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The main spoilage-related psychrotrophic bacteria in refrigerated raw milk

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ABSTRACT

Refrigerated raw milk may contain psychrotrophic microorganisms that produce thermoresistant exoproteases and lipases, which may compromise the quality of processed fluid milk and dairy products during storage. The aim of this work was to quantify and identify the deteriorating psychrotrophic microbiota in Brazilian refrigerated raw milk using genetic diversity analysis. The mean psychrotrophic count was 1.1×10^4 cfu/mL. Of the total isolates, 47.8 and 29.8% showed deteriorating activity at 35°C within 48 h and 7°C within 10 d, respectively. Among the proteolytic species, more isolated by this study were *Lactococcus lactis* (27.3%), *Enterobacter kobei* (14.8%), *Serratia ureilytica* (8%), *Aerococcus urinaeequi* (6.8%), and *Bacillus licheniformis* (6.8%). Observed among lipolytics were *E. kobei* (17.7%), *L. lactis* (15.6%), *A. urinaeequi* (12.5%), and *Acinetobacter lwoffii* (9.4%). The isolates *S. ureilytica*, *E. kobei*, *Pseudomonas* spp., and *Yersinia enterocolitica* potentially produced alkaline metalloprotease (*aprX*). Despite the low counts, a considerable portion of the psychrotrophic microbiota presented spoilage potential, which reaffirms the need for rigor in the control of contamination and the importance of rapid processing as factors that maintain the quality of milk and dairy products.

Key words: alkaline metalloprotease, lipolytic, milk spoiler, proteolytic

INTRODUCTION

The cooling of raw milk allows for control of the multiplication of mesophyll microbiota, predominantly saccharolytic microorganisms (Das et al., 2015; Erich et al., 2015). These microorganisms are responsible for the acidification and thermal instability of milk proteins, as the hydrolysis of lactose produces lactic acid as a by-product (McAuley et al., 2016).

Some mesophilic microorganisms, called psychrotrophs, adapt to refrigeration temperatures by synthesizing phospholipids and neutral lipids containing increased proportions of UFA, resulting in a reduction in the melting point of the lipids. This phenomenon serves to maintain their fluidity, thus allowing the continued functionality, solute transport, and secretion of extracellular enzymes (de Oliveira et al., 2015).

Some psychrotrophs produce and release proteases and lipases to the external environment and absorb the products of their hydrolysis (Cousin, 1982). In addition to 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 to the milk by the industry (Samarzija et al., 2012; de Oliveira et al., 2015; Baglinière et al., 2017). Prolonged action of proteases and lipases may cause organoleptic changes in fluid milk or dairy products, such as a bitter or rancid taste in cheeses or gelation and sedimentation in UHT-treated milk (Fairbairn and Law, 1986; Matéos et al., 2015; Zhang et al., 2015).

There is a global demand for dairy products with good quality and a long shelf-life. For pasteurized milk, which best preserves the nutritional and organoleptic aspects of raw milk (Andersson and Öste, 1994), there is a great expectation to increase its shelf life. For this to happen, it is necessary to keep contamination to a minimum and to control for specific microorganisms with great spoilage potential.

Several studies evaluated the composition of psychrotrophic microbiota in raw milk and its deteriorating activity (Hantsis-Zacharov and Halpern, 2007; McPhee and Griffiths, 2011; Gargouri et al., 2013; von Neubeck et al., 2015; Vithanage et al., 2016; Xin et al., 2017). However, different microbial community structures are found in raw cow milk samples from different geographical areas (Xin et al., 2017), and under good environmental hygiene conditions in obtaining raw milk, other proteolytic and lipolytic psychrotrophic microorganisms can become important for milk quality. Studies that verify the diversity of these other microorganisms are essential to determine their dairy farm of origin and

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refine hygiene practices used to further reduce these specific microorganisms in raw milk. These practices could result in minimizing the effect of proteases and lipases in processed milk, thereby increasing its shelf life, the integrity of its constituents, and consequently, the industrial yield.

The aims of this study were to quantify psychrotrophic microorganisms in Brazilian refrigerated raw milk, to verify the proteolytic and lipolytic activity of the isolates at mesophilic and psychrotrophic temperatures, to ascertain the potential of alkaline metalloprotease production, and to identify these contaminant microorganisms.

MATERIALS AND METHODS

We evaluated 20 refrigerated raw milk samples produced in the municipalities of Castro and Arapoti, in the central region of the state of Paraná, Southern Brazil. Each sample was collected from a different farm. These dairy farms were previously characterized (Ribeiro Júnior et al., 2015) and are part of the largest association of milk producers in southern Brazil. The milk produced on these farms is sent to the dairy company of the association for the production of noble dairy products and fluid milk. The samples were aseptically collected from the bulk tanks on the dairy farms and transported under refrigeration to the Laboratório de Inspeção de Produtos de Origem Animal of the State University of Londrina, Paraná, Brazil.

The raw milk samples were diluted until 10^{-3} in saline (0.9%) and peptone (0.01%) for total bacterial count (**TBC**), performed by seeding into Petrifilm AC (3M Microbiology, Maplewood, MN), followed by incubation at 35 ± 1°C for 48 h. The psychrotrophic count was performed by adding samples (0.1 mL) in duplicate on the surface of plate count agar (Oxoid, Basingstoke, United Kingdom). The plates were inverted and incubated at 7°C for 10 d.

All bacterial colonies on the plates used for counting psychrotrophs (one plaque from the duplicate) were purified in plates of standard agar and were retested on milk agar (Acumedia, Baltimore, MD) supplemented with 10% reconstituted skim milk powder solution (10%) and on tributyrin agar (HiMedia, Mumbai, India) supplemented with 1% tributyrin (HiMedia) to verify the proteolytic and lipolytic potential, respectively (Hantsis-Zacharov and Halpern, 2007). Plates were incubated for 48 h at 35°C and for 10 d at 7°C.

The isolates that presented with proteolytic or lipolytic activity (or both) were cultured in brain heart infusion broth (Acumedia, Baltimore, MD), incubated at 35°C for 48 h, and subjected to genomic DNA extraction by simple boiling as in Ribeiro Júnior et al. (2016). The products of the extraction of the isolates that presented proteolytic activity in the plates were subjected to PCR for the *aprX* gene to verify the alkaline metalloprotease production potential (Table 1).

All extracts were subjected to amplification of the internal transcript spacer (**ITS**) region 16–23S rRNA using the primers and conditions described in Table 1. The PCR was performed with approximately 50 ng of DNA template, 100 n*M* of each deoxynucleotide, 5 μL of 10× buffer, 75 mmol·L^{-1} of $MgCl_2$, 20 pmol L^{-1} of each primer, and 2.5 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Amplification was performed in a thermocycler (Aeris Thermal Cycler, Esco Micro Pte. Ltd., Singapore) and the PCR-amplified DNA samples were loaded onto a 1% agarose gel (Invitrogen) to be subjected to electrophoresis for 1 h at a constant voltage of 90 V. The gels were stained with an ethidium bromide solution of 0.2 mg/mL for 20 min before visualization. Images were saved after UV transillumination.

The amplicons of the ITS region were subjected to restriction by 2 U via the enzyme Cfo I (Promega, Madison, WI), using the reaction protocol described by the manufacturer (https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/restriction-enzymes-protocol.pdf). The amplified DNA and enzyme mixtures were incubated for 1 h at 37°C in a thermocycler. Restriction products were subjected to agarose gel electrophoresis (1.5%) for 1 h at a constant voltage of 70 V. The gels were stained with an ethidium bromide solution of 0.2 mg/mL and documented.

The amplification profiles of the ITS regions of each isolate, together with its product of restriction by the enzyme Cfo I, were used as genomic variables to construct a dendrogram of phylogenetic similarity (Ranjard et al., 2001) using Bionumerics v. 1.50 software (Applied Mathematics, Kortrijk, Belgium). The similarity matrix Dice coefficient (Dice, 1945) and the unweighted pair group mean averages algorithm (Sneath and Sokal, 1973) were used. To determine the clusters, a minimum of 70% phylogenetic similarity was used.

A representative sample from each cluster was selected for partial amplification of the 16S rRNA gene using the primers and conditions described in Table 1. The products of this PCR were purified (PureLink Genomic DNA Purification Kit, Invitrogen) and quantified (Qubit dsDNA HS Assay Kit, Invitrogen) for DNA sequencing using the Sanger method (ABI 3500 Genetic Analyzer, Applied Biosystems, Foster City, CA), which was completed in both directions.

The quality of the sequences was evaluated by the software BioEdit v. 7.2.5 (Hall, 1999), and the consensus sequences were generated by CAP 3 (Huang