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Quantification of psychrotrophic bacteria and molecular identification of *Pseudomonas fluorescens* in refrigerated raw milk

Quantificação de bactérias psicrotróficas e identificação molecular de **Pseudomonas fluorescens** *em leite cru refrigerado*

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ABSTRACT: In this study, we investigated the contamination of refrigerated raw milk produced in the western region of Paraná, southern Brazil, with psychrotrophic microorganisms, aiming to assay the proteolytic activity of the isolates and to identify *Pseudomonas fluorescens*, the main proteolytic species associated with the spoilage of milk products. Raw milk samples from 50 dairy farms were submitted to the counting of psychrotrophic microorganisms, being the microbiota characterized by its mesophilic behavior and proteolytic capacity, besides molecular identification of *P. fluorescens*. Of the samples evaluated, 94% had psychrotrophic counts ranging from 3 to 7.1 log CFU mL^{-1}, and 48.5% of these showed mesophilic behavior. Of the isolates, 48.0% had proteolytic activity in at least one evaluated temperature (21 and 30°C), and 39.3% had proteolytic activity in both temperatures. Among the 61 isolates submitted to molecular identification by polymerase chain reaction (PCR), 86.8% contained the expression of the 16S gene characteristic for *P. fluorescens*. In this study, we demonstrated that *P. fluorescens* is the most prevalent psychrotrophic bacteria species in raw refrigerated milk and their proteolytic ability poses high risks to the dairy industry.

KEYWORDS: contamination; proteolysis; refrigeration.

RESUMO: No presente estudo, investigamos a contaminação do leite cru refrigerado produzido na região oeste do Paraná, sul do Brasil, com micro-organismos psicrotróficos, visando testar a atividade proteolítica dos isolados e identificar *Pseudomonas fluorescens*, a principal espécie proteolítica associada à deterioração de produtos lácteos. Amostras de leite cru de 50 fazendas leiteiras foram submetidas à contagem de micro-organismos psicrotróficos, caracterizando-se a microbiota por seu comportamento mesofílico e sua capacidade proteolítica, além de identificação molecular de *P. fluorescens*. Entre as amostras avaliadas, 94% apresentaram contagem psicrotrófica variando de 3 a 7,1 log UFC mL^{-1} e 48,5% destas apresentaram comportamento mesofílico. Entre os isolados, 48,0% apresentaram atividade proteolítica em pelo menos uma das temperaturas testadas (21 e 30°C) e 39,3% apresentaram atividade proteolítica em ambas as temperaturas. Entre os 61 isolados submetidos à identificação molecular por reação em cadeia da polimerase (PCR), 86,8% continham expressão do gene 16S característico de *P. fluorescens*. Neste estudo, demonstramos que *P. fluorescens* é a espécie de bactérias psicrotróficas mais prevalente em leite refrigerado cru e sua capacidade proteolítica promove elevados riscos de deterioração para a indústria de laticínios.

PALAVRAS-CHAVE: contaminação; proteólise; refrigeração.

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INTRODUCTION

Raw milk is an ideal medium for the growth of contaminating microorganism due to its pH close to neutral value, high water activity, and rich nutrient and mineral content. The composition of the raw milk microbiota is very diverse and is influenced by several factors, such as farm management practices, seasons and hygienic practices and storage conditions (OLIVEIRA et al., 2015; VITHANAGE et al., 2016; PORCELLATO et al., 2018). Both Gram-positive (*Bacillus*, *Lactococccus*, *Clostridium*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Streptococcus*, *Staphylococcus*, and *Lactobacillus*) and Gram-negative bacteria (*Pseudomonas*, *Aeromonas*, *Serratia*, *Hafnia*, *Acinetobacter*, *Alcaligenes*, *Achromobacter*, *Enterobacter*, and *Flavobacterium*) have been isolated from raw milk (HANTSIS-ZACHAROV; HALPERN, 2007; VITHANAGE et al., 2016).

The prolonged refrigerated storage of raw milk may select some bacteria with psychrotolerant behavior, especially the genus *Pseudomonas*, that are responsible of the spoilage of milk and dairy products owing to their enzymatic activity (MUNSCH-ALATOSSAVA; ALATOSSAVA, 2006; NEUBECK et al., 2015; PORCELLATO et al., 2018).

According to the International Dairy Federation, psychrotrophic microorganisms are those able to grow at 7°C or less, regardless of their optimum growth temperature. Some psychrotrophic bacteria produce and release proteases and lipases to the external environment, compromising the integrity of the milk constituents and the quality of dairy products (BAUR et al., 2015; NEUBECK et al., 2015; RIBEIRO JUNIOR et al., 2018). It is widely reported that contamination of raw milk by *P. fluorescens* induces instability in ultra-high temperature (UHT) pasteurized milk during storage (GAUCHER et al., 2011; BAGLINIÈRE et al., 2012; STUKNYTĖ et al., 2016) and technological defects in cheese (MARTIN et al., 2011; CARRASCOSA et al., 2015), milk powders (CHEN et al., 2003) and others dairy products.

Besides compromising the integrity of the milk constituents, the microbial proteases and lipases are thermostable and can remain active even after the elimination of the vegetative microorganisms by heat treatments applied by the dairy industry (GLÜCK et al., 2016; VITHANAGE et al., 2016). The activities of proteases and lipases that resist the heat treatments may cause changes in quality and shelf life as well as compromise functional properties and cause flavor defects of milk products during storage (CHEN et al., 2003; MACHADO et al., 2017).

Therefore, knowledge is needed about which microorganisms are present in raw milk and their spoilage potential under different conditions. Thus, the aim of the current study was the quantification of the psychrotrophic microorganisms in Brazilian refrigerated raw milk, aiming to assay the proteolytic activity of the isolates and to identify *P. fluorescens*.

MATERIALS AND METHODS

Enumeration and isolation of psychrotrophic bacteria

Fifty dairy farms located in the western region of Paraná, southern Brazil, were selected for raw milk sampling. A single sample was collected on each farm between July 2014 and February 2015. From each raw milk sample, decimal dilutions were plated in duplicate on plate count agar (PCA, Difco™, Becton Dickinson Company, Franklin Lakes, NJ, USA) and incubated at 7°C for 10 days (COUSIN et al., 2001).

Investigation of proteolytic activity and mesophilic behavior

Four to five isolates of each plate with bacterial growth were checked for their proteolytic activity by agar diffusion assays with skim milk agar (PCA + 1.0% skim milk) and incubated at 21°C for 72 hours and 30°C for 48 hours (BEERENS; LUQUET, 1990; VITHANAGE et al., 2016). The presence of clear zones around the colonies was indicative of proteolytic activity.

The isolates were also inoculated in plate count agar and incubated at 35°C for 48 hours for the evaluation of their mesophilic behavior. The isolates were also inoculated in MacConkey agar and submitted for Gram identification to select presumptive *Pseudomonas sp.* strains.

DNA extraction and polymerase chain reaction (PCR) amplification

Isolated Gram-negative bacteria with no fermented lactose were selected for DNA extraction using the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The DNA concentration was estimated using spectrophotometry (NanoDrop™ Lite, Thermo Scientific, Wilmington, DE, USA).

Amplification of DNA 16S-specific region for *P. fluorescens* was performed using the primer set 16SPSEfluF (5'-TGCATTCAAAACTGACTG-3') and 16SPSER (5'-AATCACACCGTGGTAACCG-3'), according to SCARPELLINI et al. (2004). The PCR was performed with 12.5 µL de GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA), 1.0 µL of each primer, 9.5 µL of ultrapure water (Promega Corporation, Madison, WI, USA) and 1.0 µL of bacterial genomic DNA solution.

The amplification was performed following the thermal profile: 3 minutes at 94°C; 30 cycles of 94°C for 30 seconds; 47°C for 30 seconds; 72°C for 1 minute; and final extension of 72°C for 5 minutes. Following amplification, products were submitted to an electrophoresis run for 2 hours at 45 V in 1.5% agarose gel with GelRed™ (Biotium Inc., Hayward, CA, USA). A single DNA fragment of 850 bp was amplified for identification.

RESULTS AND DISCUSSION

Of raw milk samples presented, 94% contained psychrotrophic counts with levels ranging from 3 to 7.1 log CFU mL^{-1} (Fig. 1). Several studies evaluated the contamination by psychrotrophic bacteria in Brazilian raw milk and found high counts of this group of microorganisms which indicates flaws in production hygiene and problems in raw milk refrigeration and transport (PINTO et al., 2006; NÖRNBERG et al., 2010; MÖRSCHBÄCHER et al., 2017). ALMEIDA et al. (2017) found mean psychrotrophic counts in raw milk ranged from 5 to 7 log CFU mL^{-1} in dairy farms in the same state as this study. Although it is not a mandatory analysis for the Brazilian legislation, psychrotrophic microorganism count is very important in milk quality control. According to PINTO et al. (2006), it would be reckless to manufacture dairy products from raw milk with a psychrotrophic microorganism count higher than 6.5 log CFU mL^{-1}. Other authors do not recommend using milk with counts higher than 6 log CFU mL^{-1} (NÖRNBERG et al., 2010).

Raw milk should be stored between 2 and 4°C, before processing, to protect the nutritional and sensory qualities by the reduction of psychrotrophic growth as well as proteolytic and lipolytic activities (MACHADO et al., 2017; MELINI et al., 2017). The prompt application of a cooling treatment after milking and of cold temperatures for storage, which should be a routine practice, are not effective measures for inhibiting the growth rate of psychrotrophic bacteria. Nevertheless, the count of psychrotrophic bacteria that develop after milk collection depends on the storage period and, primally, on sanitary and hygienic conditions (HANTSIS-ZACHAROV; HALPERN, 2007; FOX et al., 2017).

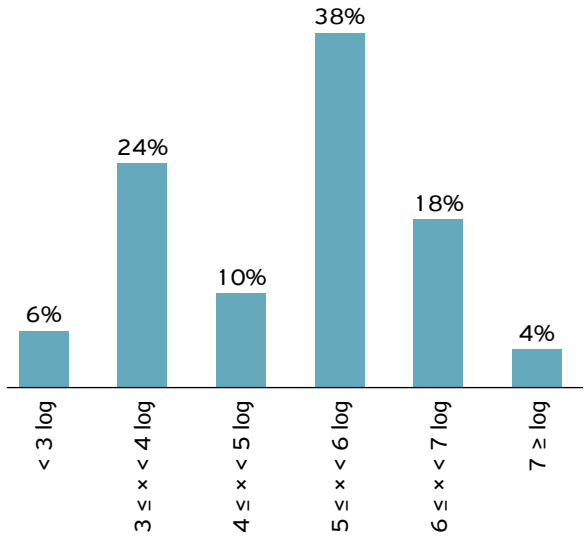

Figure 1. Psychrotrophic bacteria count (in CFU mL^{-1}) distribution in raw milk samples of Paraná western region, Brazil (n = 50).

Regarding the mesophilic behavior of the strains, 48.5% of the 229 psychrotrophic isolates evaluated were able to multiply at 35°C — temperature characteristic of mesophilic microorganisms — suggesting that these microorganisms are mesophilic (capable of adapting to cold) and able to alter their metabolism, since most of the psychrotrophic isolates present optimum temperature of multiplication between 20 and 30°C (MCPHEE; GRIFFITHS, 2011).

Among the 229 psychrotrophic isolates, 48.0% showed proteolytic activity in at least one temperature (21 and 30°C), and 39.3% showed activity in both tested temperatures (Table 1). This result resembles that of other authors who showed proteolytic activity in 40 to 45% of the psychrotrophic strains evaluated (MUNSCH-ALATOSSAVA; ALATOSSAVA, 2006; NÖRNBERG et al., 2010; RIBEIRO JUNIOR et al., 2018). The enzymatic activity of psychrotrophic bacteria are markedly influenced by incubation temperature, with 30°C being optimal (DECIMO et al., 2014).

Pseudomonas sp. isolates were submitted to the PCR confirmation by amplification of 16S-specific region for *P. fluorescens*, and 86.9% were confirmed to contain this bacterium. Thus, considering the total of isolates of psychrotrophic in this study, the percentage of *P. fluorescens* was 23.1%. Molecular methods have been frequently applied for routine identification of *Pseudomonas sp.* in raw milk and to measure their relative abundance, diversity, and proteolytic and lipolytic potential activity.

In this study 57.2% of psychrotrophic isolates were Gram-negative bacteria, and 27.5% exhibited no fermented lactose, a phenotypic characteristic of the *Pseudomonas* genus. The predominance of Gram-negative psychrotrophic in raw milk has been reported by other studies (DECIMO et al., 2014; NEUBECK et al., 2015). *Pseudomonas sp.* isolates were submitted to the PCR confirmation by amplification of 16S-specific region for *P. fluorescens*, and 86.9% were confirmed to contain this bacterium. Thus, considering the total of isolates of psychrotrophic in this study, the percentage of *P. fluorescens* was 23.1%. Molecular methods have been frequently applied for routine identification of *Pseudomonas sp.* in raw milk and to measure their relative abundance, diversity, and proteolytic and lipolytic potential activity (OLIVEIRA et al., 2015; VITHANAGE et al., 2016).

Several studies point to *Pseudomonas sp.* as the principal protease and lipase activity bacteria in refrigerated raw milk (MUNSCH-ALATOSSAVA; ALATOSSAVA, 2006; ERCOLINI et al., 2009; BAUR et al., 2015; MENG et al., 2018). WANG; JAYARAO (2001) observed a higher production of proteases by *P. fluorescens* at 22°C than at 7°C and at 32°C. Furthermore, MENG et al. (2018) found 58.2% of the isolates with extracellular peptidase activity at 10°C and 61.2% at 25°C.

Many proteases produced by strains of *P. fluorescens* are heat-resistant and retain activity even after UHT treatment. This residual activity can cause changes in the physicochemical

Table 1. Proteolytic activity of psychrotrophic isolates at different temperatures (n = 229).

	Proteolytic activity at 21°C	Proteolytic activity at 30°C	Only one temperature	In both temperatures
Psychrotrophic (n = 229)	106	93	110	90
Psychrotrophic with mesophilic behavior (n = 111)	67	63	70	60

properties of the casein micelles and lead to destabilization and gelation of UHT milk during storage (GLÜCK et al., 2016; VITHANAGE et al., 2016). Thus, adoption of appropriate practices for obtaining milk is necessary to minimize contamination of the raw material by psychrotrophic microorganisms.

CONCLUSIONS

The current study confirmed the high prevalence of proteolytic psychrotrophic bacteria, especially *Pseudomonas*, in refrigerated raw milk produced in the western region of Paraná, Brazil. This suggests the high spoilage potential of this genus under refrigeration conditions. In addition, this study confirmed that *P. fluorescens* is a major contaminating psychrotrophic microorganism in raw milk. The investigation of milk-associated psychrotrophs is required and should be encouraged in order to enhance and improve existing control methods and to ensure the quality of milk and milk-derived foodstuffs.

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