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PRESENCE OF *BACILLUS SPOROTHERMODURANS* IN RAW MILK

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PATOS DE MINAS- MG
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Artigo Científico apresentado ao curso de Engenharia de alimentos da Universidade Federal de Uberlândia Campus de Patos de Minas.

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1 Presence of *Bacillus Sporothermodurans* in Raw Milk

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ABSTRACT

The microbiological quality is important for the quality of the final products, thus the presence of *Bacillus spp.* in milk represents a problem for the dairy industry due to its sporulation capacity and resistance of the spore to heat treatment for Ultra High Temperature (UHT). The *Bacillus sporothermodurans* survives, germinate and multiplying in the stored milk and, if not were identified, can be exceed the limit determined by legislation for aerobic mesophilic microorganisms. In addition, high numbers of this microorganisms can generate changes in the quality of dairy products. Therefore, the objective of this work was evaluate the occurrence of *Bacillus sporothermodurans* in raw milk in a dairy industry in Brazil, to generate data that allow to identify the origin and frequency of these microorganisms. Of the 300 samples of raw milk samples analyzed, only 23 showed no colonies characteristic of *Bacillus sporothermodurans*. The other 277 samples (92.3%) exhibited characteristics colonies. The positive results of oxidase and esculina, negative to fermentation of glucose and growth in anaerobic conditions, indicates that the colonies were *Bacillus sporothermodurans* in all tests.

Keywords: *Bacillus sporothermodurans*, Spores, UHT milk.

1 INTRODUCTION

In accordance with the regulation of industrial and sanitary inspection of products of animal origin (BRASIL, 2017), in his article nº 235, it is understood by milk, without further specification, the product from the milking complete, uninterrupted, in conditions of hygiene, healthy cows, well fed and easy.

In agreement with Ordóñez, milk is the product generated by mammary glands of mammals females, with the function of feed the newborn. Aspect of view of physico-chemical,

the milk is a homogenous mixture with a different substances (lactose, glycerides, proteins, salts, vitamins and enzymes), which are in the emulsion, suspension or in dissolution (ORDÓÑEZ, 2005).

The main defiance of dairy industries is the improvement of the quality of raw milk. The obtaining of milk with quality should be a fundamental factor in dairy farms, once this attribute reflects directly in the price of milk paid to the producer, as well as in obtaining dairy products safe.

The microbiological quality of raw milk is related to several factors, such as the health of the cow, proper hygiene, milking equipment and binomial time and temperature of storage and transport of raw milk until the locations of processing. Taking into account these factors, it is immensely important to the microbiological control for the quality of the final products.

The microbiota of milk includes several types of bacteria that vary considerably in number and species, depending on how the milk is contaminated. The milk from an adequate management, have few microorganisms, but can suffer from contamination from the environment and man (BUSATTA et al., 2005).

Genus *Bacillus* consist in a large number of bacteria species, being reported around 347 distinct species (LPSN, 2017). The majority of species are mesophilic, however some can be thermophiles or psicrófilas (LOGAN, 1999).

The genus bacilli has as characteristics to produce endospores resistant to heat, are Gram positive and gram negative or gram variable, presenting itself in the form of sticks(BARROS, 2004).

The *Bacillus sporothermodurans*, is a type of microorganism that has been studied in various parts of the world (CATTANI, 2012; HAMMER et al., 1995; ZACARCHENCO, 1999), since it is characterized by high capacity to survive in foods treated by the UHT (Ultra

High Temperature), due to the production of heat-resistant endospores resistant to heat, which can grow and multiply in the product during storage.

Studies of the presence of *Bacillus sporothermodurans* in UHT milk conclude that considerable portions of the analyzed samples present a high count of this microorganism (CATTANI, 2012; HAMMER et al., 1995; ZACARCHENCO, 1999). In accordance with Pettersson (1996), the presence of *Bacillus sporothermodurans* in UHT milk does not interfere with the sensory characteristics and do not of the safety of products.

There is still no studies of the prevalence of *Bacillus sporothermodurans* in raw milk and it is not known what influence the growth of this microorganism in it, such as seasonality, regionality, practices of hygiene, production and genetic specificity of flocks along the year and nor what the influences of different methods of animal management. Therefore, it is necessary to study the presence of this microorganism in raw milk.

In front of the exposed, the development of this work aims to analyze the occurrence of *Bacillus sporothermodurans* in raw milk.

2 MATERIAL AND METHODS

The experiment was conducted in a Brazilian dairy industry. The raw milk samples received by the industry were analyzed with respect to quantity of *Bacillus sporothermodurans*. Total, were analyzed three hundred (300) samples of raw milk. The analyzes of the samples were carried out in accordance with adaptation of the methodology described in the Brazilian legislation (BRAZIL, 2003).

2.1 Collection and Preparation of samples

The samples were collected in each tank truck that came in the industry during the period of 30 days. These samples were identified and stored under refrigeration until the moment of analysis.

The first stage of the analysis consisted in a pre-heat treatment (thermal shock) for selection of resistant microorganisms. To perform this treatment were pipetted 10 mL of each sample tubes, identified and then taken to the bain-marie at 80 °C for 15 minutes. After having to heating, the tubes were placed in cold water (approximately 1°C) for cooling.

The samples were previously treated were pipetadas (1 mL) into sterile Petri plates. So, they were added approximately 20 mL of Brain Heart Infusion (BHI) Agar on each plate and homogenized properly, mixing the culture medium with the sample. After solidification, the plates were reversed and incubated for 72 hours at $36\pm 1^{\circ}\text{C}$.

2.2 Identification of colonies and biochemical analyzes

In accordance with the methodology (BRAZIL, 2003), when bacterial growth is due to the presence of spores of *Bacillus sporothermodurans*, it is observed an abundant growth of smooth colonies, on a regular basis, white and beige color and diameter of approximately 3mm.

The colonies characteristics were confirmed by tests of Gram staining, catalase and oxidase, growth in anaerobiosis, esculina hydrolysis, fermentation of glucose and proof of urease, in accordance with methodology (BRAZIL, 2003).

For the staining test of Gram, the colonies characteristic were collected and a smear was made on sheet, which were submitted to four steps with defined time of contact between the lamina and the reagents (crystal violet, Lugol's iodine, alcohol and safranin). Thus, when were observed Gram-positive microorganism, the catalase was tested.

For the catalase test was added a drop of 3% hydrogen peroxide in the colony under analysis and no bubbling was observed. The formation of bubbles indicates positive proof for catalase. The majority of the members of the genus *Bacillus* feature reaction of catalase positive.

When the Gram staining showed the presence of Gram-positive microorganism and proof of the catalase was positive for culture in test, the presence of *Bacillus sporothermodurans* was confirmed by means of proof of oxidase, growth in anaerobiosis, esculina hydrolysis, fermentation of glucose and production of Urease.

The test of oxidase consisted in the spread of culture with handle of platinum on the test strips for oxidase. After 10 to 20 seconds, were carried out the readings. The appearance of a blue color (N'N'N'N'-tetramethyl-parafenileno-diamine) is indicative of a positive reaction. The *Bacillus sporothermodurans* presents reaction of oxidase positive

The test of growth in anaerobiosis was performed inoculating culture in tubes containing BHI agar inclined, previously prepared, and incubated in anaerobic jar at $36\pm 1^{\circ}\text{C}$ for 72h. The *Bacillus sporothermodurans* does not grow in anaerobiosis.

For the proof of the hydrolysis of esculina, were made tubes containing esculina agar tilted, where later the culture was inoculated with platinum and handle these incubated at $36\pm 1^{\circ}\text{C}$ for 72h. The hydrolysis of esculina is evidenced by the darkening of the medium. The *Bacillus sporothermodurans* hydrolysis esculina.

The test of fermentation of glucose, the colonies were collected and inoculated in tubes containing phenol red broth-based added of glucose. Soon, the tubes were incubated at $36\pm 1^{\circ}\text{C}$ for up to 72 hours. After the step of incubation were observed if there was change of color of the phenol red indicator, where the turn to yellow coloration indicates the fermentation of sugar present. The *Bacillus sporothermodurans* does not ferment glucose.

The test of urease, culture was inoculated with handle, in tubes or plates containing urea agar, incubated at $36\pm 1^{\circ}\text{C}$ for 72h. Soon, it was observed that the growth with change of color.

The intense pink color, indicating positive for urease production. The *Bacillus sporothermodurans* does not produce urease.

3 RESULTS AND DISCUSSION

Of the 300 samples of raw milk samples analyzed, only 23 showed no colonies characteristic of *Bacillus sporothermodurans*. The other 277 samples (92.3%) exhibited colonies characteristics, as shown in Figure 1.

These colonies characteristics they passed through the test of Gram staining in which presented Gram-positive bacilli morphology (Figure 2). Afterwards was performed the catalase test where we observed the formation of bubbles (Figure 3) that is due to the decomposition of the hydrogen peroxide (H_2O_2), in water and oxygen as an indicator of the reaction, i.e., the presence of catalase enzyme or hidroperoxidase, usually this genre have positive result of catalase. Thus, the two analyzes refer to results that indicate the presence of the genus *Bacillus*.

In the proof of oxidase, after the scattering of culture on the tape oxidase, we observed the appearance of a blue color (N'N'N'N'-tetrametil-parafenileno-diamina) which is indicative of a positive reaction. Figure 4(A) shows the tapes of oxidase before the smear of microorganisms in analysis. Figure 4(B) are the tapes after the smear, with positive results. The *Bacillus sporothermodurans* have positive oxidase test.

The BHI tubes inclined, after the time of 72h to $36\pm 1^\circ C$ in jars of Anaerobiosis showed no growth (Figure 5A), confirming the presence of *Bacillus sporothermodurans*, since it does not grow in anaerobiosis. Other tubes, containing the same colonies were incubated in aerobic environment under the same conditions, and showed growth of microorganisms, as depicted in Figure 5(B).

The test of the hydrolysis of esculin occurred the darkening of the means for all 277 samples analyzed, as shown in Figure 6(B). The microorganisms *Bacillus sporothermodurans* hydrolyze the esculin. In Figure 6(A) are presented only tubes with the culture medium agar esculin.

The test of fermentation of glucose, the phenol red acts as an indicator, the phenol red Broth (Figure 7A) is recommended for use in determining the ability of organisms to ferment various carbohydrates. Thus, the means becoming orange red to yellow when the acid is produced during the fermentation of carbohydrate added. Not occurred during fermentation of glucose for none of the analyzed samples (Figure 7B), all remained in the reddish color, which indicates that the microorganisms do not ferment glucose.

The results were positive for evidence of oxidase and esculin, negative for evidence of fermentation of glucose and urease, and there was no growth in anaerobic conditions, which indicates all the colonies were confirmed as *Bacillus sporothermodurans* in all tests.

No studies were found about the presence of *Bacillus sporothermodurans* in raw milk for comparison with the present study, but there are studies reporting the presence of *Bacillus sporothermodurans* in UHT milk (SCHOCKHEN et al., 1996; ZACARCHENCO, 1999; ABOUELNAGA, 2016; CATTANNI, 2012).

Zacarchenco (1999), in a study done on samples of UHT milk, 45% of these samples presented above results of 100 CFU / mL for the count of mesophilic, being that after DNA polymorphism studies, concluded that part of the samples exist *Bacillus sporothermodurans*.

The analysis done by Neumann et al. (2010) in 511 samples of UHT milk in the regions South, Southeast and Central-west, 45% of the samples contained *Bacillus sporothermodurans* outside the legal standards. In another study conducted by Schockhen et al. (1996) with 32 samples of UHT milk marketed in the region of Ribeirão Preto, 10.5% were positive for *Bacillus sporothermodurans*.

Another study conducted with samples marketed in Alto Uruguai, in Rio Grande do Sul, identified in 88 samples of different brands analyzed, 54.4% of them contained *Bacillus sporothermodurans* (BUSATTA, 2005).

With this high prevalence of *Bacillus sporothermodurans* in UHT milk, along with the high count of this microorganism found in the present study, conclude that there is a large presence of this microorganism in milk and which can be derived from rural properties.

Then, considering that the spores are not destroyed in the pasteurization, the presence of these in raw milk indicates the need for a more effective action to control milk, ensuring that this is according to the standards of identity and quality, being excluded from the counting of mesophilic aerobic bacteria viable (BRAZIL, 2003), what is justified by pathogenic microorganisms are not considered (PETTERSSON, 1996), besides causing physical-chemical alterations and sensorial only after a count above 10^5 CFU/mL (KLIJN et al., 1997).

4 CONCLUSIONS

With the results obtained in the present study, it is concluded that there is a high prevalence of *Bacillus sporothermodurans* in raw milk, which are destroyed after the heat treatment, but unable to germinate during the shelf life of milk products, as confirmed in surveys with UHT milk. Therefore, it is necessary more studies on the *Bacillus sporothermodurans*, since they are not found characteristics in literature. It is also necessary to evaluate how and from where comes this contamination in rural properties, to provide corrective actions, always seeking the best quality food.

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FIGURE

Figure 1. Characteristics of colonies *Bacillus Sporothermodurans*.

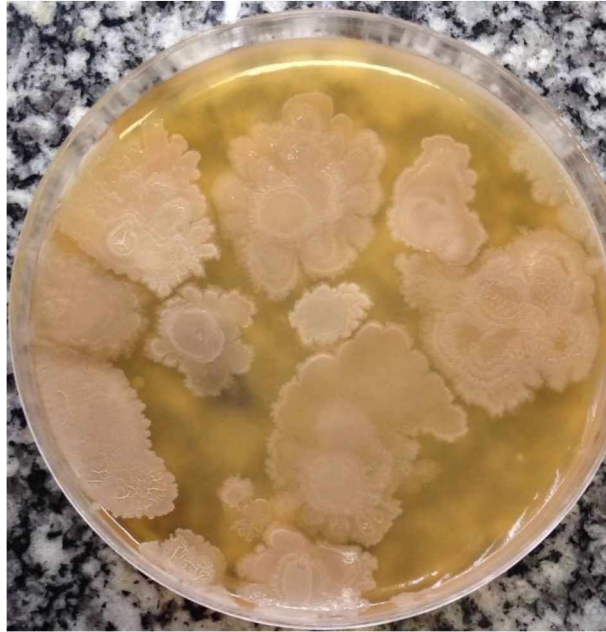


Figure 2. Microscopic image of *Bacillus* Gram positive.

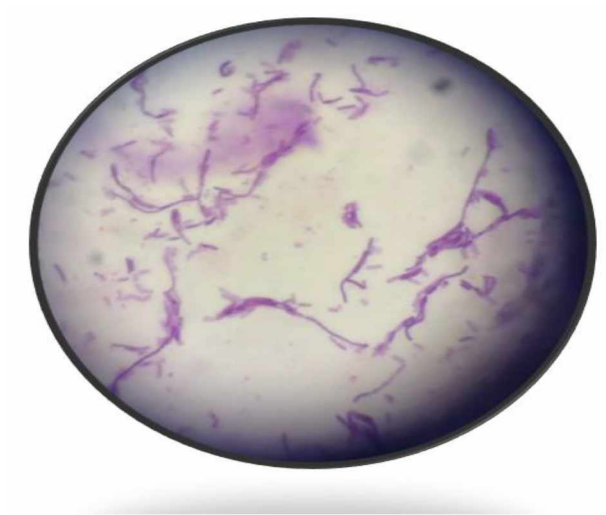


Figure 3. Proof of catalase in colonies characteristics.



Figure 4. (A) Shows the tapes of oxidase before the smear of microorganisms in analysis. (B) are the tapes after the smear, with positive results.

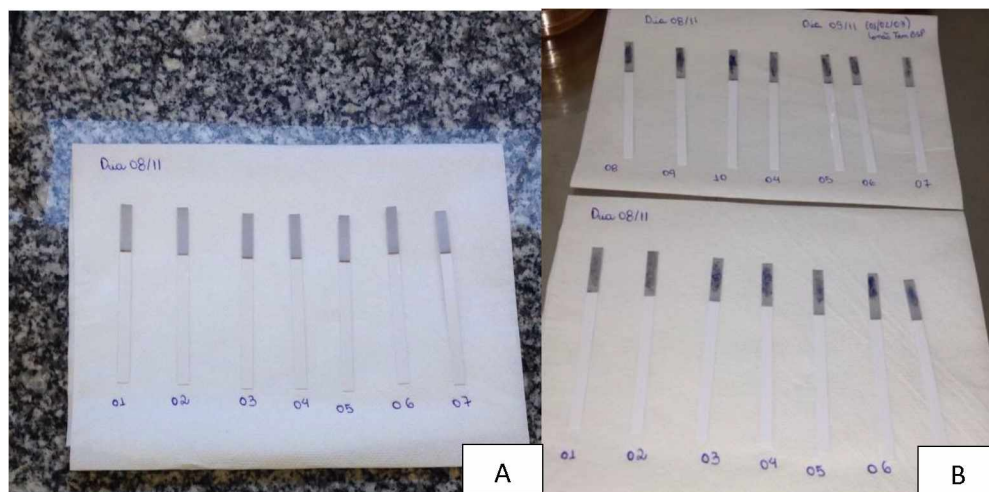


Figure 5. Jar of anaerobiosis (A) used for incubation of microorganisms and (B) comparison between growth in anaerobiosis and aerobic medium.

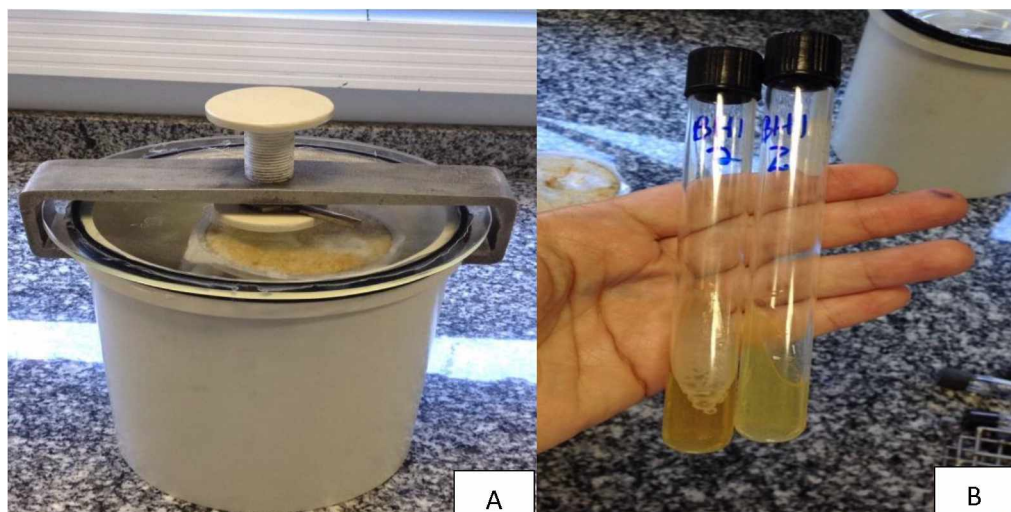


Figure 6. (A) Half tilted agar esculina and (B) medium after incubation.

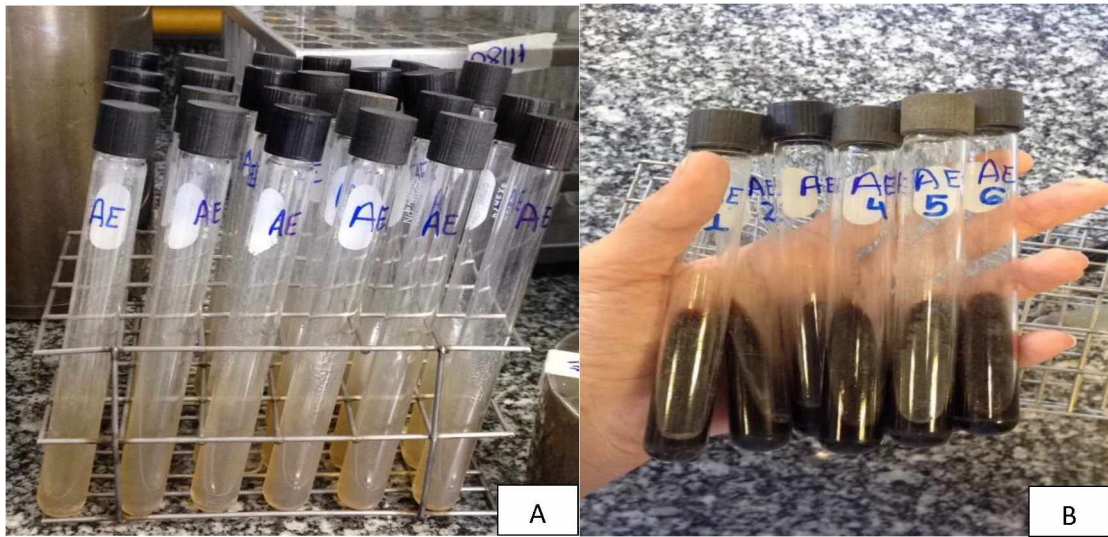


Figure 7. (A) Tubes with phenol broth- red; (B) after incubation of the inoculu.

