. This fact can be explained due to the variation of the microbial load present in raw milk and in the manufacturing unit, existing between the two studies.

Strains of B. licheniformis, one of the species found in Southeast in this study, are known for their ability to cause deterioration in dairy products due to the production of proteolytic and lipolytic enzymes that compromise the organoleptic and functional properties of these products. In addition, they have the ability to form biofilms in the processing plants, being considered by some researchers, the species of Bacillus predominant in the dairy industries (CRIELLY et al., 1994; DE JONGHE et al., 2010; REGINENSI et al., 2011; DHAKAL et al., 2014). Ten isolates (8.8%) were identified as being B. licheniformis, similar result was found by Cosentino et al. (1997) that evaluated several dairy products from Sardinian regarding the incidence of bacteria of the genus Bacillus. Among these products, evaluated 60 samples of UHT milk, of which 27 strains of Bacillus were isolated and identified, the species found were B. sphaericus, B. pumilus, B. licheniformis, B. laterosporus, B. coagulans and B. brevis. Of the species characterized 11.1% were of B. licheniformis.

Another species of Bacillus found was B. velezensis, corresponding to 14.9% of the isolates evaluated. This bacterium was isolated in the Vélez River in Malaga, Spain by Ruiz-García et al. (2005) and usually is not found in food. For these researchers it is a species belonging to the group B. amyloliquefaciens, but for others it is a later heterotypic synonym of this bacterium (WANG et al., 2008). Through sequencing, 29 isolates (25.4%) were identified as B. amyloliquefaciens. This bacterium was isolated in samples of UHT milk from all companies evaluated in the Southeast region, which may indicate that it is part of the autochthonous microbiota of this region. McKillip et al. (2016) identified isolates of these bacteria in organic UHT milk and proved that it is a biofilm producer in amounts exceeding those of the B. cereus ATCC strain used as control. In addition, virulence genes were present in B. amyloliquefaciens isolates that was isolated.

As the milk samples quality was totally unknown and it was not possible to predict which types of microorganisms would be present, two different heat treatments were applied. The first treatment - T1: 80 ºC / 10 min (WESTHOFF, 1981; WEHR et al., 2004) - was chosen because it is the most used to proceed with the spore count, since it is able to kill the vegetative cells present in the medium. The second treatment (T2: 100 ºC / 30 min), because it was more intense, was applied in order to verify if the binomial would be able to select a different and more resistant microbiota than in the first case. However, through the sequencing results there was no difference between the microorganisms from the two treatments. There was also no difference between the types of bacteria found in UHT milk samples without heat treatment and those treated thermally for spore research. All species were found in all conditions.

In this study, we observed that different spore-forming bacteria were isolated in UHT milk samples from the same dairy company. Several factors may explain this fact, including the level of initial contamination of the raw material, temperature and transport time from raw milk to the dairy companies, hygienic conditions of processing line, and particular environmental conditions of each raw material producer region, as well as of the processing unit (CHAVAN et al., 2011).

4. CONCLUSION

The results showed high frequency of spore-forming bacteria in the analyzed samples, absence of B. sporothermodurans strains, and a high biodiversity among the isolates obtained.

Through the typical characteristics of the identified strains, such as the ability to form biofilms and produce thermoresistant spores, we can suggest that the problems of the batches, to which the samples belonged, may be associated with the presence of biofilms in the processing line, as well as, with the use of raw material with high microbiological count for the production of UHT milk.

Although it was known that these were samples of UHT milk from batches with problems, the presence of species capable of causing food-borne diseases, such as B. cereus, reinforced the need to implement quality measures along the chain productive.

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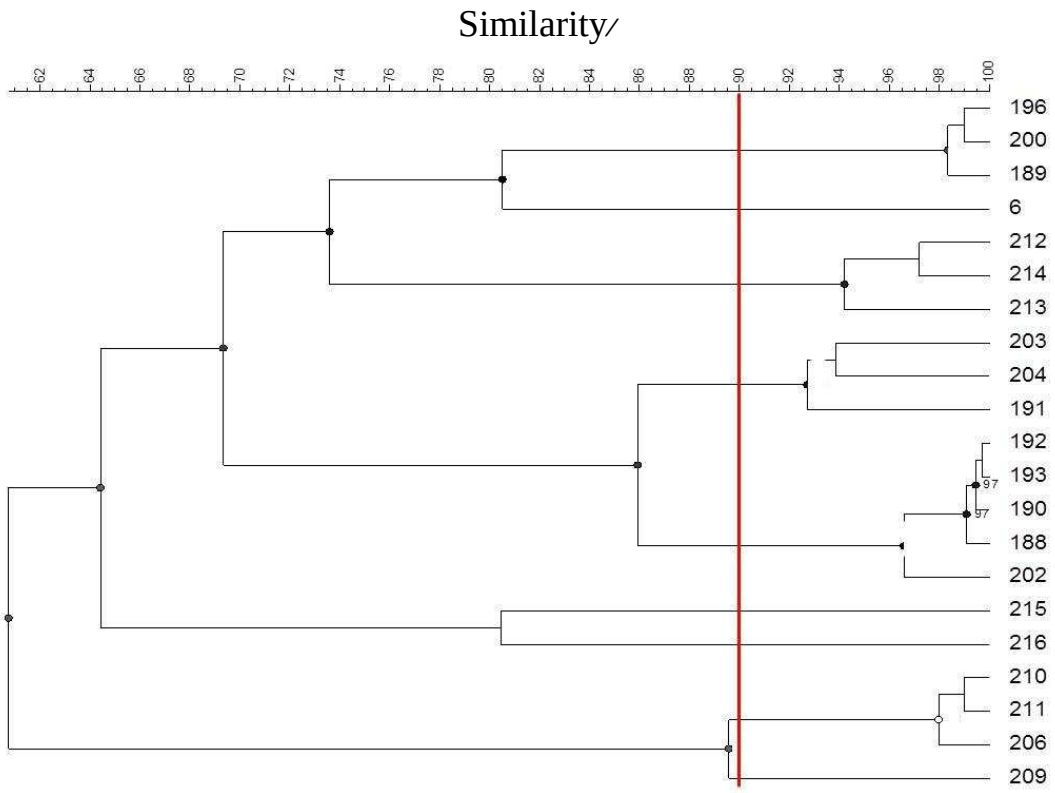
ATTACHMENT

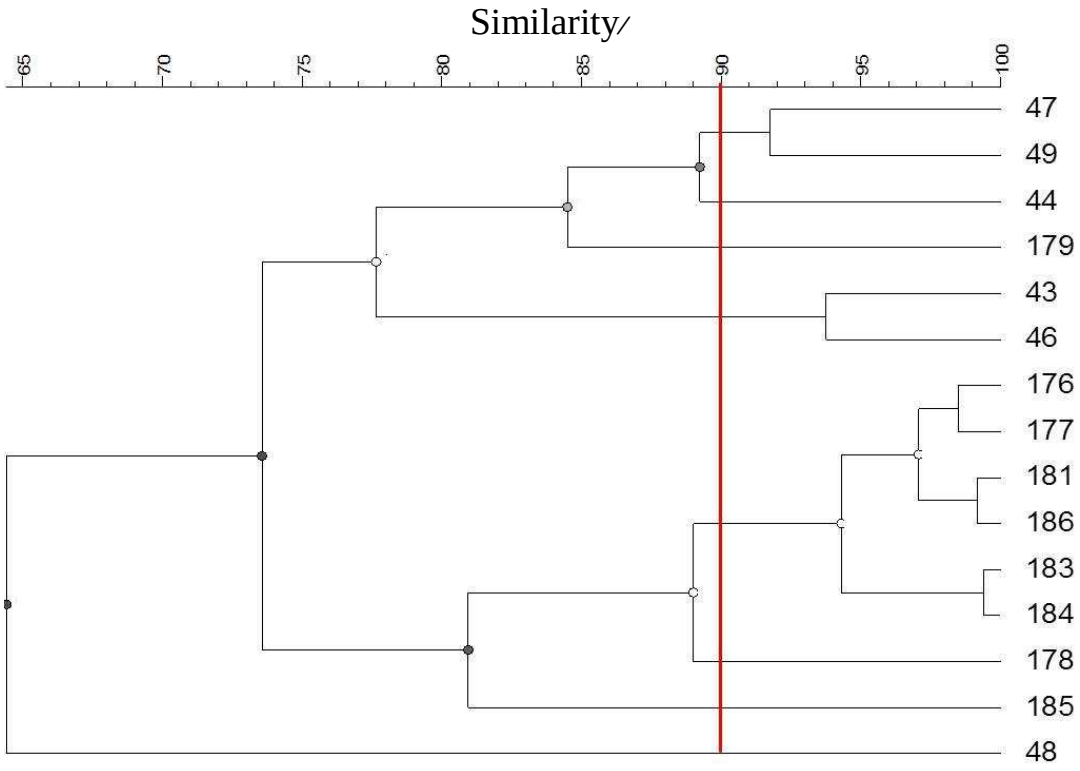
Annex 1. Distribution of UHT milk samples according to the population of microorganisms.

Population (CFU·mL^{-1})	**Number of UHT milk samples by dairy company**							
	A	B	C	D	E	F	G	**Total**
< 1	1	24	17	1	10	0	15	**68**
1 - 10^{1}	0	10	7	0	3	0	3	**23**
10^{1} - 10^{2}	7	4	1	0	1	1	1	**15**
10^{2} - 10^{3}	7	1	2	4	0	5	2	**21**
10^{3} - 10^{4}	3	3	13	35	0	0	3	**57**

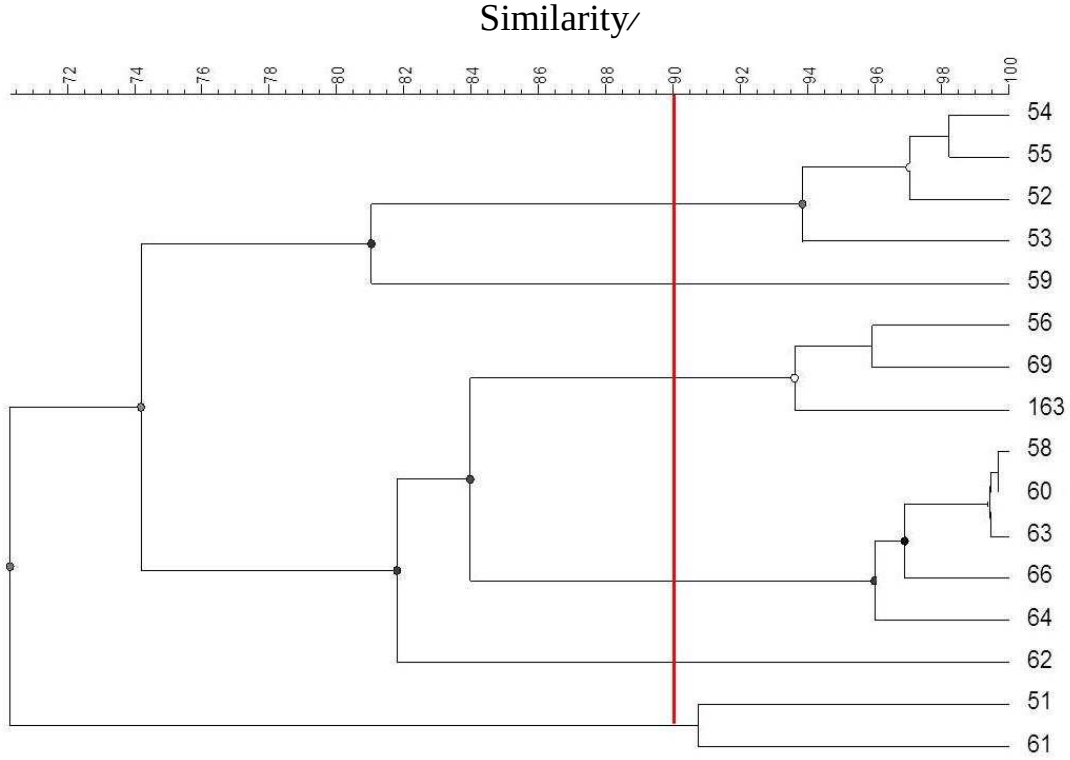
Annex 2. Dendrogram based on UPGMA clustering (Pearson coefficient) of Rep-PCR profiles of isolated strains, isolated from UHT milk samples from dairy company "A".



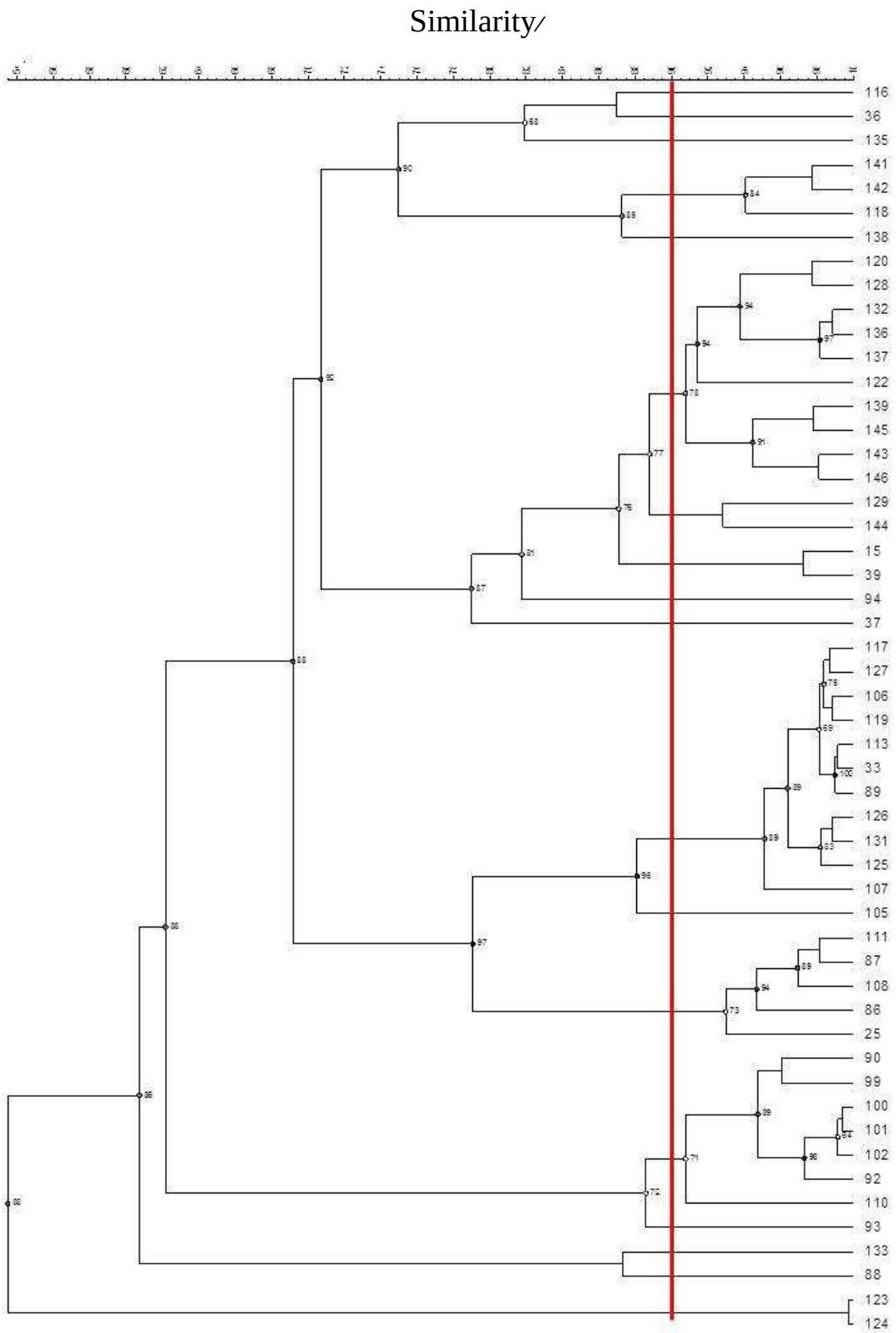
Annex 3. Dendrogram based on UPGMA clustering (Pearson coefficient) of Rep-PCR profiles of isolated strains, isolated from UHT milk samples from dairy company "B".



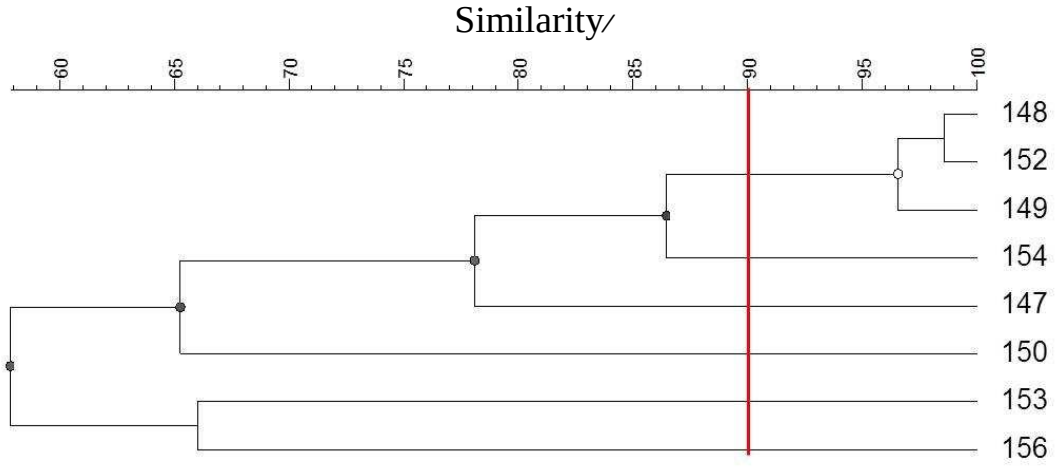
Annex 4. Dendrogram based on UPGMA clustering (Pearson coefficient) of Rep-PCR profiles of isolated strains, isolated from UHT milk samples from dairy company "C".



Annex 5. Dendrogram based on UPGMA clustering (Pearson coefficient) of Rep-PCR profiles of isolated strains, isolated from UHT milk samples from dairy company "D".



Annex 6. Dendrogram based on UPGMA clustering (Pearson coefficient) of Rep-PCR profiles of isolated strains, isolated from UHT milk samples from dairy company "G".



Annex 7. Percentage of the identity, according to BLAST software - NCBI, of the strains obtained by the 16S rDNA sequencing.

Strain	Classification	% similarity
36	Bacillus cereus	100
37	Bacillus cereus	97
49	Bacillus amyloliquefaciens	100
52	Bacillus proteolyticus	100
56	Bacillus cereus	80
59	Bacillus proteolyticus	100
61	Bacillus cereus	99
88	Bacillus cereus	100
94	Bacillus velezensis	99
102	Bacillus cereus	99
105	Bacillus licheniformis	97
110	Bacillus subtilis	77
116	Bacillus cereus	100
117	Bacillus amyloliquefaciens	100
133	Bacillus subtilis	100
138	Bacillus subtilis	86
141	Bacillus cereus	96
150	Bacillus subtilis	92
154	Bacillus cereus	99
156	Bacillus cereus	81
186	Bacillus licheniformis	79
190	Bacillus velezensis	99
212	Bacillus amyloliquefaciens	99
215	Bacillus cereus	99
216	Bacillus cereus	85

Annex 8. Species of Bacillus found in each of the dairy companies.

Dairy Companies	Species of Bacillus
A	B. amyloliquefaciens, B. cereus, B. licheniformis, B. subtilis and B. velezensis
B	B. amyloliquefaciens, B. cereus, B. licheniformis and B. subtilis
C	B. amyloliquefaciens, B. cereus and B. proteolyticus
D	B. amyloliquefaciens, B. cereus, B. licheniformis, B. proteolyticus, B. subtilis and B. velezensis
E	B. amyloliquefaciens
F	B. cereus
G	B. cereus, B. subtilis and B. velezensis

GENERAL CONCLUSION

Through this study it was possible to compare molecular techniques and biochemical tests. And although biochemical tests provide us with many interesting information about the metabolism and behavior of microorganisms, they are time consuming and laborious and if the microorganism has been subjected to some adverse conditions, it can completely change its metabolism and phenotype in this way, a false positive result can be obtained.

The high incidence of thermoresistant spore-forming bacteria, even from problematic samples, gives us as future perspectives the search for more effective conservation methods against this class of microorganisms. Parallel to this, we must always seek the producers' awareness about the importance of the quality of the raw material.

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