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ULTRA HIGH TEMPERATURE MILK: MICROBIOLOGICAL QUALITY, AGE GELATION AND A PROPOSAL OF AN ALTERNATIVE METHOD FOR MICROBIAL DETECTION

Thesis 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 *Doctor Scientiae*.

Adviser: Antônio Fernandes de Carvalho

Co-adviser: Solimar Gonçalves Machado

VIÇOSA - MINAS GERAIS
2024

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
M838u
2024

Moreira, Isabella Maria Fernandes Botelho, 1993-
Ultra high temperature milk: microbiological quality, age gelation and a proposal of an alternative method for microbial detection / Isabella Maria Fernandes Botelho Moreira. – Viçosa, MG, 2024.
1 tese eletrônica (77 f.): il. (algumas color.).

Texto em inglês.
Orientador: Antônio Fernandes de Carvalho.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Tecnologia de Alimentos, 2024.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2024.422
Modo de acesso: World Wide Web.

1. Leite - Microbiologia. 2. Leite - Qualidade. 3. Geleificação. 4. Agentes estabilizantes. 5. Análise multivariada. I. Carvalho, Antônio Fernandes de, 1964-. II. Universidade Federal de Viçosa. Departamento de Tecnologia de Alimentos. Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos. III. Título.

CDD 22. ed. 664.024

Bibliotecário(a) responsável: Alice Regina Pinto Pires CRB-6/2523

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APPROVED: February 27, 2024.

Assent:

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Data: 02/08/2024 10:11:44-0300
Verifique em https://validar.iti.gov.br

Antônio Fernandes de Carvalho
Adviser

ACKNOWLEDGEMENTS

To God for always being my support in difficult times throughout my journey.

To my parents, Simoncele and Elizete, for all their love, dedication, encouragement and trust over all these years. To my brother, Tito, for always being by my side, even at long distances, advising, relaxing and supporting my dreams.

To Grandpa Miguel (in memoriam) and Grandma Léa (in memoriam), for always dedicating their lives to all of us grandchildren. Thank you, for all your availability and welcome, always making us believe that all our dreams are possible to be achieved. Without you I would be nothing, all my gratitude and love in the world for you. This journey has a huge piece of both of you.

To my godmother Ildete, for being my second mother and for understanding me so well that words are unnecessary and unable to express the immensity of my gratitude for having someone like her in my life.

To my godchildren, especially Mallu, who besides being goddaughter and cousin, is my person. Thank you for always being here for me.

To my colleagues at the INOVALEITE laboratory, for all the conversations, coffees, scientific exchanges, moments of joy and celebrations over these almost 10 years. In particular, Lu, Frida, Jaque and Rafa for their partnership in the development of these projects and the moments of letting off steam in the “coffee with tears”.

To my friends from the various volleyball teams (LUVE, AAAE, Amigas, Lespa, Espartanas) and from the “peladinhas” too, for being so present and an escape valve for moments of mental fatigue.

The city of Viçosa that welcomed me at the age of 18 and was my home for more than a decade and provided me with such happy and incredible experiences and moments that I cannot put into words.

To friends “from Viçosa” who were and are my support in moments of greatest fragility, fear, anxiety and vulnerability. In particular, Falqueto, Max, Dani, Lucas, Hugo, Lari, Pedro, Jean, Patty, Rodrigo and Ju, who, even with physical distancing, continue to be present daily and at every opportunity to meet, they make every effort to be with me and celebrate my achievements. I love you and I'm grateful for holding my hand in the most difficult moments in recent years, I dare say that without you it would be impossible to have reached this moment. You are the best, the family I chose for myself.

To my co-advisor, Sol, for all shared knowledge during this work.

To my advisor, Professor Antônio Fernandes, for the opportunity to work on this team and be his oriented, for the concern, friendship and shared wisdom.

To the Federal University of Viçosa, for the opportunity to complete the postgraduate course.

To Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, DF, Brazil) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, Belo Horizonte, MG, Brazil), for granting the scholarship.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

"*Voici mon secret. Il est très simple: on ne voit bien qu'avec le cœur. L'essentiel est invisible pour les yeux.*"

Antoine de Saint-Exupéry

ABSTRACT

MOREIRA, Isabella Maria Fernandes Botelho, D.Sc., Universidade Federal de Viçosa, February, 2024. **Ultra high temperature milk: microbiological quality, age gelation and a proposal of an alternative method for microbial detection.** Adviser: Antônio Fernandes de Carvalho. Co-adviser: Solimar Gonçalves Machado.

The UHT (Ultra High Temperature) milk is a food that has stood out in the market due to its ease of transport, storage conditions and long shelf life. Due to this, it is becoming increasingly important to study this product in order to guarantee the quality and food safety of the consumer. The objective of this study was to evaluate three different lines within the "UHT milk universe": the microbiological quality of the product, the action of stabilizing salts on the gelation process caused by bacterial proteases and an alternative and faster methodology for microbial detection. Microbiological quality was determined through the evaluation of 184 samples of Brazilian UHT milk for the presence of vegetative cells of aerobic mesophilic bacteria and spores. The 335 isolates obtained were purified, phenotypically characterized and subjected to molecular analyzes in order to determine the biodiversity between them. By constructing a phylogenetic tree, it was possible to identify bacteria belonging to the genus *Bacillus* sp. that in addition to being capable of forming spores, some strains in the group also secrete proteolytic and lipolytic enzymes that compromise the quality of the product throughout its shelf life. Based on this, the second project aimed to evaluate the action of phosphate and citrate salts on the gelation process in UHT milk samples intentionally inoculated with strains of *Pseudomonas fluorescens* 07A, a strain that produces the AprX protease, for a period of 18 h at 20 °C. In this project, five stabilizing salts (polyphosphate (T1), pyrophosphate (T2), citrate (T3), tripolyphosphate (T4) and monophosphate (T5)) were evaluated for 30 days in three repetitions in samples of UHT milk produced through reconstitution of skimmed milk powder. Through physical-chemical analyzes (pH, thermal stability in the oil bath, azocasein assay, SDS-page, protein content) and visual evaluation of the gel formed at the bottom of the sample bottles, it was possible to determine that the treatment added with tripolyphosphate (T4) was that showed greater efficiency in reducing the speed gelation of samples. Finally, the last project came from the demand for less laborious and faster methods for detecting microbial contamination in food. The BD BACTEC™ FX is currently used for the rapid detection of microbial growth in blood

samples. The project was divided into two stages in which, in the first, samples of UHT milk intentionally inoculated with strains of *Bacillus spizizenii* ATCC19659 were evaluated, and in the second, industrial samples of UHT milk were evaluated. Through the results obtained, it was verified that the equipment was very promising due to its ease of use, shorter contamination detection time and greater sensitivity when compared to conventional methodologies. The results obtained in all three projects were very satisfactory, but they are just the beginning of a range of possibilities for advances and discoveries that permeate the main object of the study, UHT milk.

Keywords: UHT milk; Quality; Age Gelation; Stabilizing Salts; Alternative Analysis Methods.

RESUMO

MOREIRA, Isabella Maria Fernandes Botelho, D.Sc., Universidade Federal de Viçosa, fevereiro de 2024. **Leite ultra-alta temperatura: qualidade microbiológica, gelificação e proposta de método alternativo para detecção microbiana.** Orientador: Antônio Fernandes de Carvalho. Coorientadora: Solimar Gonçalves Machado.

O leite UHT (Ultra High Temperature) é um alimento que vem ganhando cada mais mercado devido a sua facilidade de transporte, condições de armazenamento e longa vida de prateleira. Devido a isto, se torna cada vez mais importante o estudo deste produto com o intuito de garantir a qualidade e a segurança alimentar do consumidor. O objetivo deste estudo foi avaliar três diferentes linhas dentro do "universo leite UHT": a qualidade microbiológica do produto, a ação de sais estabilizantes sobre o processo de geleificação causado por proteases bacterianas e uma metodologia alternativa e mais rápida para detecção microbiana. A determinação da qualidade microbiológica se deu através da avaliação de 184 amostras de leite UHT brasileiro para a presença de células vegetativas de bactérias mesófilas aeróbias e esporos. Os 335 isolados obtidos foram purificados, caracterizados fenotipicamente e submetidos a análises moleculares a fim de se determinar a biodiversidades entre eles. Através da construção de uma árvore filogenética foi possível identificar bactérias pertencentes ao gênero *Bacillus* sp. que além de serem capazes de formar esporos, algumas estirpes do grupo, também secretam enzimas proteolíticas e lipolíticas que comprometem a qualidade do produto ao longo do *shelf life*. Baseado nisto, o segundo projeto teve por objetivo avaliar a ação de sais de fosfato e citrato sobre o processo de geleificação em amostras de leite UHT intencionalmente inoculados com cepas de *Pseudomonas fluorescens* 07A, cepa produtora da protease AprX, e incubadas por um período de 18 h à 20 °C. Neste projeto foram avaliados, durante 30 dias em três repetições, cinco sais estabilizantes (polifosfato (T1), pirofosfato (T2), citrato (T3), tripolifosfato (T4) e monofosfato (T5)) em amostras de leite UHT produzidos por meio da reconstituição de leite em pó desnatado. Através das análises físico-químicas (pH, estabilidade térmica no banho de óleo, ensaio de azocaseína, SDS-page, análise de proteína) e da avaliação visual do gel formado no fundo das garrafas das amostras, foi possível determinar que o tratamento adicionado de sais de tripolifosfato (T4) foi o que apresentou maior eficiência na diminuição da velocidade de geleificação das amostras. Por fim, o terceiro e último projeto partiu da demanda por métodos menos

laboriosos e mais rápidos para a detecção de contaminação microbiana em alimentos. O BD BACTEC™ FX é atualmente utilizado para a detecção rápida do crescimento microbiano em amostras de sangue. O projeto foi dividido em duas etapas nas quais, na primeira foram avaliadas amostras de leite UHT inoculadas intencionalmente com *Bacillus spizizenii* ATCC19659, e na segunda foram avaliadas amostras industriais de leite UHT. Através dos resultados obtidos, verificou-se que o equipamento se mostrou bem promissor devido a facilidade de uso, menor tempo de detecção da contaminação e maior sensibilidade quando comparado com as metodologias convencionais. Os resultados obtidos em todos três projetos foram muito satisfatórios, mas são apenas o começo de uma gama de possibilidades de avanços e descobertas que permeiam o objeto principal do estudo, o leite UHT.

Palavras-chave: Leite UHT; Qualidade; Geleificação; Sais Estabilizantes; Métodos de Análises Alternativos.

LIST OF ILLUSTRATIONS

Chapter 1: Phylogenetic characterization and biodiversity of spore-forming bacteria isolated from Brazilian UHT milk

Chapter 2: Enhancing UHT Milk Safety: A Novel Approach to Microbial Contamination Detection Using the BD BACTEC™ FX System

Chapter 3: Evaluation of the activity of phosphate salts on the gelation process in UHT milk samples added with *Pseudomonas fluorescens* producing AprX protease

LIST OF TABLES

Chapter 2: Enhancing UHT Milk Safety: A Novel Approach to Microbial Contamination Detection Using the BD BACTEC™ FX System

Chapter 3: Evaluation of the activity of phosphate salts on the gelation process in UHT milk samples added with *Pseudomonas fluorescens* producing AprX protease

SUMMARY

GENERAL INTRODUCTION

Microorganisms make up a very important class of contaminants in food, because besides being able to spoil food, some of them are also responsible for causing diseases in humans. According to the World Health Organization, bacteria and viruses are responsible for the majority of food-borne outbreaks worldwide (WHO, 2020).

Among the food raw material, milk stands out as a food rich in nutrients, which makes it an ideal environment for the development of many microorganisms, both spoilage and pathogenic, which includes heat-resistant spore-forming bacteria.

In the dairy industries, heat treatments, such as pasteurization and UHT (Ultra High Temperature), are responsible for eliminating some these contaminating microorganisms present in the raw material. The UHT eliminates all vegetative cells of microorganisms present in the milk, as well as a large number of spores, except for heat resistant spores (FRANCO et al., 2008; MUCIDAS, 2010; TRONCO, 2010; FEAM, 2011, MENEZES et al., 2014) and some thermoresistant enzymes.

The proteolytic enzymes secreted by the bacteria contaminating the milk act on the casein micelles, destabilizing them, favoring the gelation process throughout the shelf life of UHT milk. Therefore, in addition to the critical points inherent to processing (guaranteeing the application of the appropriate time/temperature combination, aseptic packaging conditions, use of sterile packaging), the potential presence of heat resistant spores, enzymes-producing bacteria and post-processing contamination are the main challenges for maintaining commercial sterility and quality of UHT milk products.

Based on the need to ensure food quality and safety, in order to minimize protein instability during heat treatment, stabilizing salts, mainly phosphate and citrate, are added to the milk as chelating agents (CHEN et al., 2012). Furthermore, the food industries have been looking for alternatives such as faster methods for the detection of potential contaminants (ZHAO et al., 2014; SARAVANAN et al., 2020).

This work was developed with the objective of determining the microbiological quality and the main contaminating groups of UHT milk samples evaluated; evaluate

the action of some stabilizing salts on the gelling phenomenon, as well as testing an alternative method for microbial detection.

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GOALS

General Objective

Determine the microbiological quality of Brazilian UHT milk samples; enable the use of a rapid alternative methodology using a blood culture system for microbial detection in UHT milk, in addiction the evaluation of the action of different phosphate salts in the age gelation process.

Specific Objectives

- To detect the presence of spores and spore-forming bacteria in Brazilian UHT milk samples, to characterize the presence of *B. sporothermodurans* strains through phenotypic and genotypic analyses and to determine the genetic biodiversity among the isolates through phylogenetic analysis;
- To carry out tests that scientifically justify the application of the blood culture system (BD BACTEC™ FX) for the analysis of UHT milk in quality control;
- To evaluate the effect of citrate and phosphate salts on the phenomenon of gelation of UHT milk samples from reconstituted powdered milk and purposely inoculated with AprX-producing *Pseudomonas fluorescens* strain.

Chapter 1: Phylogenetic characterization and biodiversity of spore-forming bacteria isolated from Brazilian UHT milk

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Article published in the Brazilian Journal of Microbiology
https://doi.org/10.1007/s42770-023-01063-6

Chapter presented according to the final format of the journal.

ABSTRACT

Among the milk contaminating microorganisms, those which are able to form heat-resistant spores are concerning, especially for dairy companies that use ultra-high temperature (UHT) technology. These spores, throughout storage, can germinate and produce hydrolytic enzymes that compromise the quality of the final product. This study evaluated 184 UHT milk samples from different batches collected from seven Brazilian dairy companies with a possible microbial contamination problem. The bacteria were isolated, phenotypically characterized, clustered by REP-PCR and identified through 16S rDNA sequencing. The presence of *Bacillus sporothermodurans* was verified using biochemical tests (Gram staining, catalase and oxidase test, 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-shapes, were submitted to molecular analyses in order to determine the microbial biodiversity existing among them. The isolates obtained were grouped into 16 clusters, four of which were composed of only one individual. A phylogenetic tree was constructed using the sequences obtained from the 16S rDNA sequencing and some reference strains of species close to those found using BLAST search in the NCBI nucleotide database. Through this tree, it was possible to verify the division of the isolates into two large groups, the *Bacillus subtilis* and the *Bacillus cereus* groups. Furthermore, most isolates are phylogenetically closely related, which makes it even more difficult to identify them at the species level. In conclusion, it was possible to assess, in general, the groups of sporulated contaminants in Brazilian UHT milk produced in the regions evaluated. In addition, it was also possible to determine the biodiversity of spore-forming bacteria found in UHT milk samples, thus opening up a range of possible research topics regarding the effects of the presence of these microorganisms on milk quality.

Keywords: UHT milk, spore-forming bacteria, *Bacillus* sp., milk quality, biodiversity.

1. Introduction

Considering the availability of formal fluid milk, in Brazil, about 6,977 billion liters (26.1%) were designated for the Ultra High Temperature (UHT) milk sector in 2020, which was just below fluid milk used for cheese (33.8%) and powdered milk (26.6%) production. UHT milk consumption in Brazil has increased significantly and currently accounts for about 87% of the country's fluid milk consumption [1]. This fact can be explained by the long shelf life of the product and its ease in storage, the practicality of the packaging and the fact that the cold chain is eliminated during the internal logistics and distribution of the product.

Ultrapasteurization or UHT treatment consists of a heat treatment in continuous flow (130 – 150 °C/2 – 4 s), followed by cooling at 32 °C and aseptic packing in sterile and hermetically sealed packages [2]. This legislation establishes that UHT milk must not contain microorganisms capable of multiplying under normal conditions of storage and distribution [2]. This heat treatment process is able to eliminate all vegetative microorganism cells present in milk, as well as a large number of spores; except for heat resistant spores, such as is in the case of *Geobacillus stearothermophilus, Bacillus subtilis, Bacillus megaterium, Bacillus coagulans, Bacillus licheniformis, B. cereus*, and *B. Sporothermodurans* spores - bacterium that produce one of the most heat resistant spores [3–6].

Thus, there is a great possibility that heat-resistant spores can germinate and, after germination, the bacteria can multiply during product storage and distribution; making it unfit for consumption and at odds with the standards of legislation [7]. In addition, after germination, the bacteria can multiply, achieve high counts, and produce hydrolytic enzymes that reduce product quality; mainly texture change and production of off flavors [3, 8, 9].

Based on this, the objective of this study was to detect the presence of spores and spore-forming bacteria in UHT milk samples from three Brazilian regions. Furthermore, to characterize the presence of *B. sporothermodurans* strains through phenotypic and genotypic analyses and, finally, determine the genetic biodiversity among the isolates through phylogenetic analysis.

2. Materials and methods

2.1. UHT milk samples

A total of 184 UHT milk samples from batches with possible microbial contamination and collected from seven Brazilian dairy companies (A, B, C, D, E, F and G) were analyzed over six months (June to November).

2.2. Microbiological analyses of UHT milk samples and determination of spore-forming bacteria presence

Immediately upon receipt, the UHT milk samples were incubated for seven days at 36 ± 1 °C. Subsequently, the samples were evaluated for the occurrence of changes in product characteristics (coagulation, flocculation, desorption, off-odor or other). When evident change occurred, no further analyzes was performed with the sample in question, and the result was reported as "altered product after incubation at 36 ± 1 °C for 7 days".

Samples that did not display modification after incubation were ten-fold diluted in tubes containing 9 mL of 0.1% (w/v) peptone saline solution until 10^{-2} dilution. The dilutions were then pour plated onto brain heart infusion (BHI; Kasvi, São José dos Pinhais, PR, Brazil) agar and nutrient agar free of yeast extract (Kasvi), in duplicate. The plates were incubated at 30 ± 1 °C for 72 hours. After incubation, the colonies that formed on the plates were counted and the results of aerobic mesophilic counts were expressed in $CFU \cdot mL^{-1}$ [10].

To determine the presence of spore-forming bacteria, one aliquot of approximately 10 mL of each UHT milk sample was collected in sterile test tubes. Each of the aliquots was subjected to heat treatment (80 °C/10 min) [11], to eliminate all vegetative forms of the microorganisms present, and to allow for the detection of spores.

After heat treatment, the aliquots were diluted (up to 10^{-2}) in 0.1% (w/v) peptone saline solution and pour plated, in duplicate, using BHI agar (Kasvi) and nutrient agar free of yeast extract (Kasvi); and then followed by incubation at 30 ± 1 °C for 72 hours. The colonies formed on the plates were counted, after incubation, and the results of spore-forming bacteria expressed in $CFU \cdot mL^{-1}$.

Ten percent of the colonies were randomly selected from each BHI agar plate, referring to the count of aerobic mesophiles and germinated spores, followed by purification through the streak plate method on Plate Count Agar (PCA; Kasvi). The pure cultures were stored at − 80 °C in BHI broth (Kasvi) supplemented with 30% (v/v) glycerol.

2.3. Phenotypic analyses of isolates

Each isolate from the UHT milk samples was submitted to biochemical tests, according to the methodology described by Normative Instruction nº 62 of 2003; with regards to phenotypic characterization to identify the presence of *B. sporothermodurans.* The following tests were performed: Gram staining, catalase and oxidase test, anaerobic growth, glucose fermentation, esculin hydrolysis, nitrate reduction and urease test.

After performing the phenotypic tests, the isolates that showed bacilli morphology through Gram staining were cultivated in BHI broth (Kasvi), and subsequently subjected to molecular analysis.

2.4. Molecular biodiversity analyses

2.4.1 DNA extraction

A pellet of activated isolate culture was obtained by centrifugation at 10.000 × *g* for 10 min. The DNA of each isolate was extracted and purified using the DNA Purification Wizard® Genomic kit (Promega Corp., Madison, WI, USA). The quality and the concentration of the extracted DNA were measured on a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the DNA concentration was standardized to 100 ng/µL.

2.4.2. Clustering of isolates by Rep-PCR

Rep-PCR analyses were performed according to the protocol described by Dal Bello et al. [12]; using a single universal primer $(GTG)_5$ (5'-GTGGTGGTGGTGGTG-3'). PCR reactions contained 12.5 µL of Go Taq Green Master Mix 2x (Promega), 1.0 µL of the primer (100 pMol), 100 ng DNA and ultra-pure water (Promega) for a final volume of 25 µL. PCR amplification was carried out in a thermal cycler, and the cycle

used was 95 °C for 5 min as initial step, 95 °C for 30 s, annealing at 40 °C for 30 s and 65 °C for 8 min 30 cycles, and a final extension step of 65 °C for 16 min [12].

The PCR products were visualized on 2% (w/v) agarose gel stained with GelRed (Biotium Inc., Hayward, CA, USA) and left for 2 h at a constant voltage of 75 V in 0.5 × TBE buffer, and developed using an LPIX transilluminator (Loccus Biotechnology, São Paulo, SP, Brazil). The banding profile was analyzed using BioNumerics software 6.6 (Applied Maths, Kortrijk, Belgium). 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 isolate of each group generated by Rep-PCR analyses was randomly selected and subjected to 16S rDNA gene amplification using 8f (5"-CACGGATCCAGACTTTGATYMTGGCTCAG-3") and 1512r (5"-GTGAAGCTTACGGYTAGCTTGTTACGACTT-3") primers [13], which gives 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 (0.1 µM), 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 °C for 5 min as initial step, 94 °C for 20 s, annealing at 54 °C for 20 s and 68 °C for 2 min for 35 cycles, and a final extension step of 72 °C for 7 min. The products obtained from the PCR reaction were sequenced by Macrogen Inc. (South Korea). The obtained sequences were then aligned with 16S rRNA gene sequences present in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) of the National Center for Biotechnology Information (NCBI) using BLAST software (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST).

2.4. Phylogenetic analyses

A phylogenetic tree was constructed from the partial 16S rRNA sequences with MEGA 11 X version 11.0.10 [14], using MUSCLE [15] to align the sequences. Reference 16S rRNA gene sequences were retrieved from NCBI's databases. The evolutionary history was inferred using the Maximum Likelihood method [16], with bootstrap analyses of 1,000 replicates [17], and the Hasegawa-Kishino-Yano model

[18] using a discrete Gamma distribution (+*G*) with 5 rate categories. The Interactive Tree Of Life (iTOL v.6.4) web-based tool was used to edit the phylogenetic tree (https://itol.embl.de/) [19].

3. Results

3.1. Microbiological quality of UHT milk samples

After incubation for seven days at 36 ± 1 °C, only one UHT milk sample from company B, presented gelation and was not considered for the following steps, due to it already being considered unsatisfactory, and of an unacceptable quality [20].

Regarding the microbiological quality, approximately 51% of the analyzed UHT milk samples presented viable aerobic mesophile counts above 100 CFU·mL^{-1} (Figure 1), which was the maximum limit allowed by current legislation at the time the samples were produced [2].

Most samples were found to be above the limit (100 CFU·mL^{-1}) established by legislation and are classified as non-standard, in relation to the aerobic mesophile and spore-forming bacteria counts after heat treatment. Only company C presented non-standard samples that did not have any spore counts after heat treatment.

Dairy company "F" presented the worst results and the smallest variability, which is shown by the size of the rectangle in the boxplot. One hundred percent of the samples from dairy F were outside the standards established by the legislation. Followed by, companies "D" and "A", with 97.5% and 94.4% of the samples, above the limits, respectively (Figure 1). Furthermore, 57.1% of 183 evaluated samples presented microbial counts after heat treatment (80 °C/10 min).

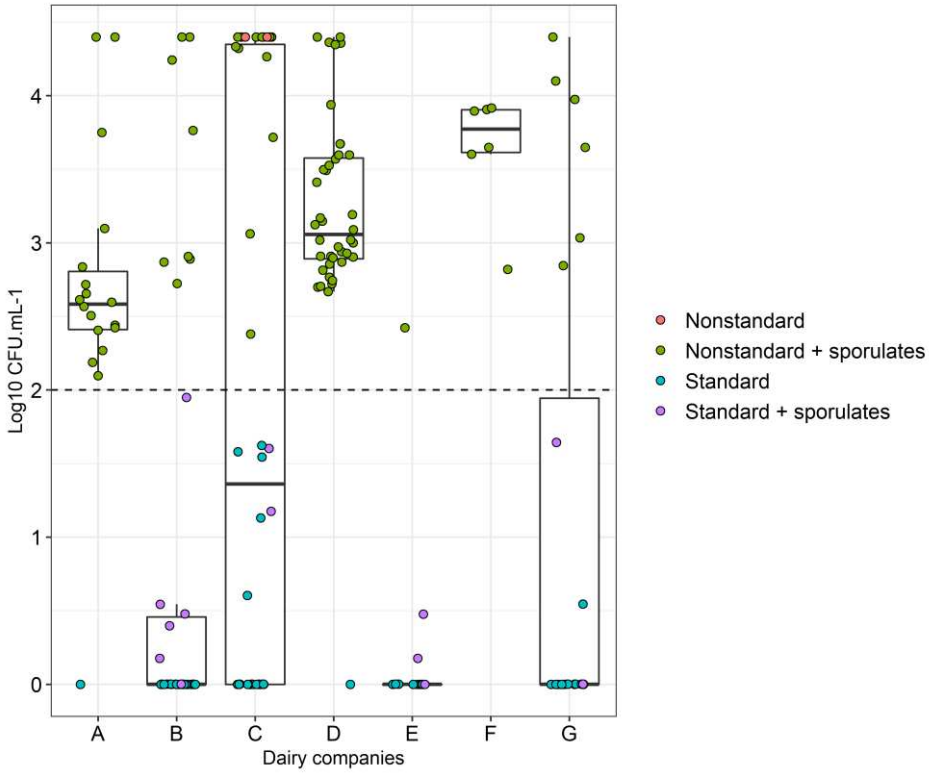

Figure 1. Boxplot containing an overview of the aerobic mesophilic and spore counts in the evaluated UHT milk samples, from each production company. The black dashed line indicates the limit of aerobic mesophiles allowed by legislation (100 CFU·mL^{-1}). **Nonstandard + sporulates:** UHT milk samples with total counts above the limit determined by legislation (100 CFU·mL^{-1}) and the presence of spore-forming bacteria; **Nonstandard:** non-standard samples that did not show counts of spore-forming bacteria; **Standard + sporulates:** samples with total count within the limits determined by legislation and the presence of spore-forming bacteria; and **Standard:** samples with total count within the standard determined by legislation without the presence of spore-forming bacteria.

After enumeration of microorganisms in the UHT milk samples, a total of 890 isolates were obtained, of which 231 (25.9%) were isolated from heat-treated samples; therefore, spore-forming bacteria.

3.2. Phenotypic analyses of isolates

According to the Normative Instruction nº 62 of 2003, colonies of *B. sporothermodurans* present gram-positive, rod shaped 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 [10]. Therefore, to be considered a strain of *B. sporothermodurans*, the isolates must exhibit all characteristics.

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 the methodology used, none of the 890 isolates were identified as *B. sporothermodurans*.

Even though a negative result was obtained regarding the presence of *B. sporothermodurans*, 335 isolates were presented bacilli morphology and were subjected to molecular analyses, to obtain more information regarding their taxonomy. This choice was made by the decision to keep the focus of the study on the possible representatives of the *Bacillus* genus; due to the ability of some species to form thermoresistant spores.

3.3. Molecular biodiversity analyses

Rep-PCR analyses revealed a highly varied band profile, indicating a high diversity among the isolates. Even though the similarity between some isolates was very high, there was no case where it was 100%. Considering a level of 90% similarity, the dendrogram generated 25 clusters. Of the 25 clusters formed, 11 were formed by single strain; 11 clusters with 2 to 9 isolates and 3 clusters with 12 to 17 isolates.

Regarding the geographic region, of the 25 groups obtained, 18 were composed only of isolates from the Southeast, 1 isolate from the Midwest, 5 isolates from the Southeast and Midwest regions, and 1 isolate from each of the three regions; and no group was formed, exclusively, by isolates from the Southern region (Figure 3).

Considering the clusters, there was a high diversity even within a single production line among isolates collected from a single company. Between the isolates of each company (from "A" to "G"), 9, 8, 6, 18, 1, 1 and 6 clusters were formed, respectively (Figure 2). Except for companies "E" and "F", which formed a cluster with only one isolate, the others would require a level between 50 and 70% of similarity for all the isolates to be grouped into a single cluster.

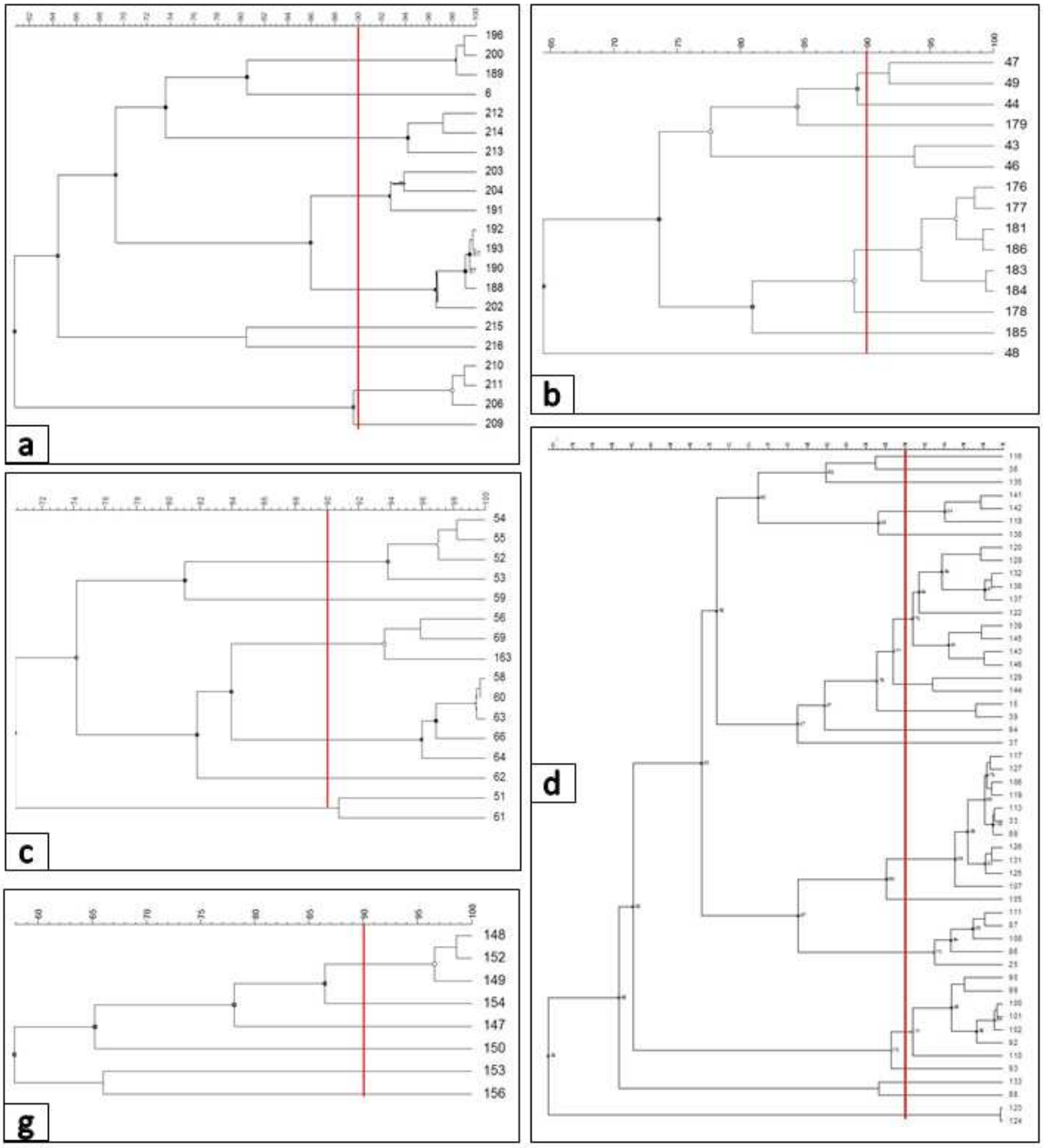

Figure 2. Dendrograms generated from REP-PCR print data of isolates from UHT milk samples of companies (a), (b), (c), (d) and (g). Similarities between profiles were calculated using Pearson's correlation. The dendrograms were constructed using the Unweighted Peer Group Methodwith Arithmetic (UPGMA). The highlighted red line indicates the 90% similarity limit considered for clustering.

Within each cluster of the 25 formed through Rep-PCR analyses, isolates were randomly selected for identification through 16S rDNA sequencing. Of the 25 strains sent for sequencing, 16 sequences were selected for construction of the phylogenetic tree due to the quality of the sequences obtained.

Considering a 95% similarity for the identification of the genus and 97% for the identification at the species level [21], the remaining 16 strains were identified as bacteria belonging to *Bacillus* genus. Although 16S rDNA sequencing was not possible to identify the strains to the species level. However, there were some species with which the sequences matched better. They were *B. cereus, B. thuringienses, B. proteolyticus, B. subtilis, B. licheniformis, B. amyloliquefaciens* and *B. velezensis*.

3.4. Phylogenetic analyses

In the present study, the taxonomic relationship of the obtained isolates was inferred through phylogenetic analysis. The phylogenetic tree (Figure 3) was constructed using the 16S rRNA gene sequences of the 16 selected strains, and 7 reference strains (belonging to the *Bacillus* groups that were close matches, according to BLAST), and *Pseudomonas putida* as an outgroup.

The numbers above and below tree branches correspond to the bootstrap support, which was performed with 1000 replications [17]. These numbers indicate the percentage of confidence of the formed clades. The higher the percentage, the more support given to the clades. In general, values above 70% are considered reliable [22].

When observing the phylogenetic tree, and considering the minimum value of 70% for the formation of clades, the sequences grouped into two main clades. The first is composed of strains from the *B. subtilis* species group (*B. subtilis, B. licheniformes, B. amyloliquefaciens* and *B. velezensis*) and the evaluated strains 212, 150, 94, 49, 190 and 117; and the second clade is composed of the *B. cereus* species group (*B. cereus, B. proteolyticus* and *B. thuringiensis*) and the evaluated strains 88, 59, 102, 52, 116, 215, 154, 36, 141 and 61. The clade formed by individuals of the *B. subtilis* group was subdivided into three further clades, the first being composed of just *B. licheniformis*, the second of *B. subtilis*, *B. velezensis* and *B. amyloliquefaciens*, and the last one formed by the 6 isolates evaluated in the study, mentioned above. While the *B. cereus* clade was not subdivided, forming a single clade composed of *B. proteolyticus*, *B. thuringiensis*, *B. cereus* and the isolates 88, 59, 102, 52, 116, 215, 154, 36, 141 and 61. The formation of this clade reaffirms the phylogenetic proximity between these species, which had already been evidenced through the sequence analyses obtained in the 16S rDNA sequencing.

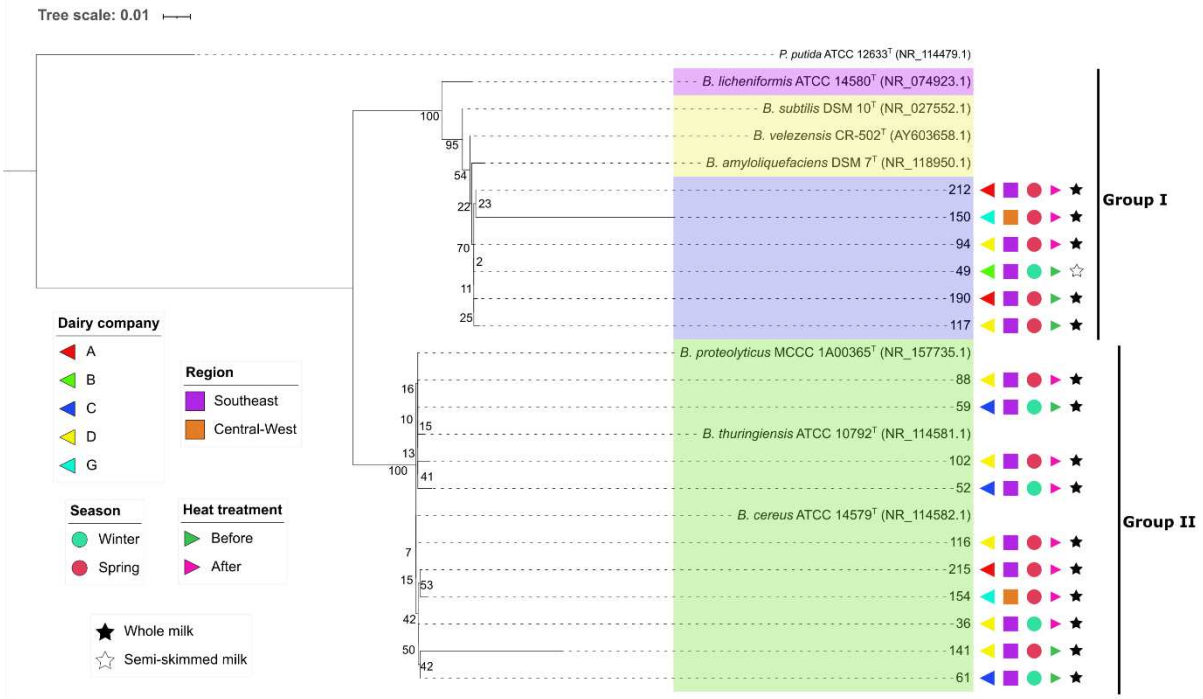

Figure 3. Phylogenetic analysis by Maximum Likelihood method: Evolutionary history was inferred using the Maximum Likelihood method and Hasegawa-Kishino-Yano model [18]. The tree with the highest log likelihood (-4572.85) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.2803)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA11 [14]. Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 1000 times to generate majority consensus tree, and the background colors identify the groups formed through these percentages (>70%). *Pseudomonas putida* 12633T was used as outgroup.

4. Discussion

Although there is no correlation between bacterial counts and spoilage potential, it is important to assess the quality of UHT milk by analyzing the contamination of the final product; as required by legislation. In this analysis, the average counts of contaminating mesophiles and spore-forming bacteria in the UHT milk samples ranged from 0 to 3.7 log CFU/mL, which represents a high variability of its microbiological contamination (Figure 1). This high variability is confirmed in other studies that evaluated the quality of UHT milk in Brazil. Reported percentages of samples with viable aerobic mesophiles counts outside the limit allowed by legislation varied between 24 - 41.2% [23–25]. Pinto et al. [26] also evaluated samples of Brazilian UHT milk for the presence of spore-forming bacteria, and among 20 milk brands

evaluated, 45% presented sporulated bacteria counts. In addition, 18.7% of the brands had sporulated counts greater than 100 CFU·mL^{-1}.

It is important to note that in 2018, MAPA (Ministério da Agricultura, Pecuária e Abastecimento) established a Manual of official methods for the analysis of food of animal origin (Normative Instruction N° 30/2018). In this manual, the microbiological quality of UHT milk is determined through the Commercial Sterility Test for Low Acid Foods - pH > 4.6 - and the results are expressed as "positive" or "negative" for the presence of microorganisms [27].

Furthermore to the microbiological quality of the raw milk, the presence of contaminating microorganisms in UHT milk may be associated with failures in the production line, especially during: packaging; insufficient heat treatment; and issues in piping sanitation procedures. All of which favors microbial adhesion and biofilm formation, as well as failures in packaging asepsis procedures [5, 6, 9, 28–30].

The presence of bacteria from the *Bacillus* genus, specifically, is more associated with raw milk with a high spore count and/or post-process contamination, due to the presence of biofilms in the pipes [31–33]. The presence of *Bacillus* spp. in UHT milk, confirmed by this study, reaffirms the importance of the use of quality raw material and the lowest level of contamination possible; since some spore-forming microorganisms, besides producing heat resistant enzymes and forming biofilms, are considered pathogenic and represent a great risk to the health of the consumer, as is the case with *B. cereus* [34].

Despite the high levels of contamination and the large number of isolates belonging to the *Bacillus genus*, it was not possible to detect *B. sporothermodurans* in the analyzed samples. In studies with Brazilian UHT milk samples similar results were found regarding the absence of this bacterium [26, 35]. However, some researchers did find this bacterium in Brazilian UHT milk samples. Zacarchenco et al. [7] evaluated 100 samples of UHT milk from 6 Brazilian states and identified 24 isolates of *B. sporothermodurans*. Busatta et al. [29] and Pereira et al. [36] 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. [37] evaluated 41 samples that were taken at different stages

during UHT milk manufacturing and the presence of *B. sporothermodurans* was identified only in raw milk samples. This difference between the results can be explained by the different techniques used to identify *B. sporothermodurans*. Among the studies carried out with Brazilian UHT milk samples, the presence of this species was confirmed based on the morphological characteristics and/or thermal resistance of the isolates, or on grouping isolates with a control strain belonging to *B. sporothermodurans* using RAPD typing technique. As presented throughout this article, species from the Bacillus genus are very close phylogenetically, which makes differentiation based only on the techniques used in these studies very difficult.

Through this study it was possible to compare molecular techniques and biochemical tests. Although they are simple and provide us with many interesting information about the metabolism and behavior of microorganisms, 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 microorganisms through these techniques becomes completely unfeasible.

As shown in the results obtained through molecular techniques and phylogenetic analysis, the isolates belonging to the *Bacillus* genus, despite not having been identified at the species level, are phylogenetically close to some species of the genus, which is observed through the phylogenetic tree presented in Figure 3.

The branches of the tree are associated with the transmission of genetic information from one generation to the next. In other words, the evolution of the descendants in relation to the node from which it is derived. The longer the branch, the greater the genetic change [38]. In general, except for isolates 141 and 150, the other individuals are evolutionarily very close to their ancestors, which indicates small genetic differences between them.

There was no pattern regarding the grouping of strains according to region, company, or season in which they were isolated. Furthermore, before and after heat treatment at 80 °C/10 min, the bacteria also subdivided between the main clades (Group 1 and Group 2). These results give us a general idea about the contaminants

in Brazilian UHT milk. There is a high possibility that this is a common group of bacteria that do not depend on the region and season in which they were isolated.

Among the species associated with the isolates, 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 [3, 31, 39]. *B. cereus* strains were also found in other studies with Brazilian UHT milk samples [40–43]. In addition to UHT milk, this species has already been isolated from several dairy products such as: raw milk, pasteurized milk, milk powder, ice cream and fermented milk [31, 40, 41, 44–47].

Liu et al. [48] and Lorenzo et al. [3], identified *B. proteolyticus* and *B. thuringiensis* as a new species of the *B. cereus* group, respectively. *B. proteolyticos* strains are capable of producing proteinases that hydrolyze starch and casein [48]. In turn, *B. thuringiensis*, like *B. cereus*, is pathogenic and has been isolated in farm environments, and in dairy products such as raw milk, cream, pasteurized milk and cheese [45, 49]. These species were also associated with some sequences of the isolates evaluated in this study. This fact draws attention because, associated with the toxigenic potential of *B. cereus*, such bacteria have a high potential for deterioration. The strains associated with these species were mainly isolated from UHT milk samples from dairy companies in the Southeast.

Some isolates from the Southeast and Central-West UHT milk samples showed great similarity with *B. subtilis*. This species is related with the deterioration of various foods, which mainly include dairy products such as raw, pasteurized and UHT milk [50]; as it is capable of producing a variety of enzymes (proteolytic, aminolytic, lipolytic and fibrolytic), a number of metabolites and volatile substances with antifungal activity, and bacteriocins [51–53]. Pinto et al. [26], in a study on the microbiological quality of Brazilian UHT milk, also reported the presence of *B. subtilis* corresponding to 68% of the 46 isolated strains.

B. licheniformis was associated with the strains isolated in the Southeast and it is known for its ability to cause deterioration in dairy products, due to the production

of proteolytic and lipolytic enzymes that compromise the sensorial and functional properties of these products. In addition, it has the ability to form biofilms in processing plants, and is considered by some researchers as the predominant *Bacillus* species in dairy industries [31, 54–56]. Together with *B. subtilis* and *B. cereus*, these are the most predominant mesophilic aerobic spore-forming bacteria in raw milk [57].

B. velezensis and *B. amyloliquefaciens* are another species of the *B. subtilis* group [58–60] associated with evaluated isolate sequences. In recent studies they demonstrated the ability of *B. velezensis* strains to form biofilms and produce proteolytic and lipolytic enzymes that, consequently, compromise the quality of products in dairy industries [61, 62]. McKillip et al. [63] identified isolates of *B. amyloliquefaciens* in organic UHT milk and proved that it is a biofilm producer in amounts exceeding those of the *B. cereus* ATCC 14579 strain used as control.

It was possible to notice that most species of the *Bacillus* genus were genetically associated with the isolates found in the evaluated UHT milk samples, and are producers of enzymes capable of hydrolyzing milk constituents and causing its destabilization. It is likely that one of these species was responsible for the gelation of the sample from company B. 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 the action of proteolytic enzymes produced by some bacteria. Kappa-casein is easily degraded by these proteases because it is located on the micelle surface. When it is hydrolyzed, a destabilization of the casein micelle can lead to milk coagulation [64]. The time required for gel formation over UHT milk storage is dependent on the extent of contamination of raw milk, however, each bacterium has its predilection to produce such enzymes, which leads us to conclude that the bacterial count is not always directly linked to the amount of enzyme produced [65].

Due to the typical characteristics of the *Bacillus* species, to which the isolates were phylogenetically close, it can be suggested that the problems associated with the batches to which the samples belonged to may be linked to the presence of biofilms in the processing line, as well as the use of raw milk with high microbiological and spore count for UHT milk production.

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. It is the only way possible to minimize the problems associated with microbiological contamination in UHT products and ensure the consumer has a high-quality product.

5. Conclusion

Through the results obtained, it can be concluded that spore-forming bacteria of the *Bacillus* genus comprise an important group of contaminants in UHT milk in Brazil. Although there was a possibility that the evaluated UHT milk samples belong to batches with problems, the presence of isolates phylogenetically close to species capable of causing food-borne diseases, such as *B. cereus*, gives us future perspectives to search for faster detection and identification methods for these contaminants. In addition, more effective conservation methods, compatible with the reality of the dairy industries, against this class of microorganisms must also be evaluated. At the same time, one should always seek to raise awareness among producers about the importance of raw material quality.

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Chapter 2: Enhancing UHT Milk Safety: A Novel Approach to Microbial Contamination Detection Using the BD BACTEC™ FX System

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Manuscript submitted and under analysis for publication in Journal of Food Protection

Chapter presented according to the final format of the journal.

ABSTRACT

The demand for effective detection methods to ensure the safety and quality of Ultra-High Temperature (UHT) milk remains crucial in the food industry. Traditional microbial detection techniques are time-consuming and labor-intensive, prompting the need for rapid and sensitive alternatives. This study evaluates the efficacy of the BD BACTEC™ FX system for microbial detection in UHT milk samples. The investigation comprises two stages: a controlled laboratory experiment using spiked UHT milk samples and an industrial-scale assessment of commercial UHT milk. Results indicate that the BD BACTEC™ FX system exhibits heightened sensitivity compared to conventional methods, detecting microbial contamination in a significantly shorter time frame (6 - 13 h). Taxonomic identification of contaminants reveals the presence of *Cellulomonas* spp. and *Enterococcus* spp. in the samples. The findings emphasize the critical importance of robust detection techniques in ensuring the safety and quality of UHT milk products.

1. Introduction

Ultra-high temperature (UHT) milk plays a pivotal role in the food industry due to its longer shelf life and convenience for consumers. UHT treatment involves heating milk to temperatures ranging from 135 to 150°C for 2 to 10 s (De Souza et al., 2023), effectively killing harmful bacteria and microorganisms while preserving its nutritional value. This process allows UHT milk to remain safe for consumption without refrigeration for several months, making it a popular choice for consumers worldwide. However, despite its advantages, ensuring the safety and quality of UHT milk remains a paramount concern.

Considering the imperative to uphold food quality and safety standards, the presence of microorganisms in UHT milk has emerged as a growing concern for the dairy industry. Consequently, there is a pressing need for innovative approaches to address this challenge within quality programs, including the development of alternative methods for the swift detection of potential contaminants (Zhao et al., 2014; Saravanan et al., 2020). Ensuring the efficacy of UHT treatment in eliminating microbial contamination capable of proliferating during shelf life is a mandatory requirement for all low-acid products (Council Directive, 1992; Codex Alimentarius, 2004; Brazil, 1997; Brazil, 2018).

Despite the efficacy of heat treatment, UHT milk may exhibit non-sterility attributed to two primary factors. Firstly, thermoduric spore-forming bacteria inherent in raw milk can withstand the treatment process (Boor & Murphy, 2005). Examples include *Bacillus cereus*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus megaterium* and *Geobacillus stearothermophilus* (Pinto et al., 2018; Alonso et al., 2021; Moreira et al., 2023). Secondly, recontamination of microorganisms into processed milk post-treatment leads to a diverse array of non-spore-forming microorganisms, with common offenders including coliform bacteria, *Staphylococcus* spp., and *Pseudomonas* spp. (Boor & Murphy, 2005, Chen et al., 2011).

The necessity to mitigate contamination risks throughout processing, packaging, and distribution stages emphasizes the critical need for robust quality control measures and efficient detection techniques to uphold public health standards

and instill consumer confidence in dairy products. Traditional microbial detection methods rely on culturing microorganisms on specific media, often necessitating over a week to yield conclusive results. This approach is not only time-consuming but also labor-intensive (Zhao et al., 2014; Wang et al., 2018; Foddai & Grant, 2020; Jayan et al., 2020). Consequently, industries face increased expenditures due to prolonged product storage and the associated maintenance costs for storage facilities.

Combining all these factors mentioned so far with the intention of offering a product of assured safety and extended shelf life, there is a growing demand for effective methods and rapid detection of microbial contamination (Foddai & Grant, 2020; Griesche & Baeumner, 2020; Saravanan et al., 2020). In recent years, scientists have been actively engaged in the development of methods designed to streamline the detection of microbial contamination in UHT milk. Noteworthy advancements in this endeavor encompass various categories of techniques.

Several studies have been performed using molecular techniques, such as PCR and its variants, to investigate contamination in UHT milk (Abouelnaga et al., 2016; Cattani et al., 2016; Roumani et al., 2021). However, these techniques have one common limitation as most of these approaches require the detection of specific target microorganism. Concurrently, cellular detection methods, which consider flow cytometry assay, were developed (Gunasekera et al., 2000; Liu et al., 2021). Nonetheless, cellular detection techniques require high-cost instrument and training of specialists. Additionally, bacteriophage-based approaches have been explored for their utility (Foddai & Grant, 2020; O'Sullivan et al., 2020), but different bacteriophages are required to infect various foodborne microorganisms.

Commercial systems based on colorimetric detection of pH changes, CO_2 production and O_2 consumption have been studied as attractive alternatives to existing conventional milk-quality detection methods (Diep et al., 2019; Poghossian et al., 2019). BD BACTEC™ FX system, developed by Becton, Dickinson and Company (BD, New Jersey, USA), is used for the rapid detection of the microbial growth in blood samples and relies on the detection of the fluorescence emitted by a sensor in the bottom of disposable test vials containing culture medium. Despite being widely used for blood cultures, this system had never been applied to detect microbial contamination in food. Therefore, the development of a cost-saving, fast, sensitive, and

in-situ detection technique for the early detection of microbial contamination in UHT milk is essential for addressing food safety challenges and upholding industry standards. Then, this work aimed scientific evidence for application of BD BACTEC™ FX for a rapid and sensitive microbial detection in UHT milk.

2. Material and Methods

2.1. Bacterial growth conditions and cell suspension

The preliminary stage involved the use of *Bacillus spizizenii* ATCC19659 strain (formerly *B. subtilis* ATCC19659) as the target microorganism in spiked UHT milk samples. The strain was retrieved from the stock culture stored at -80°C through aseptic inoculation in Brain Heart Infusion (BHI, BD Difco™, New Jersey, USA) broth (1% v/v), followed by overnight incubation at 37°C. Following overnight incubation, microbial cells were harvested via centrifugation (Heraeus Megafuge 8R, Thermo Fisher Scientific, Waltham, USA) at 3260 *g* for 15 minutes at 4°C and subsequently washed with a saline solution (0.85% w/v). The concentration of the microbial cell suspension in the saline solution was adjusted to 10^7 CFU/mL using a UV–Vis Spectrophotometer (UV-5100, Global Tarde Technology, China) at a wavelength of 600 nm (Optical Density at 600 nm = 0.100). The concentration of the cell suspension was confirmed by plating serial dilutions on BHI agar using the pour plate method. Colonies were counted after 24 h of incubation at 37°C. Prior to each experiment, the cell suspension with standardized concentration was diluted in saline solution to achieve the desired population.

2.2. Influence of milk stabilizing salts supplementation on microbial detection by BD BACTEC™ FX system

To determine whether the presence of stabilizing salts in UHT milk affects microorganism detection outcomes using the alternative methodology, two commercial brands of UHT milk were evaluated: one containing sodium citrate, sodium mono-, di- and triphosphate and another with no additives listed in the ingredients. UHT milk samples from both commercial brands were aseptically diluted in distilled water at varying concentrations of milk to water ratio (no dilution, 1:2, 1:5, 1:8). The diluted UHT milk samples were inoculated with 10^1 and 10^2 CFU/mL of *B. spizizenii* ATCC19659 cell suspension. Immediately after inoculation, bacterial counting of spiked milk

samples was performed using pour plate method and BHI agar. Following incubation at 37°C for 24 h, the concentrations of inoculated strain in UHT milk samples were expressed in CFU/mL. Concurrently, microbial detection in UHT milk samples (diluted and undiluted) was carried out using BD BACTEC™ FX system in aerobic condition (BD BACTEC™ Standard Aerobic Medium, BD, New Jersey, USA) following the manufacturer's instructions. Briefly, 8 mL of the milk sample was inoculated into the vial containing specific culture medium (BD BACTEC™ Standard Aerobic Medium) using a syringe. The vial inoculated with the samples were incubated in the BD BACTEC™ FX system at 35 ± 1°C for up to 5 days. This experiment was repeated at least twice.

2.3. Comparison between conventional methodology and BD BACTEC™ FX system for detecting *B. spizizenii* ATCC19659 in UHT milk

The experiment was conducted in original 1 L bottles of whole UHT milk. Ten bottles of commercial UHT milk from to the same batch were intentionally contaminated with 1 mL of *B. spizizenii* ATCC19659 cell suspension at concentrations ranging from 10^0 to 10^4 CFU/mL in duplicate. Two samples without the addition of microbial cell suspension were used as negative control.

An 8 mL volume of each UHT milk sample was inoculated into BD BACTEC™ FX disposable test vials (BD BACTEC™ Standard Aerobic Medium) and incubated in the BD BACTEC™ FX system at 35 ± 1°C for up to 5 days. At the same time, the concentration of *B. spizizenii* in UHT milk samples were assessed by conventional technique based on plating the samples on BHI agar using the pour plate method. After incubation at 37°C for 24 h, *B. spizizenii* counts were expressed as log CFU/mL. The time required for the BD BACTEC™ FX system to detect each sample as positive was recorded.

2.4. Microbiological analysis of commercial UHT milk samples

To evaluate the implementation of the BD BACTEC™ FX system for microbial detection in UHT milk samples at an industrial scale, 153 bottles of commercial whole UHT milk were assessed. Sampling was conducted every 15 days, resulting in a total of 6 sets of samples (Batch I – VI) (Figure 1). During each sampling event, samples

from the beginning, middle, and end of the production batch were collected to ensure the representativeness of the specimen.

During this stage, 153 whole UHT milk samples were evaluated, with 102 samples subjected to conventional methodology based on Normative Instruction N.30 (Brazil, 2018), while the remaining 51 samples were examined using the BD BACTEC™ FX system following the manufacturer's instructions under both aerobic and anaerobic conditions (BD BACTEC™ Standard Aerobic and BD BACTEC™ Standard Anaerobic media) (Figure 1). For conventional microbiological analysis, UHT milk samples were incubated at two different temperatures (36°C ± 1°C and 55°C ± 1°C) for 10 and 7 days, respectively, followed by visual inspection. Samples that tested positive by either approach (conventional and/or alternative method) underwent purification steps of contaminating microorganisms, followed by taxonomic identification through 16S rDNA sequencing and MALDI-TOF (Matrix-Assisted Laser Desorption Ionization – Time of Flight) analysis (Figure 1).

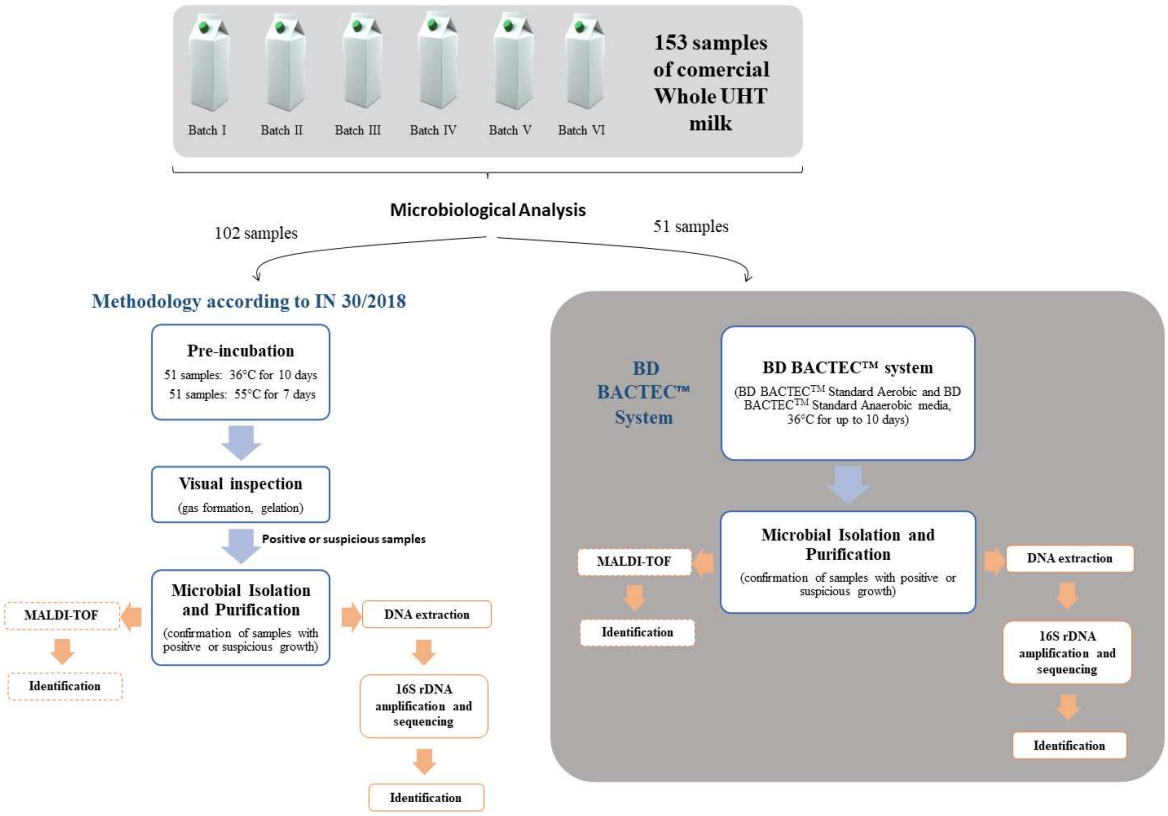

Figure 1. Flow chart of procedures employed in the microbiological analysis of UHT milk samples.

2.4.1. Taxonomic Identification

All test vials in which bacterial presence was detected during the incubation period in the BD BACTEC™ FX system, as well as all UHT milk samples that exhibited any visual alterations (gas production, gelation, sedimentation) during incubation using the conventional methodology, underwent microbial purification and taxonomic identification through 16S rDNA sequencing and MALDI-TOF analysis.

For microbial isolation, the contents of each positive BD BACTEC™ FX vial and the UHT milk samples exhibiting visual changes were spread onto Plate Count Agar (PCA) and incubated for 24 h at the temperature corresponding to the conducted test (36°C ± 1°C or 55°C ± 1°C). Colonies exhibiting growth were isolated and purified. In cases where positive samples analyzed through the conventional method did not yield microbial contaminants upon PCA plating, a 1 mL aliquot of the spoiled UHT milk was added to 9 mL of BHI broth followed by incubation under the initial assay conditions (36°C ± 1°C or 55°C ± 1°C). This step aimed to facilitate the recovery of these bacteria for subsequent identification analyses. Consequently, successful recovery, isolation, and purification of these microorganisms were achieved.

2.4.1.1. 16S rDNA sequencing

The isolates obtained from positive samples were cultured in BHI broth for 24 h at the temperature corresponding to the test conducted in the BD BACTEC™ system or conventional technique (36°C ± 1°C or 55°C ± 1°C). The DNA of the isolates was extracted and purified by Wizard® Genomic DNA Purification kit (Promega Corp., Madison, WI, USA) following the manufacturer's instructions. The quality and the concentration of the extracted DNA were measured in NanoDrop™ Lite Spectrophotometer (Thermo Scientific, Massachusetts, EUA) and the concentration was standardized to 100 ng/µL**.**

After the DNA extraction, the isolates were subjected to 16S rRNA 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), 10 µM of each primer (100 mol/L), 100 ng DNA and ultra-pure water (Promega) to a final volume

of 25 µL. PCR amplification were carried out in a thermal cycler and the cycle used were 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. The amplicons obtained after PCR were sent to ACT Gene (Alvorada, Rio Grande do Sul, Brazil) for purification and sequencing. The sequences obtained were treated at MEGA X and 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 Basic Local Alignment Search Tool software (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST).

2.4.1.2. Taxonomic identification by MALDI-TOF

For MALDI-TOF analysis, approximately 5 isolated colonies of each bacterium were selected and resuspended in 300 microliters of sterilized distilled water, followed by the addition of 900 microliters of ethanol. Subsequently, the previously prepared samples were sent to the Biological Institute of São Paulo for mass spectrometry analysis (MALDI Biotyper® for Microbial Research, Bruker, USA).

2.5. Statistical Analysis

All statistical analyses were performed using R software, version 4.3.0 (R Core Team, 2023), in conjunction with Rstudio version 2023.03.0 Build 386 (RStudio Team, 2020). The packages utilized included 'ExpDes.pt', 'stats', and 'ggplot2' for data visualization (R Core Team, 2023; Ferreira et al., 2013; Wickham, 2016).

Detection times for the BD BACTEC™ FX System applied to the microbiological analysis of spiked UHT milk samples were analyzed through an experiment conducted in a completely randomized design with two replications. This experiment employed a 2 × 4 × 2 factorial arrangement, comprising two types of UHT milk (with and without stabilizing salts), four dilutions (0, 1:2, 1:5, 1:8), and two inocula (10^1 and 10^2 CFU/mL of *B. spizizenii* ATCC19659). The results were evaluated through analysis of variance (ANOVA) and Tukey's test at a significance level of 0.05. Furthermore, correlation analysis using Pearson's correlation coefficient was conducted to examine the relationship between the log CFU/mL counts of *B. spizizenii* ATCC19659 and the detection time.

3. Results and Discussion

3.1. BD BACTEC™ FX system for detecting *B. spizizenii* ATCC19659 in UHT milk

The initial stage of this investigation simulated microbial contamination to juxtapose conventional and alternative methodologies for its detection in commercially sterile milk. For this purpose, *B. spizizenii* ATCC19659 was used as contaminant in UHT milk samples given that *Bacillus* spp. are spore-forming bacteria commonly related to microbiological contamination of this dairy product (Pinto et al., 2018; Alonso et al., 2021; Moreira et al., 2023). As the alternative method proposed by this study is based in microbial metabolism detection, the initial inquiry concentrated on examining the potential impact of product additives, such as stabilizing salts, on detection time of *B. spizizenii* ATCC19659 intentionally added to UHT milk samples.

A statistically significant difference was observed between UHT milk samples with and without stabilizing salts only when there was no dilution, and the samples were inoculated with 10^1 CFU/mL of *B. spizizenii* ($p < 0.05$). In this context, UHT milk supplemented with stabilizing salt demonstrated the shortest detection time (Figure 2). For both types of UHT milk, samples with the highest inoculum, 10^2 CFU/mL, showed shorter detection times in all dilutions ($p < 0.05$). The addition of stabilizing salts (citrates and phosphates) to UHT milk is not mandatory, but it is done with the aim of increasing the thermal stability of the protein during the UHT heat treatment, minimizing sedimentation and, consequently, improving UHT plant working time without cleaning stops and maintaining the stability of the milk throughout the shelf life (Chen et al., 2012). As many companies use this strategy to increase the shelf life of UHT milk, the possibility of using the alternative methodology in a wider range of products is an important point to be highlighted. Thus, it was verified that the detection times recorded for the detection of *B. spizizenii* in UHT milk samples with and without stabilizing salts during this study were significantly different (Figure 2).

When analyzing the dilutions of UHT milk samples supplemented with stabilizing salts, it was observed that, for both 10^1 CFU/mL and 10^2 CFU/mL inocula, there was no significant difference in detection time between the dilutions ($p > 0.05$). However, considering UHT milk samples without stabilizing salts, when these samples were inoculated with 10^1 CFU/mL, the sample without dilution showed a longer detection

time, not significantly different from the 1:2 dilution. The 1:5 and 1:8 dilutions showed shorter detection times, with 1:5 not differing significantly from 1:2 ($p > 0.05$). Regarding the 10^2 CFU/mL inoculum, the 0, 1:2 and 1:5 dilutions demonstrated longer detection times, while the 1:8 dilution showed a shorter time, not statistically different from the 1:5 dilution ($p > 0.05$).

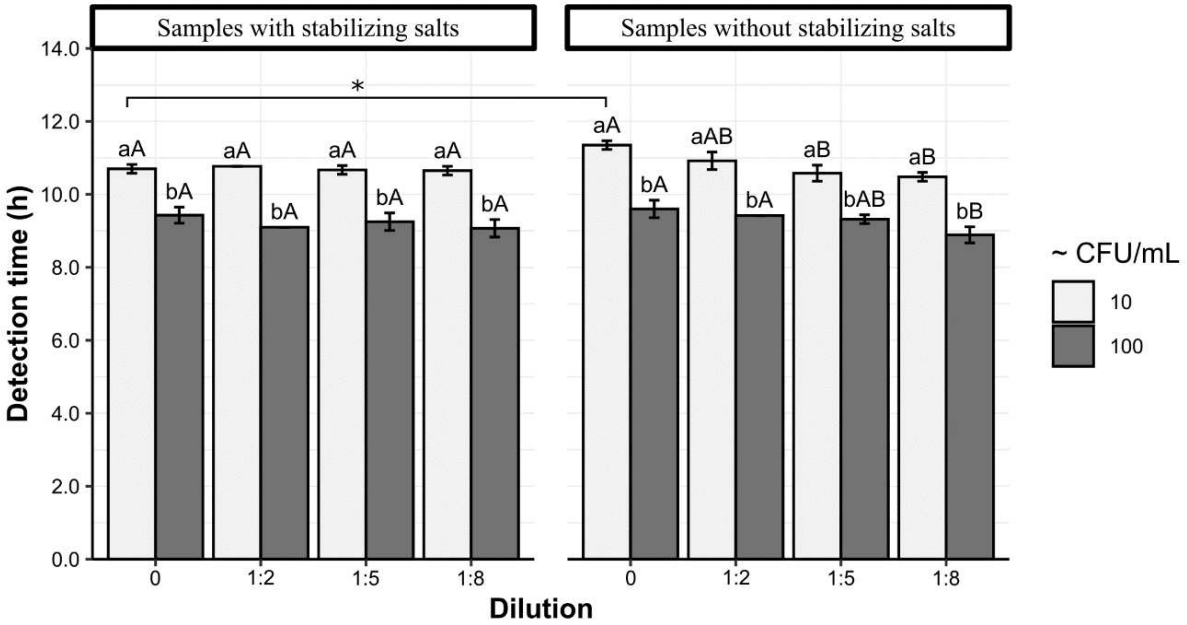

Figure 2. Detection time of *B. spizizenii* ATCC19659 contamination by the BD BACTEC™ FX System. Bars with different letters represent significant differences within each type of UHT milk samples. Different lowercase letters represent statistical difference ($p < 0.05$) between inocula (10^1 and 10^2 CFU/mL), within each dilution. Different capital letters represent statistical difference ($p < 0.05$) between dilutions, within each inoculum. The asterisk represents the significant difference between samples of UHT milk types (* $p < 0.05$).

The longer detection time observed for samples with higher milk concentration and without the addition of stabilizing salts may be attributed to the stabilizing role of additives in milk proteins, preventing sedimentation and age gelation. Chelating salts enable the control of ionic calcium concentration and pH in milk. The reduction in the level of ionic calcium in milk by stabilizing salts is associated with an increase in its heat coagulation time and a decrease in sedimentation occurrence post-UHT treatment due to the diminished calcium-mediated aggregation of k-casein-depleted casein micelles (Souza et al., 2023). The presence of aggregates may interfere with the detection of fluorescence emitted by the components of the gel deposited at the bottom of the BD BACTEC™ FX test vials, which react with CO_2 resulting from the microbial metabolism. While the results indicated a statistical difference in the

detection times of contaminated UHT milk samples based on their composition, all detection times remained under 12 h.

In the further analysis, the detection time of alternative method for detection of microbiological contamination of UHT milk samples inoculated with five different levels (10^0 to 10^4 CFU/mL) of *B. spizizenii* were recorded. The lowest *B. spizizenii* count (10^0 CFU/mL) inoculated into UHT milk was detected by the alternative method after 13 h of incubation (Figure 3). The detection time of spore-forming bacteria in dairy products using other alternative solutions, such as Greenlight (Mocon, Minneapolis, USA), Bact/Alert (bioMérieux, Marcy l'Etoile, France), and Soleris (Neogen, Lansing, USA), is longer (Diep et al., 2019), making the BD BACTEC™ FX system a promising tool for batch release of UHT milk.

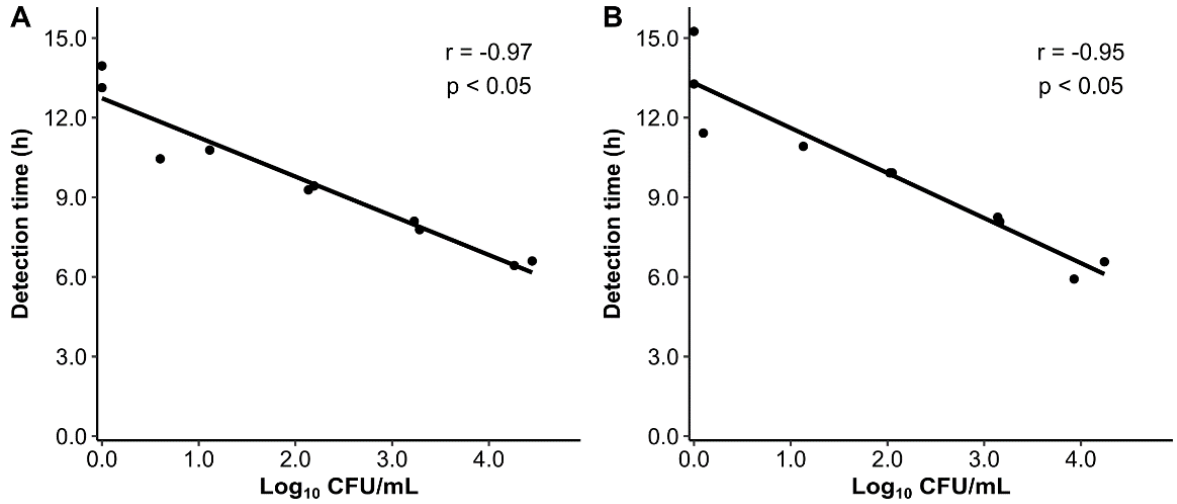

Figure 3. Scatter-plot showing the relationship between detection time and *B. spizizenii* counts present in UHT milk samples using the BD BACTEC™ FX System. (A) milk with stabilizing salts, and (B) milk without stabilizing salts. Pearson's correlation coefficient (r) was calculated, indicating a significant correlation ($p < 0.05$).

Correlation analysis (Figure 3) revealed a robust inverse relationship between detection times and the log CFU/mL of *B. spizizenii* in UHT milk, exhibiting coefficients of -0.97 (milk with stabilizing salts, Figure 3A) and -0.95 (milk without stabilizing salts, Figure 3B). Hence, the reduction in *B. spizizenii* concentration strongly corresponds to the increase in detection time required by the BD BACTEC™ FX System for sample analysis. The findings regarding microbial contamination detection in spiked UHT milk samples underscore the BD BACTEC™ FX System's potential for serving as a rapid alternative method. To substantiate this potential, the alternative method was employed to evaluate commercial samples on an industrial scale.

3.2. UHT milk sterility assessed by BD BACTECTM FX System

In this study, we examined the efficacy of the BD BACTEC™ FX system for microbial detection in commercial UHT milk samples at an industrial scale using aerobic and anaerobic conditions (BD BACTEC™ Standard Aerobic and BD BACTEC™ Standard Anaerobic media). A total of 153 samples of commercial whole UHT milk were subjected to assessment. Contaminants were detected in only two out of the six evaluated batches (I – VI), either through the conventional methodology, the alternative methodology, or both (Table 1). Through data analysis, it is possible to note that BD BACTEC™ system was more sensitive in the detection of contaminants, since all samples were evaluated by both methodologies; however, their presence was mostly detected by the alternative methodology. The detection of microbiological contamination through the conventional methodology relied on the observation of physical changes in the UHT milk samples (such as coagulation) following the incubation period (7 or 10 days). Despite the observable alteration in the physical characteristics of the samples, the plating methodology failed to ascertain the population present in each sample. Following a 24-hour incubation period, no colonies formed on the plates. Consequently, aliquots were sequentially inoculated into BHI broth in an effort to retrieve these bacteria for molecular identification analyses. Subsequent to this procedure, it became feasible to recover, isolate, and purify these cultures.

Samples from two evaluated batches were contaminated (Batch IV and Batch V). The contaminated samples named from A to F belong to batch IV, and the samples named G to K belong to batch V. A and C samples were produced at the beginning of the production batch IV and B and D at the middle (Table 1). The microbial contamination was detected in these samples (from A to D), belonging to the same batch (IV), using both conventional and alternative methodologies. Although consistent with the conventional methodology, the BD BACTEC™ system detected the presence of microbial contamination in a much shorter time (nearly 13 h) (Table 1). While the standard methodology requires at least 7 days for the sample to be incubated before the analysis, in some cases, with about 7 h of incubation in the BD BACTEC™ system, it was already possible to detect microbiological contamination. From an operational perspective and taking into account the time required by each of the assessed

methods, along with the demands originating from the food sector, the proposed alternative methodology emerged as a promising approach.

Table 1. Detection of microbiological contamination in commercial UHT milk samples using BD BACTECTM FX system and conventional methodology.

Samples	Batch	Production period	Methodology	Detection time	CFU/mL
A	IV	Beginning	BD BACTEC™ Standard Aerobic	12.63 h	3.5×10^9
B		Middle	BD BACTEC™ Standard Aerobic	12.28 h	2.2×10^9
C		Beginning	Conventional Methodology (36°C)	10 days	ND
D		Middle	Conventional Methodology (36°C)	10 days	ND
E		End	Conventional Methodology (36°C)	10 days	ND
F		End	Conventional Methodology (55°C)	7 days	ND
G	V	Beginning	BD BACTEC™ Standard Aerobic	06.65 h	1.4×10^5
H		Beginning	BD BACTEC™ Standard Aerobic	06.28 h	1.4×10^5
I		Middle	BD BACTEC™ Standard Aerobic	06.93 h	3.6×10^6
J		End	BD BACTEC™ Standard Aerobic	07.23 h	1.0×10^6
K		End	BD BACTEC™ Standard Aerobic	07.38 h	8.0×10^6

* ND - Microbial growth was not detected by the plate counting technique.

3.2.1 Taxonomic identification of microbial contaminants detected in commercial UHT milk samples

The commercial UHT milk samples that exhibited microbiological contamination detected by both methods were evaluated to isolate the contaminant for subsequent taxonomic identification through 16S rRNA gene sequencing and mass spectrometry. Out of the 11 samples subjected to sequencing, only one (sample F) could not be identified even at the genus level because DNA extraction was not successful using the approach employed in this study. The remaining isolates were identified at the genus level by 16S rRNA gene sequencing and mass spectrometry. Samples A to E, belonging to the batch VI, were classified as members of the genus *Cellulomonas*, while samples G to K, belonging to the batch V, were assigned to the genus *Enterococcus* (Table 2).

The genus *Cellulomonas* comprises mesophilic, Gram-positive, aerobic, or facultative anaerobic bacteria (Stackebrandt & Schumann, 2015). Found naturally in various environments such as animal rumens, activated sludge, and cellulose-rich locations (Ndongo et al., 2018), these bacteria are renowned for their cellulose-degrading capabilities facilitated by a series of specific enzymes they secrete. Strains

from this genus have previously been isolated from meat, olives, starchy foods, and raw milk (Rajoka & Malik, 1999). The latter emerges as a likely primary source of the contamination issue observed in the samples under investigation in this study. No documented instances exist in the literature of *Cellulomonas* species surviving UHT treatment, thereby casting suspicion on contamination post-heat treatment.

Table 2. Percentage of the identity, according to BLAST software - NCBI, of the strains obtained by the 16S rDNA sequencing.

Samples	Taxonomic identification	% similarity
A	*Cellulomonas* sp.	95.63
B	*Cellulomonas* sp.	100.00
C	*Cellulomonas* sp.	100.00
D	*Cellulomonas* sp.	100.00
E	*Cellulomonas* sp.	100.00
F	ND	ND
G	*Enterococcu*s sp.	100.00
H	*Enterococcu*s sp.	100.00
I	*Enterococcu*s sp.	100.00
J	*Enterococcu*s sp.	99.86
K	*Enterococcu*s sp.	100.00

ND: not determined.

Enterococcus spp. are mesophilic, gram-positive, facultative anaerobic bacteria, naturally inhabiting diverse environments including water, soil, vegetation, and various foods, particularly those of animal origin, as well as the gastrointestinal tract of humans and animals (De Fernando, 2011). In addition to its wide environmental presence, its ability to endure adverse cultivation conditions such as salinity, pH, and temperature variations makes this bacterium a common food contaminant, posing a significant food safety concern due to its association with infections and foodborne illnesses (Giraffa, 2014). Similar to *Cellulomonas* sp. strains, contamination of samples by *Enterococcus* sp. likely occurred subsequent to UHT heat treatment and the initial source of this contamination could range from raw materials.

4. Conclusion

The study underscores the efficacy of the BD BACTEC™ FX system as a rapid and sensitive method for detecting microbial contamination in UHT milk. By detecting contaminants such as *B. spizizenii*, *Cellulomonas* spp. and *Enterococcus* spp. in spiked and in commercial samples, this research provides insights that the proposed alternative method has great potential to be applied in the food industry to certify the

commercial sterility of UHT milk. Future studies should be conducted to assess the use of this method for other commercially sterile foods, as well as validation according to standards required by internationally recognized regulatory agencies.

5. Funding Sources

The authors received research support from Becton, Dickinson and Company that provided supplies and BD BACTECTM FX system for carry out this study.

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Chapter 3: Evaluation of the activity of phosphate and citrate salts on the gelation process in UHT milk samples added with *Pseudomonas fluorescens* producing AprX protease

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1. Introduction

UHT (Ultra High Temperature) milk is the product obtained through a heat treatment of 130/150 °C for a period of 2 to 4 seconds, followed by refrigeration at a temperature below 32 °C and filling under aseptic conditions in sterile and hermetically closed packages (BRASIL, 1997).

From the storage of raw milk under refrigeration for a long time, the presence of psychrotrophic bacteria became a problem for the dairy chain. These microorganisms are able to multiply under refrigeration temperatures (VIDAL & NETTO, 2018). Bacteria of the genus *Pseudomonas* are important examples of psychrotrophs in the dairy chain.

A characteristic of most psychrotrophs is the production of proteolytic and lipolytic enzymes. Such enzymes are produced mainly during the log phase and the beginning of the stationary phase (OLIVEIRA, et al., 2015). Within the genus *Pseudomonas*, the species *Pseudomonas fluorescens* stands out for the production of the enzyme AprX, a protease responsible for destabilizing milk casein micelles, thus causing the phenomenon of UHT milk gelation (LONGHI et al., 2020).

The AprX is characterized by its ability to partially resist UHT heat treatment, thus being classified as a heat resistant enzyme (MATÉOS et al., 2015). This protease easily hydrolyzes casein micelles into peptides and later into amino acids, reducing the shelf life of the final product. Phosphate and citrate salts are widely known for their stabilizing role in UHT milk. The addition of these salts is made in order to minimize protein instability during UHT treatment and avoid milk gelation during its shelf life (CHEN et al., 2012).

Considering the importance of phosphate salts for the commercialization of UHT milk, this work aims to evaluate the effect of these salts on the phenomenon of gelation of UHT milk samples from reconstituted powdered milk and purposely inoculated with AprX-producing *P. fluorescens* strain.

2. Materials and methods

The study was carried out in partnership with the company Israel Chemicals Ltd (ICL), in three repetitions (R1, R2 and R3) and in each of them the samples were evaluated during 30 days.

2.1. Preparation of reconstituted milk powder 10% (w/v)

The reconstituted skimmed milk powder (RSMP) was prepared in a concentration of 10% (w/v), using previously autoclaved water. Ten liters of RSMP, divided into 2 Schott flasks of 5 L, were used for each of the seven treatments.

2.2. *Pseudomonas fluorescens* 07A inoculum preparation

P. fluorescens 07A strain was isolated from a milk sample (PINTO et al., 2006) and is part of the culture bank of the Laboratório de Pesquisa em Leite e Derivados (INOVALEITE/UFV). This bacterium was activated in BHI (Brain Heart Infusion) broth and incubated at 25 °C for 24 hours. After this time, an aliquot of 1% (v/v) was transferred to 100 mL of autoclaved reconstituted milk powder, which was incubated at 25 °C for 24 hours.

2.3. Heat treatment (90 °C / 60 s)

The heat treatment was carried out in the UHT processing system (ARMFIELD LTD RINGWOOD; U.K.) at the ICL headquarters, located in the city of São José dos Campos/SP. This thermal treatment aimed to inactivate plasmin, an enzyme naturally present in milk, so that there were no interferences in the study. After the treatment, the milk was placed in sterile Schott flasks of 5 L.

2.4. Inoculation of *P. fluorescens* 07A

For the purpose of slightly delay the gelation process observed in the preliminary tests, it was decided to dilute the inoculated sample with *P. fluorescens* 07A in a proportion of 1:2 after incubation. Therefore, for each treatment, only one of the 5 L flasks was inoculated with this bacterium. In order to obtain an initial population of 10^5 CFU/mL, 5 mL of the inoculum (population of 10^8 CFU/mL - confirmed by plating the inoculum) was added in only one of the Schott of each treatment. The flasks were incubated at 20 °C for 18 hours in a climatized chamber. Immediately after inoculation

and 18 hours later, surface plating was performed to verify the initial and final population of *P. fluorescens* in the samples.

2.5. Addition of stabilizers

After incubation time, the two 5 L Schott flasks, corresponding to each of the treatments, were mixed and citrate and phosphate salts were added according to the dosages determined by the company, as shown below:

T1: Polyphosphate (0.08%) + inoculum
T2: Pyrophosphate (0.04%) + inoculum
T3: Citrate (0.1%) + inoculum
T4: Tripolyphosphate (0.1%) + inoculum
T5: Monophophate (R1: 0.032%; R2: 0.037%; R3: 0.060%) + inoculum
T6: Control: no phosphate + inoculum
T7: Control: no phosphate, no inoculum

The addition was made gradually, under agitation and with the aid of a pHmeter, the pH of the mixture was monitored.

2.6. Ultra High Temperature (UHT) treatment

At the ICL headquarters, the UHT treatment was performed in the UHT processing system and the binomial of 135.3 °C for 4.1 seconds was used. At the end of the line, inside a laminar flow hood, samples of each treatment were collected in 250 mL aseptic bottles.

2.7. Shelf life

The bottles of each of the treatments were placed in BOD at 25 °C, at Inovaleite/UFV, to monitor the shelf life and evaluate the gelation process. The samples were evaluated for 30 days and the following analyzes were performed:

a. Total aerobic mesophilic count

The methodology used was spread plate as described by the American Public Health Association (APHA, 2001). The analysis was performed in duplicate and the plates were incubated at 32 °C for 24 hours. Only counts ranging from 30 to 300 colonies were considered (CLARK, 1965; SCHORTEMEYER et al., 1996).

b. Spores Count

For spore counting, the pour plate methodology was used as described by APHA (APHA, 2001). A heat treatment of 80 °C for 10 minutes was applied to the samples, in order to inactivate the vegetative cells present and induce the germination of possible spores present in the sample. The analysis was performed in duplicate and the plates were incubated at 32 °C for 24 hours. Only plates that presented counts between 30 and 300 colonies were also considered (CLARK, 1965; SCHORTEMEYER et al., 1996).

Both results of the total aerobic mesophilic count and the spore count were expressed in colony forming units per milliliter (CFU/mL) and Equation 1 was used to calculate the result.

$$CFU = \frac{n \,.\, d^1}{v} \qquad (1)$$

Where:

n = Number of colonies counted on the plate;

d = Inverse of plated dilution;

v = Sample volume plated (mL).

c. pH analysis

The analysis of the hydrogen ion potential of the samples was performed in triplicate using a Kasvi pHmeter.

d. Thermal stability in the oil bath

The thermal stability of the samples was performed in an oil bath. 5 mL of sample was added to a test tube and this was immersed in oil at a temperature of 140 °C. When the tube was immersed, the time until the appearance of lumps (indicating the destabilization of the milk) was timed.

e. Proteolytic Activity – Azocasein Assay

Proteolytic activity was determined following the methodology of Charney & Tomarelli (1947) with modifications. Azocasein solution (SIGMA-ALDRICH, USA) 3% (w/v) was used as substrate and 20% (v/v) trichloroacetic acid as precipitating agent.

Neutralization was performed with 1 N sodium hydroxide solution and the absorbance reading was performed in triplicate in a spectrophotometer at a wavelength of 440 ηm.

f. Evaluation of casein hydrolysis by SDS-page

The samples were submitted to evaluation by SDS-page in 12% acrylamide gel following the methodology of LAEMMLI (1970) with modifications. The 1 mm thick gels were produced using milli-Q water, 30% acrylamide, 1.5 M Tris HCl buffer pH=8.8, 25% SDS, TEMED and 10% ammonium persulfate. The samples were acidified with 3 M HCl, centrifuged at 3260 x g for 10 minutes at 7 °C and resuspended with 0.5 M Tris HCl buffer, pH=9.0. After acidification the samples were diluted 20 times before being applied to the running gel. The volumes of 5 µl protein standard, 3 µl marker and 8 µl of samples were added to each well of the gel. The run was performed at 110 V/90 minutes. The gel was stained with a coomassie blue dye solution.

g. Protein Content

The protein content was determined using the Kjeldahl Method, in which the sample is oxidized by sulfuric acid, with the presence of catalysts, in the hot digestion. The digestion product goes to a distillation with 40% NaOH (m/v), where the formation of NH_3 occurs. The ammonia formed is captured by boric acid and the solution with the complex formed goes to titration with 0.1 M HCl. The titration ends with the appearance of a light pink color (PEREIRA et al., 2001).

Using the technique, it is possible to determine the content of total nitrogen (TN), protein nitrogen (PN) and non-protein nitrogen (NPN). The contents are expressed by the equations below:

$$\%\,TN = \frac{(A-B)\,.\,C\,.\,fc\,.\,1{,}4}{V} \qquad (2)$$

$$\%NPN = \frac{(A-B)\,.\,C\,.\,fc\,.\,1{,}4}{V} \qquad (3)$$

$$\%PN = (\%\,NT) - (\%NPN) \qquad (4)$$

Where:

A = volume of HCl spent on sample titration (mL);

B = volume of HCl spent in the blank titration (mL);
C = concentration of HCl used in the solution (mol/L);
fc = correction factor of the HCl solution;
V = sample volume, considering the dilution performed.

3. Results and Discussion

In the first repetition, the samples were evaluated at times 0 h, 15 and 30 days. However, it was noticed that even with the dilution of the samples inoculated with *P. fluorescens* 07A in the proportion of 1:2, the gelation process was more intense in some treatments, in view of this, in repetitions 2 and 3 a time of analysis with 7 days of shelf life was added. The results presented were obtained through the mean of the three repetitions of the experiment.

The presence of aerobic mesophilic and spore forming was not detected in the UHT milk samples evaluated throughout the study. This result was expected considering that the UHT treatment is capable of eliminating all vegetative forms of microorganisms present in milk, as well as most of the sporulated ones, with the exception of heat-resistant spores (LOPEZ-BREA et al., 2017).

Considering the absence of bacteria and acidifying substances in the UHT milk samples, it was expected that there would be only a small variation in the pH values of the samples over time, as shown in the Table 1.

Table 1. Results of pH.

	Storage time (days)			
Treatments	**0**	**7**	**15**	**30**
T1	6.53[a]	6.54 [a]	6.54 [a]	6.51 [a]
T2	6.60 [a]	6.62 [a]	6.62 [a]	6.54 [a]
T3	6.75 [a]	6.84 [a]	6.74 [a]	6.67 [a]
T4	6.69 [a]	6.71 [a]	6.66 [a]	6.61 [a]
T5	6.68 [a]	6.70 [a]	6.66 [a]	6.60 [a]
T6	6.60 [a]	6.65 [a]	6.58 [a]	6.52 [a]
T7	6.68 [a]	6.72 [a]	6.63 [a]	6.58 [a]

Mean values followed by the same letter do not differ from each other at the 5% level.

Through the statistical analysis ANOVA (analysis of variance) of the pH data over time for each of the treatments, considering a significance level of 5%, it was found that there was no significant result, that is, there was no evidence of a difference between the means.

Although there is no statistically significant difference, it is possible to notice a subtle drop in samples pH values throughout the shelf life (Table 1). Other researchers found results similar to those found in this work, for them the cause of this drop might be associated with proteolysis. Anema (2017) evaluated 10 samples of UHT milk, from reconstituted skimmed milk powder, over a shelf life of 10 months, and observed a decrease of up to 0.2 in pH values when compared to those obtained at time zero. While Zhang et al. (2020) obtained pH variations of up to 0.6 during the shelf life of UHT milk samples under study, in addition, it was observed that the gelled samples had a lower pH than the samples that did not form a gel.

For other authors, the decrease in pH values of UHT milk samples during shelf life is associated with factors other than proteolysis, such as, for example, the degradation of lactose, the release of ions from protein-protein interactions, dephosphorylation of caseins and the formation of acids during the steps of the Maillard reaction (REAL et al., 2013; STRÖHER et al., 2021).

With proteolysis, in addition to variations in pH values throughout the shelf life, the milk is destabilized and consequently loses its thermal stability. The thermal stability test indicates the milk ability to withstand heat treatment without forming a clot. It is a test normally carried out on raw milk and is capable of determining the quality of the raw material, considering that thermally unstable milk causes losses to industries due to lower yield, technological problems, such as clogging of pasteurization and UHT systems, in addition to compromising the quality of the final product.

Coagulation occurs due to the destabilization of milk and this in turn can occur due to several factors, which includes the activity of bacterial proteases. As in the case of this study the contamination was intentional, it was expected that throughout the shelf life, as the AprX protease acted on the samples, the thermal stability of the milk would decrease, which can be seen in the Figure 1.

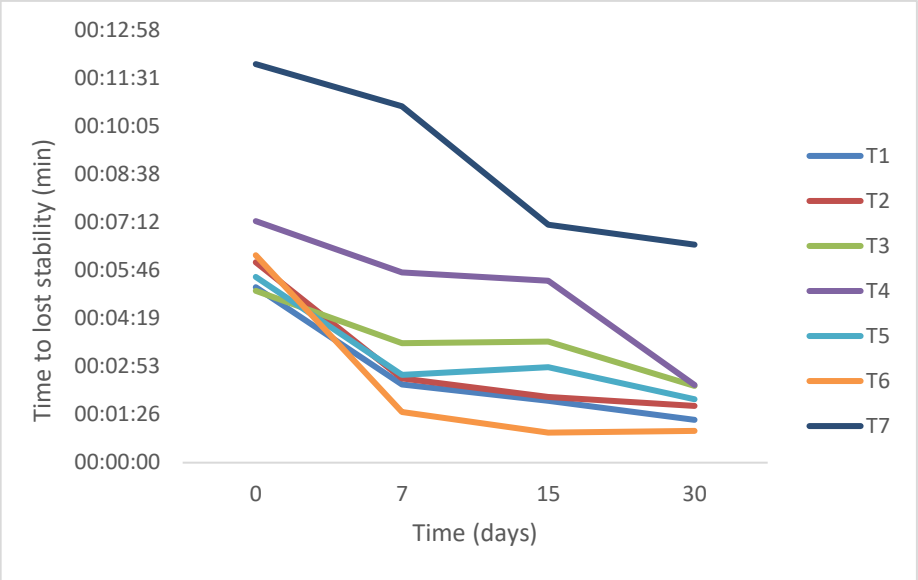

Figure 1. Heat stability of the treatments by oil bath test (140 °C). Mean results expressed according to the time (hr:min:seg) required for the presence of floccules.

Studies carried out by Recio et al. (2000) demonstrated that the AprX protease acts on the casein micelle in a similar way to chymosin, a protease widely used in cheese manufacturing. However, the bacterial protease has lower specificity than chymosin and can hydrolyze, primaly, the bonds 103-104, 104-105, 106-107 and 107-108. By cleaving the glycosylated portion of the protein, there is a decrease in electrostatic and steric repulsion between the molecules, thus favoring aggregation and consequent gelation. The hydrolysis of α and β-casein fractions, even though they are not carried out as a priority stimulate gelation by destabilizing the internal part of the casein micelles (ZHANG et al., 2018).

As we can see in Tables 2-4, over the shelf life, due to the presence of AprX protease in the medium, an increase in protein fractions is expected due to proteolysis of the proteins present. Result that is consistent with the study carried out by Gaucher et al. (2011), in which UHT milk samples were evaluated throughout the shelf life in relation to the content of protein fractions (NCN and NPN). After 92 days, the NCN content of UHT milk previously contaminated with P. fluorescens increased, approximately, 10 times and the NPN content increased five times compared to the control sample.

With the gelation over time, even with the homogenization of the samples before the protein analysis, the protein clusters were formed and that resulted in a little data variation obtained, mainly, of the total nitrogen content of the samples.

Table 2. Results obtained for non-protein nitrogen (NPN) in protein analysis. Results expressed in %.

	Storage time (days)			
Treatments	**0**	**7**	**15**	**30**
T7	0.197^{a}	0.206^{a}	0.231^{a}	0.234^{a}
T4	0.195^{a}	0.255^{a}	0.227^{a}	0.283^{a}
T5	0.205^{a}	0.294^{a}	0.258^{a}	0.334^{ab}
T3	0.196^{a}	0.252^{a}	0.237^{a}	0.336^{ab}
T2	0.191^{a}	0.251^{a}	0.290^{a}	0.405^{ab}
T6	0.223^{a}	0.276^{a}	0.370^{a}	0.462^{b}
T1	0.196^{a}	0.285^{a}	0.347^{a}	0.544^{b}

The Tukey test was applied to compare means. Mean values followed by the same letter do not differ from each other at the 5% level.

Table 3. Results obtained for non-casein nitrogen (NCN) in protein analysis. Results expressed in %.

	Storage time (days)			
Treatments	**0**	**7**	**15**	**30**
T7	0.384^{a}	0.417^{a}	0.445^{a}	0.533^{a}
T4	0.429^{a}	0.535^{a}	0.483^{ab}	0.716^{ab}
T5	0.470^{a}	0.557^{a}	0.517^{ab}	0.747^{b}
T3	0.411^{a}	0.592^{a}	0.588^{abc}	0.875^{b}
T2	0.456^{a}	0.598^{a}	0.703^{abc}	0.870^{b}
T1	0.542^{a}	0.718^{a}	0.813^{b}	1.018^{b}
T6	0.472^{a}	0.691^{a}	0.866^{bc}	0.942^{b}

The Tukey test was applied to compare means. Mean values followed by the same letter do not differ from each other at the 5% level.

An analysis of variance (ANOVA) was performed on the results obtained through protein analysis to determine whether or not there was a significant difference in the action of phosphates on proteolysis during shelf life.

We can see that, for the NPN and NCN fractions, the treatments differed statistically from each other, with treatments T7 (negative control – without addition of inoculum and stabilizing salts) and treatment T4 (0.1% tripolyphosphate + inoculum) presenting the best results compared to treatments T6 (positive control – addition of inoculum only) and T1 (0.08% polyphosphate + inoculum), which performed the worst. This indicates that, in the face of the protein hydrolysis process induced by the addition of a highly proteolytic strain of *P. fluorescens*, the stabilizing salt of treatment 4 was able to slow down this process and maintained the UHT milk sample with percentages of nitrogen fractions similar to the negative control. On the other hand, based only on this analysis, the addition of other stabilizing salts is

not justified, since the samples did not differ statistically from the sample added only by the proteolytic strain, indicating that there was no improvement in the stability of the milk samples throughout the shelf life. The results found for the total nitrogen (Table 4) did not show a statistically significant difference as expected.

Table 4. Results of total nitrogen (TN). Results expressed in %.

	Storage time (days)			
Treatments	**0**	**7**	**15**	**30**
T1	3.193[a]	3.189[a]	3.314[a]	3.195[a]
T2	3.141[a]	3.098[a]	3.290[a]	2.968[a]
T3	3.168[a]	3.128[a]	3.192[a]	3.189[a]
T4	3.185[a]	2.927[a]	3.192[a]	3.117[a]
T5	3.223[a]	2.944[a]	3.217[a]	2.968[a]
T6	3.202[a]	3.035[a]	3.168[a]	3.003[a]
T7	3.190[a]	3.175[a]	3.216[a]	3.148[a]

Mean values followed by the same letter do not differ from each other at the 5% level.

Another method that was used to determine proteolic activity after the addition of inoculum to UHT milk samples was the azocasein assay. However, as mentioned by Aguilera-Toro et al. (2023), it is difficult to compare the results of proteolytic activity obtained through azocasein assays, due to the diversity of methodologies found in the literature, which mainly involves sample preparation and enzyme action time. As it is a quantity directly dependent on the incubation time. The results obtained are not comparable to those found by Baur et al., (2015), Andreani et al. (2016) and Aguilera-Toro et al. (2023), as the conditions and time for the enzyme to act on the samples in the present study were longer, 16 h compared to 2 h, 1 h and 1 h, respectively.

The peak observed in the azocasein graphs (Figure 2) is characterized by the momentary increase in the stability of the three-dimensional structure of the enzyme caused by the UHT treatment. The modification of the structure makes the molecule more active, that is, it makes it able to cleave more substrate in the same time interval. As the protease cannot sustain this modification over time, we observed a drop in its activity and a consequent drop in the curve in the graph.

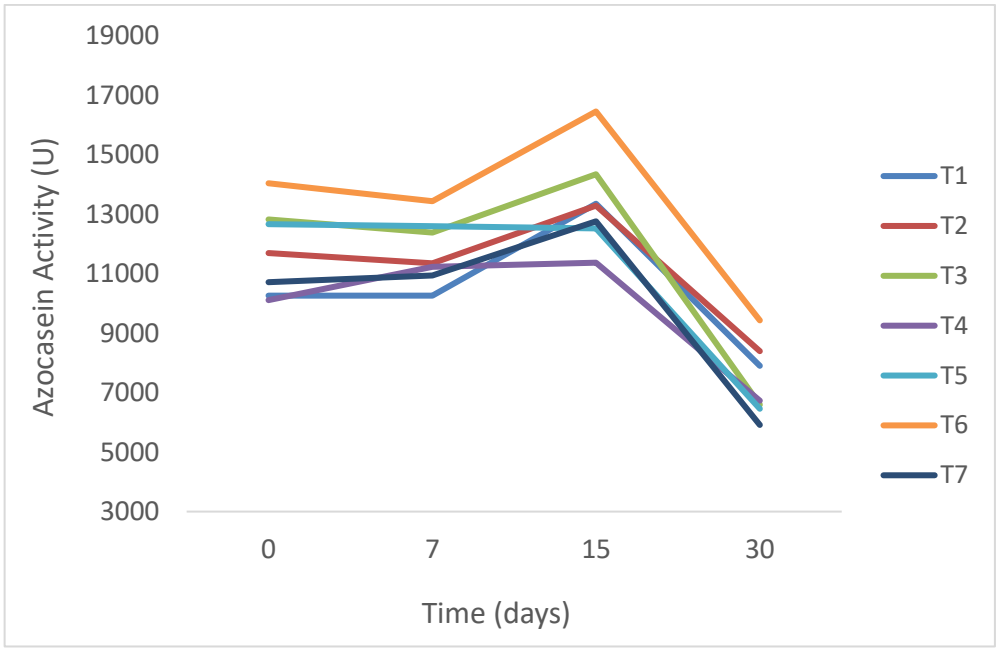

Figure 2. Results of proteolytic activity by azocasein test (U) during shelf life.

In addition to the azocasein test, proteolysis can be evaluated using the SDS-page technique, which consists a type of electrophoresis performed under denaturing conditions for the separation of proteins based on their molecular weight. Through this technique it is possible to separate the fractions of the casein micelle and the serum proteins and from the visualization of the formed bands to determine if these fractions are being degraded or not, over time. As the band disappears from the gel, it is indicative of the degradation of the respective protein fraction.

There is no consensus on which fraction of casein the AprX protease hydrolyzes primarily. Some authors defend its non-specificity (GAUCHER et al., 2011), while others describe that both the kappa and beta fractions are hydrolyzed primarily (ZHANG et al., 2018). Through the images of the SDS-page gels in this study (Figure 3), we can see that throughout the shelf life the band that disappeared first, indicating the hydrolysis of the protein fraction, was that referring to kappa casein, followed by beta and lastly the alpha.

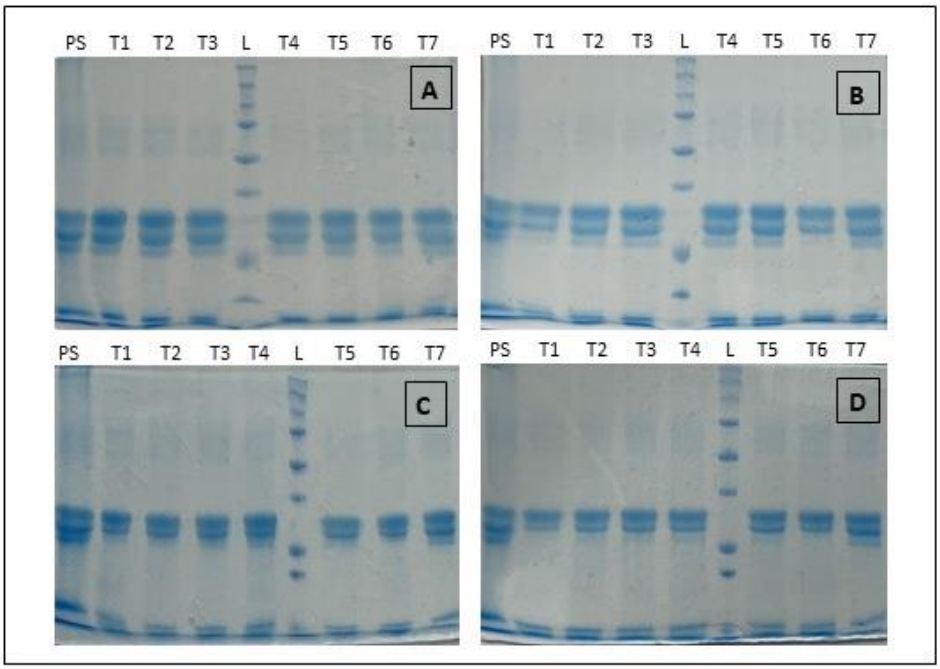

Figure 3. SDS-gel. A: t = 0 h; B: t = 7 days; C: 15 days and D: t = 30 days. PS: Protein Standard; L: Protein Ladder. The process was similar in the three repetitions.

The dosage of monophosphate (T5) used varied in the three repetitions and, due to this it was not possible to compare the results among themselves or with the other treatments, since there is no guarantee of the reproducibility of the obtained data.

It was found that in all three repetitions, treatments 3 and 4 (T3 and T4) showed slower protein hydrolysis compared to the others, indicating that the stabilizing salts (citrate and tripolyphosphate, respectively) used in these treatments managed to delay more effectively the gelation caused by the protease AprX. On the other hand, treatments 1 and 2 (T1 and T2) were those in which the salts used did not show good effectiveness in preventing gelling, when compared to the others. These results were also evident from the visual evaluation of the UHT milk bottles (Figure 4).

Furthermore, the denaturation of proteins, as well as the consequent formation of complexes, are directly associated with the time and intensity of heat treatment applied to the milk. The longer the heating time and temperature, the greater the denaturation of whey proteins and their association with casein micelles (CHAVAN et al., 2011).

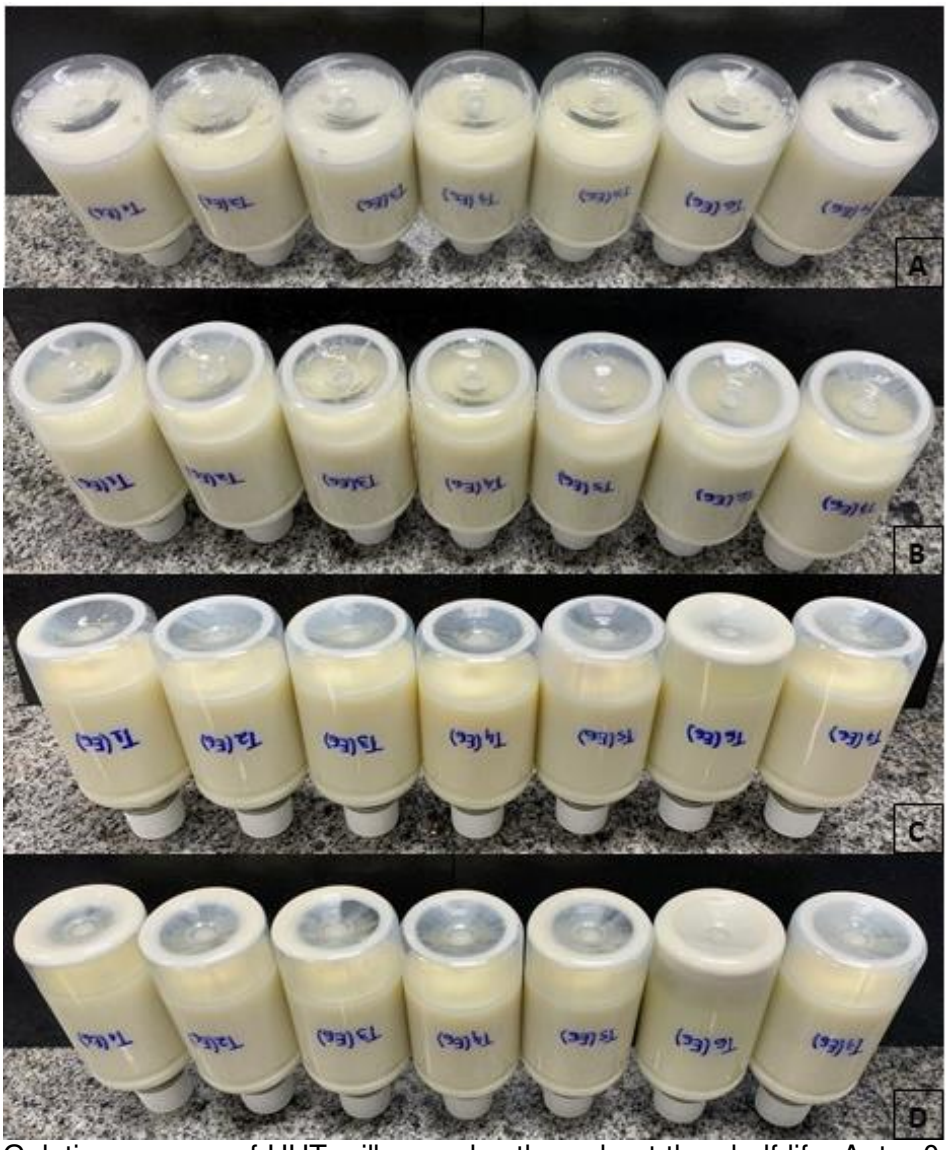

Figure 4. Gelation process of UHT milk samples throughout the shelf life. A: t = 0 h; B: t = 7 days; C: t = 15 days and D: t = 30 days. The process was similar in the three repetitions.

Through the analysis of the images of samples, over the shelf life, it is possible to observe that the sample 6 (T6) showed a greater deposit at the bottom of the UHT milk bottle at the end of the 30 days, followed by samples 1 (T1) and 2 (T2). The other samples, despite also showing gel formation, the gelling process occurred less intensely.

The difference between the gelation speed of the samples may be associated with the inoculum and salts presence. Law et al. (1977) evaluated samples of UHT milk produced with different levels of contamination in the raw material and found that samples with counts below $8x10^6$ CFU/mL had a shelf life of around 140 days, while samples produced with milk with an initial contamination in the order of $5x10^7$ CFU/mL

showed signs of gelation from the twelfth day of shelf life. After all, the most important thing, and what was possible to observe, is that regardless of the time taken to start gelation, the same results were attributed to the treatments in the three repetitions.

We can observe that the positive (T6) and negative (T7) controls presented gelation consistent with expectations. The treatment with the presence of inoculum and without the addition of salts was the sample that gelled the fastest, while the treatment without the addition of inoculum and salts presented gelation slightly superior to the best treatment (T4), indicating that the phosphate added in T4 presented an improvement in relation to the gelation time of standard milk.

The experiments were carried out in a controlled manner and using a single strain of bacteria with a high proteolytic capacity, and due to this it was possible to observe these results more quickly in relation to the potential of the citrate and phosphate salts tested. To extrapolate the results obtained, new experiments would be necessary considering a more diverse microbiota.

4. Conclusion

Based on the results, the best stabilizing salts results were obtained for treatment T4 (tripolyphosphate - 0.1%). On the other hand, the worst results were treatments T1 and T2. Among the worst results obtained, T1 (polyphosphate - 0.08%) stood out as being the one that destabilized it the fastest. It is important to emphasize that the tests were carried out under extrapolated conditions of microbial count and, consequently, amounts of AprX protease that would normally be found in this type of product. Under usual conditions, it is possible that these salts are able to delay the gelation process more effectively and guarantee a stable product during its shelf life.

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GENERAL CONCLUSION

Through the three projects developed over this study, it was possible to perceive the infinity of possibilities and the need for further development in the various areas that permeate the central theme "Quality of UHT milk".

It was possible to have an overview of the microbiological quality of this product, as well as the genera of microorganisms that pose a risk to both - food safety and the quality of the final product. Parallel to this, and based on these two factors, we sought to create a rapid alternative method for detecting the presence of these biological contaminants, which even presented a very promising result, as well as tools that could maintain the stability of the raw material and guarantee, consequently, the production of a quality product.

It is important to highlight that these results obtained constitute only the beginning of an important line of studies about these new tools, which have also been developed by other authors, and which help us in the search for knowledge and application of the same in favor of improvements in society.