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PROTEOLYTIC POTENTIAL OF PSYCHROTROPHIC MICROORGANISMS ISOLATED FROM FRESH CHEESE

Potencial proteolítico de bactérias psicrotróficas isoladas de queijo fresco

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ABSTRACT

Given the economic importance of the dairy industry, product quality should be a constant concern. Some sensorial and technological properties of milk and dairy products are related to the spoilage contaminant microbiota, which is mainly represented by psychrotrophic proteolytic bacteria producing thermoresistant enzymes. Fresh artisanal cheeses are generally produced with raw milk, so it is of great importance to know the microbiota of these products. Therefore, the objective of this work was to count and isolate the psychrotrophic proteolytic bacteria and to evaluate the spoilage potential of these microorganisms isolated from fresh cheeses handcrafted and commercialized in Salinas, MG, Brazil. After the isolation of psychrotrophic proteolytic bacteria present in fresh cheese, the isolates were characterized in terms of Gram staining, shape, and oxidase activity. The proteolytic activity of the isolates was confirmed by a spot halo test using specific agar. The results highlighted the predominance of Gram-positive psychrotrophic proteolytic bacteria when the spoilage microbiota of the fresh cheese samples was evaluated. Considering the proteolytic potential, 34% of the isolates were considered highly proteolytic. The results showed that the initial contamination of fresh cheeses may interfere with the microbiological quality of these products.

Keywords: proteolysis; spoilage; quality; raw milk.

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RESUMO

Devido a importância econômica da indústria de laticínios, a qualidade dos derivados lácteos deve ser uma preocupação constante. Algumas propriedades sensoriais e tecnológicas do leite e derivados estão relacionadas à microbiota deterioradora contaminante, representada, principalmente, por bactérias psicrotróficas proteolíticas que produzem enzimas termoresistentes. Os queijos frescos produzidos artesanalmente são geralmente fabricados com leite cru, por isso, é de grande importância conhecer a microbiota desses produtos. Portanto, o objetivo deste trabalho foi enumerar e isolar as bactérias psicrotróficas proteolíticas além de avaliar o potencial deteriorador desses microrganismos isolados de queijos frescos artesanais comercializados em Salinas, MG, Brasil. Após o isolamento das bactérias psicrotróficas proteolíticas presentes no queijo fresco, os isolados foram caracterizados em termos de coloração de Gram, forma e atividade de oxidase. A atividade proteolítica dos isolados foi confirmada pela formação de halo de hidrólise em meio de cultura específico. Os resultados destacaram a predominância de bactérias proteolíticas psicrotróficas Gram-positivas quando foi avaliada a microbiota deterioradora das amostras de queijo fresco. Considerando o potencial proteolítico, 34% dos isolados foram classificados como altamente proteolíticos. Os resultados mostraram que a contaminação inicial de queijos frescos pode interferir na qualidade microbiológica destes produtos.

Palavras-chave: deterioração; qualidade; leite cru; proteólise.

INTRODUCTION

One-third of the milk produced in Brazil is used to make cheese, which represents one of the most appreciated dairy products in the country. In 2017, the production of cheese reached one million tons with a rise of 2% over the previous year (EMBRAPA, 2018). According to EMBRAPA Gado de Leite (2018), in 2017, Brazil imported 32 thousand tons of cheese, especially mozzarella, processed, and semi-hard cheese. Thus, the universe of cheese (between local production and importation) moves about $ 18 billion per year.

Given the increased production of milk and dairy products and the importance of these products for the national balance, there is a need to expand the dairy market, as well as improving the quality of products offered to the consumption.

The safety and microbiological quality of milk and, consequently, its derivatives, are related to the contaminating microbiota. Milk contamination has a diverse origin and has been the subject of several studies that describe the udder of dairy animals, the milking and processing environment, the utensils, and equipment used in the milk processing stages as the main sources of contamination (SANTOS *et al.*, 2018; VELÁZQUEZ-ORDOÑEZ *et al.,* 2019; ZACHARSKI *et al.,* 2018). However, several studies point to raw milk as a potential source of cheese contamination (KOUSTA *et al.,* 2010). Therefore, artisanal cheeses, which are generally made from raw milk, are often evaluated for microbiological contamination (KAMIMURA *et al*., 2019).

Some microorganisms present in milk are known to pose risks to customer health (BIANCHI *et al*., 2013). In addition to these pathogenic microorganisms, some microorganisms present in milk and dairy products, such as psychrotrophic microorganisms, may contribute to the development of technological problems (SØRHAUG; STEPANIAK, 1997).

Psychrotrophic microorganisms are mostly inactivated by the heat treatments

commonly used in the dairy industry. However, these microorganisms can produce hydrolytic enzymes, such as proteases and lipases, even when exposed to lower temperatures (MAZIERO *et al*., 2010). These enzymes may be heat resistant and hydrolyze milk proteins and fats even afterheat processing (MARCHAND *et al*., 2017). For these reasons, the deterioration by psychrotrophics may exist in cheeses produced from pasteurized milk arising from high contamination in raw milk or post-processing contamination. However, this problem is even more relevant in products made from raw milk since the product is not subjected to heat treatment during its processing.

The activity of microbial proteases in cheese production results in some sensorial changes such as bitter taste, which is produced mainly by the action of its residual activity on highly hydrophobic portions of casein. The accumulation of extremely hydrophobic peptides may be the cause of bitterness in cheese (GARCIA; PENNA, 2010).

To minimize the negative effect of contamination by proteolytic psychrotrophic bacteria, it is important to know the diversity of this microbiota to propose control strategies. As evidenced by Machado *et al.* (2017), the biodiversity of the deteriorative proteolytic psychrotrophic microbiota may vary depending on the geographical location of the region producing milk and dairy products.

Artisanal cheeses, mainly fresh artisanal cheeses, have been extensively studied concerning the pathogenic microbiota, but little is known about the spoilage microbiota. Until the date, there are no studies in the literature focused on the before mentioned microbiota in dairy products produced in Salinas, located in the north of Minas Gerais. The study of this microbiota can contribute to the development of strategies aiming to avoid economic losses and technological issues for the industry. Thus, this study aimed to isolate and evaluate the spoilage potential of the proteolytic psychrotrophic bacteria of raw fresh milk cheese with the perspective of contributing to the production of quality dairy products.

MATERIAL AND METHODS

Sampling

Initially, a survey was carried out with fresh cheese producers, who sold their dairy products at the Salinas Municipal Market, based on data obtained from the Salinas, MG, Department of Agriculture and Environment, in partnership with the Company of Technical Assistance and Rural Extension (EMATER). Subsequently, cheeses produced from raw milk were selected. Samples of fresh artisanal cheese produced from raw milk, without an inspection seal, marketed by this group were collected considering a representative part of the total of producers. To determine the sample size (M), the following equations were used:

$$M_0 = \frac{\left[Z^2_{\frac{\alpha}{2}} * p * q \right]}{E^2} \qquad \text{(Equation 1)}$$

$$M = \frac{\left[M_0 * N \right]}{\left[M_0 + (N-1) \right]} \qquad \text{(Equation 2)}$$

Where: M_0 = number of individuals in the sample; $Z_{\alpha/2}$ = critical value corresponding to the desired confidence level; p = population proportion of individuals belonging to the category we are interested in studying; q = population proportion of individuals who do not belong to the category of individual we are not interested in studying; E = margin of error or estimated maximum error and N = population (BUSSAB; MORETTIN, 2017).

Nine cheese samples were collected from nine different producers between April and June 2018. The maximum time between cheese production and sample collection was 24 h. After collection, the samples were placed in isothermal boxes with ice and taken to the laboratory. During the experiment, the cheese samples were stored at 7 °C for seven days.

Enumeration of proteolytic psychrotrophic and psychrotrophic microorganisms

The analyzes were performed at 0, 2, 5, and 7 days (t_0, t_2, t_5, t_7) of cheese storage. Sample preparation was performed according to the procedures described by Frank *et al*. (1992). The counting of proteolytic psychrotrophic and psychrotrophic microorganisms was performed according to Pinto *et al*. (2015) with changes in the incubation conditions. After serial dilutions in peptone-salt solution (0.85% w/v NaCl; 0.1% w/v bacteriological peptone), samples were plated onto Plate Count Agar (PCA) supplemented with 2% w/v skimmed milk powder. After inoculation, plates were incubated at 7 ± 1 °C for 10 days.

The counting of all visible colonies after the incubation period represented the population of psychrotrophic. Protease-producing colonies formed a clear halo of hydrolysis around them. These protease-producing colonies were enumerated to express the proteolytic psychrotrophic population. The population of both microbial groups was expressed in CFU/g of cheese.

Isolation of proteolytic psychrotrophic bacterial cultures

The Petri dishes which presented between 25 and 250 colonies of psychrotrophic bacteria were used to isolate proteolytic microorganisms. A total of 10% of the protease-producing colonies, with different morphology, were isolated by streaking them onto PCA supplemented with 2% w/v skimmed milk powder. To ensure the purity of the isolates, one single colony of each isolated microorganism was streaked three successive times. The isolates were stored at -20 °C in cryogenic tubes containing 20% glycerol and 80% Brain Heart Infusion broth (BHI).

Characterization of Gram stain, shape, and oxidase activity

The isolates, which were stored at -20 °C, were reactivated in BHI broth followed by incubation at 30 °C for 24 h. Proteolytic psychrotrophic isolates were Gram-stained as described by Pelczar *et al*. (1981). Gram stain and cell shape were observed under a binocular microscope (Bioblue, BB 4260 Euromex, Arnhem, Netherlands) with 1000× magnification. The oxidase test was performed from colonies taken from a solid medium according to Ramos *et al*. (2013) in oxidase tape (Laborclin®, Brasil).

Evaluation of proteolytic potential

Proteolytic psychrotrophic isolates were evaluated for their ability to produce proteases according to Frank *et al*. (1992) with modifications in the incubation conditions. The proteolytic activity was evaluated in PCA supplemented with 2% skimmed milk powder. The isolates were reactivated in BHI at 30 °C for 24 hours. The isolates were inoculated into the surface of Petri dishes containing the above culture medium with the aid of a platinum needle. The plates were incubated at 30 °C for 24-48 hours. After the incubation period, a clear halo of casein hydrolysis was present in the medium. The halo was measured with a pachymeter and the isolates were classified as slightly proteolytic and highly proteolytic when the measured halo diameter

was smaller or larger than the mean value, respectively (DE JONGHE *et al*., 2011).

RESULTS AND DISCUSSION

Enumeration of proteolytic psychrotrophic and psychrotrophic microorganisms

The counts of psychrotrophic and psychrotrophic proteolytic microorganisms of fresh cheeses evaluated during the storage period of 7 days are shown in Figure 1. The count of psychrotrophic bacteria varied between 1.8×10^7 CFU/ g and 8.9×10^7 CFU/ g over the storage period. Therefore, there was no considerable increase in the psychrotrophic population (Figure 1) since high counts, above 10^7 CFU/ g, have already been detected at time 0 (t_0). Concerning proteolytic psychrotrophic microorganisms, the initial count was 5.8 log CFU/ g and the final population, after 7 days of storage at 7 °C, was 6.9 log CFU/ g. There was an increase of approximately 1 log cycle (Figure 1).

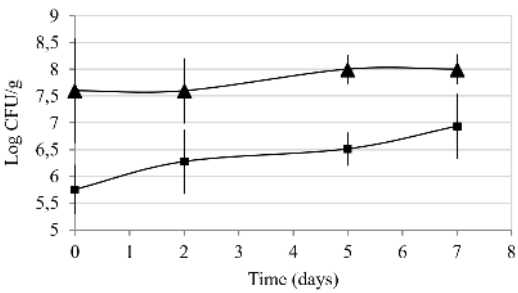


Figure 1 – Evolution of psychrotrophic (triangle) and proteolytic psychrotrophic (square) counts (log CFU/g) in fresh cheese samples over 7 days of storage at 7 °C. The bars represent the standard deviation

Despite the high psychrotrophic count after 7 days of storage, it is not possible to state that the product is unsuitable for consumption based only on this information, since the Brazilian legislation does not contemplate the count of psychrotrophic microorganisms in dairy products. It is also worth noting that the shelf life of fresh cheeses, such as Minas Frescal cheese, may be longer than the 7 days they were stored during this study. According to Oliveira *et al*. (2016), generally, the Minas Frescal cheese has a storage period of approximately 21 days when refrigerated.

Silva *et al*. (2016) evaluated the count of psychrotrophic and psychrotrophic proteolytic microorganisms in Mozzarella cheese, a high moisture cheese, as is the fresh cheese analyzed in this work. These authors showed that the population of the microorganisms described previously did not show significant changes due to the duration of storage. However, they showed an increase in the population at the end of the 60 days of storage under refrigeration, reaching values of 2.8×10^3 CFU/ g for psychrotrophics and 9.1×10^3 CFU/ g for proteolytic psychrotrophics.

Rosa *et al.* (2005) evaluated samples of Minas Frescal cheese stored at 4 °C, the initial count reached the value of 4.7×10^5 CFU of aerobic psychrotrophics/ g. Sangaletti *et al.* (2009), on the other hand, reported changes in the populations of psychrotrophic, lipolytic psychrotrophic, and proteolytic psychrotrophic bacteria with a final average increase in the population close to 8 log cycles between the 1st and the 30th day of storage at 4 °C of Minas Frescal cheese. In this study, the initial psychrotrophic count in cheeses was 7.6 log CFU/g, which represents a higher count than those found by Sangaletti *et al.* (2009), which analyzed Minas Frescal cheeses with initial counts between 2.5 and 4.2 log CFU/g. Teider Junior *et al.* (2019) demonstrated a variation in the counts of psychrotrophic microorganisms of 3.5×10^7 to 6.8×10^9 CFU/g in inspected Minas Frescal cheese samples.

The presence of psychrotrophic microorganisms in high counts can compromise the quality of the product due to the proteolytic and lipolytic activities of some bacteria that belong to this microbiota (SILVEIRA *et*

al., 1998). Thus, the enumeration of the proteolytic psychrotrophic microbiota was necessary.

The population of proteolytic psychrotrophic bacteria contaminating the cheeses analyzed in this research was growing during storage at 7 °C (Figure 1). Sangaletti *et al.* (2009) highlighted the proteolytic psychrotrophic microbiota present in Minas Frescal cheeses stored at 4 °C was initially between 1.0 and 2.1 log CFU/g. The same author detected an increase in this microbiota that reached values between 7.7 and 11.4 log CFU/g after 30 days of storage at 4 °C. The results of the present study corroborate the results of Sangaletti *et al.* (2009) and demonstrate that fresh cheeses can be a very favorable environment for the growth of the evaluated deteriorating microbiota.

The great variation concerning the counts of psychrotrophic and proteolytic psychrotrophic in fresh cheeses found in this work and the studies developed by Rosa *et al.* (2005), Sangaletti *et al.* (2009), and Silva *et al.* (2016) may be related to the microbiological quality of the raw material, the hygienic-sanitary conditions during processing and the post-processing contamination. The cheeses analyzed in this work were produced from raw milk as opposed to the cheeses analyzed by the authors mentioned previously that were produced with pasteurized milk.

The use of raw milk to produce fresh cheese is not permitted under Brazilian law. However, Yoon *et al.* (2016) highlight that the diversity of microorganisms present in raw milk contributes to the manufacture of a variety of cheeses with different sensory characteristics such as taste and texture. The lack of pasteurization of milk in cheese production preserves indigenous bacteria responsible for some important physical and chemical characteristics of the product (COSTANZO *et al*., 2020). However, unpasteurized milk may present a microbiological risk, as pathogenic microorganisms, that may be present. However, these authors pointed out that the microbial population present in cheeses made with raw milk can control the pathogenic microbiota despite the lack of consensus regarding the antagonistic effects of these microorganisms (YOON *et al*., 2016).

Isolation, characterization of Gram stain, shape, and oxidase activity

In the case of the found microbiota be different from that found in other types of cheese previously analyzed, it would be interesting to know some characteristics of the contaminating microorganisms of fresh cheeses produced with raw milk. Thus, 50 proteolytic psychrotrophic isolates were obtained. Among these, 72% were classified as Gram-positive and all isolates presented the coccoid form.

According to Sørhaug and Stepaniak (1997), psychrotrophic microorganisms can be rods, coconuts, vibrios, spore-forming or not, Gram-negative or Gram-positive, aerobic or anaerobic. However, Cousin (1981) stated that psychrotrophic bacteria are, for the most part, Gram-negative and are found in environments where temperatures are constantly between 15 °C and 20 °C. The highest proportion of Gram-negative bacteria in the psychrotrophic microbiota of chilled raw milk has also been evidenced by several studies (ARCURI *et al*., 2008; DE JONGHE *et al*., 2011; HAHNE *et al*. 2019; RIBEIRO JÚNIOR *et al*., 2018; PERIN *et al.,* 2012; RASOLOFO *et al*., 2010; VON NEUBECK *et al*., 2015). Analyzing these studies, there is a consensus that bacteria of the genus *Pseudomonas* are predominantly found in refrigerated raw milk.

Unlike traditional cheeses, usually made with raw milk, most cheeses produced in large industries are obtained from pasteurized milk.

Although it is well described in the literature that the spoilage microbiota of chilled raw milk is mainly composed of Gram-negative bacteria, in this study a predominance of Gram-positive bacteria was found in fresh cheeses stored under refrigeration.

Recent studies involving the study of the biodiversity of the spoilage microbiota of chilled raw milk (HAHNE *et al*., 2019; RIBEIRO JÚNIOR *et al*., 2018; VON NEUBECK *et al*., 2015) showed the genus *Pseudomonas* as predominant but also reported the detection of species belonging to the genera *Lactococcus* and *Streptococcus* in the deterioration of this type of raw material.

The results presented by these studies (HAHNE *et al*., 2019; RIBEIRO JÚNIOR *et al*., 2018; VON NEUBECK *et al*., 2015) corroborate the hypothesis that the highest proportion of Gram-positive psychrotrophic microorganisms found in cheese samples is due, probably, to the presence and predominance of lactic acid bacteria in the analyzed samples. Lactic acid bacteria (LAB) are a group of Gram-positive microorganisms, which do not form spores and which generally grow under microaerophilic conditions or are strictly anaerobic in addition to some groups having a proteolytic activity (JAY, 2005). LAB profiles change according to some factors, for example, the type of cheese, production processes, and ripening conditions (ALMEIDA *et al.* 2020). The LAB genera most commonly found in cheese are *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* e *Enterococcus* (BRUNO; CARVALHO, 2009).

In addition to decreasing the pH, LABs act as competitors for pathogens, producers of antimicrobial compounds, and contribute to the depletion of carbohydrate substrates in favor of lactic acid production (CALIFANO; BEVILACQUA, 2000). In a study carried out by Almeida *et al.* (2020), the authors identified LAB as the main microbial group in Serro cheese produced from raw milk, with *Lactococcus* being the most frequently identified genus, followed by *Streptococcus* and *Lactobacillus* (ALMEIDA *et al.*, 2020). Despite the predominance of LAB, the microbiota of artisanal cheeses showed a greater variability due to the use of raw milk and the absence of standardized production procedures *(*KAMIMURA *et al., 2019)*.

The predominance of Gram-negative microorganisms in pasteurized milk and its derivatives can be explained due to the need to keep the milk refrigerated before and after pasteurization, in addition, it is necessary to produce it in the most possible hygienic way to keep the milk microbial count at low rates. These determinations lead to a population of lactic acid bacteria in milk and to deterioration at low temperature by proteolytic and lipolytic psychrophilic Gram-negative bacteria, such as *Pseudomonas* and *Acinetobacter* species (MARTIN *et al.*, 2018; VON NEUBECK *et al.*, 2015).

A possible source of contamination of pasteurized milk may be the aerobic spore-forming bacteria that have origin in the raw milk and are able to survive pasteurization, but there is evidence that approximately 50% of the milk supply have contamination with Gram-negative thermolabile bacteria that have their origin in the processing plant environment, recontaminating the fluid milk after pasteurization (MARTIN *et al*., 2018).

In an attempt to obtain more information about the psychrotrophic microbiota with the potential for damaging the fresh cheeses, the oxidase test was performed. Among the 50 proteolytic psychrotrophic isolates, 92% (n = 46) did not show oxidase activity. Among the isolates identified as negative oxidase, approximately 78% (n = 36) were Gram-positive. Discrepant results were found by Ramos *et al*. (2013) who describe that 73.33% of psychrotrophic microorganisms found in Minas Frescal cheese stored at 4 °C showed

positive results for oxidase activity. The positive result for the oxidase test indicates the production of cytochrome oxidase, which is an enzymatic system that is related to the cytochromes of the respiratory chain of some microorganisms (KOUSSÉMON *et al.*, 2001).

Silva (2011) evaluated lactic acid bacteria isolated from São Jorge DOP cheese. This author isolated 83 strains that showed negative oxidase activity. The results obtained by Silva (2011) and Ramos *et al*. (2013), added to those found in this study, reinforce the possibility of LAB predominance in the composition of the proteolytic psychrotrophic microbiota of fresh cheeses stored under refrigeration.

Evaluation of proteolytic potential

The spoilage potential of the dominant proteolytic psychrotrophic microbiota was assessed by measuring the casein hydrolysis halo. The results were presented in Figure 2. A total of 34% of the isolated microorganisms had a halo size greater than the average value, which was 6.5 mm and, therefore, was considered highly proteolytic. The proportion of very proteolytic psychrotrophic isolates founded in this study was lower than that described by Machado *et al*. (2015), who evaluated the deteriorating microbiota of cold raw milk. These authors determined that 76.6% of the proteolytic psychrotrophic isolates had a mean hydrolysis halo greater than the mean value of 8.3 mm. The most proteolytic isolates identified by molecular techniques in the above-mentioned study belonged to the genera *Pseudomonas* and *Serratia*, which may explain the higher proportion of very proteolytic isolates compared to the proportion found in this study (34%).

It is known that proteolytic enzymes produced by psychrotrophics can cause several technological problems in the dairy industry (SØRHAUG; STEPANIAK, 1997). To date, studies have prioritized the characterization of proteolytic enzymes produced by Gram-negative psychrotrophics. However, the characterization of enzymes produced by Gram-positive psychrotrophic microorganisms and the impacts that these enzymes can have on dairy products should also be explored.

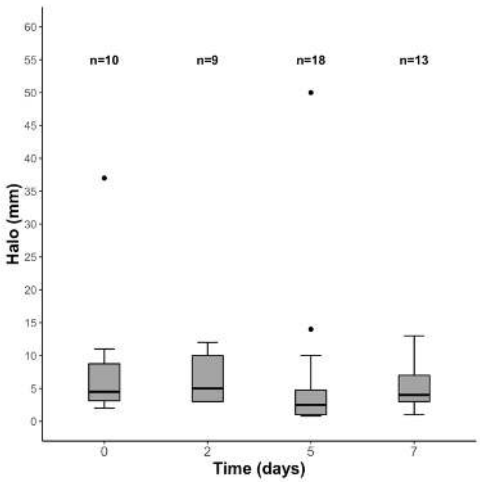


Figure 2 – Spoilage potential, determined by the diameter of the casein hydrolysis halo of the dominant proteolytic psychrotrophic isolates obtained from fresh cheese samples stored at 7 °C for 7 days. The results are presented with the number of test isolates (n) in each time. The solid line inside the box marks the median. The box represents the 25th and 75th percentiles. Supports above and below the box indicate the 10th and 90th percentiles. Outliers are shown as points

CONCLUSION

The proteolytic psychrotrophic microbiota present in fresh cheeses manufactured from raw milk, represented predominantly by Gram-positive bacteria, differs from the deteriorating microbiota

of Minas Frescal cheeses produced with pasteurized milk that have a predominance of Gram-negative bacteria. This predominant microbial population in the analyzed fresh cheeses has a spoilage potential which can compromise the quality of the final product.

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