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Genetic diversity of thermoduric spoilage microorganisms of milk from Brazilian dairy farms

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ABSTRACT

When correctly pasteurized, packaged, and stored, milk with low total bacterial counts (TBC) has a longer shelf life. Therefore, microorganisms that resist heat treatments are especially important in the deterioration of pasteurized milk and in its shelf life. The aim of this work was to quantify the thermoduric microorganisms after the pasteurization of refrigerated raw milk samples with low TBC and to identify the diversity of these isolates with proteolytic or lipolytic potential by RFLP analysis. Twenty samples of raw milk were collected in bulk milk tanks shortly after milking in different Brazilian dairy farms and pasteurized. The mean thermoduric count was 3.2 ($\pm$4.7) $\times$ 10^2 cfu/mL (2.1% of the TBC). Of the 310 colonies obtained, 44.2% showed milk spoilage potential, 32.6% were proteolytic and lipolytic simultaneously, 31% were exclusively proteolytic, and 48 (36.4%) were only lipolytic. Regarding the diversity, 8 genera were observed (*Bacillus*, *Brachybacterium*, *Enterococcus*, *Streptococcus*, *Micrococcus*, *Kocuria*, *Paenibacillus*, and *Macrococcus*); there was a predominance of endospore-forming bacteria (50%), and *Bacillus licheniformis* was the most common (34.1%) species. Considering the RFLP types, it was observed that the possible clonal populations make up the microbiota of different milk samples, but the same milk samples contain microorganisms of a single species with different RFLP types. Thus, even in milk with a high microbiological quality, it is necessary to control the potential milk-deteriorating thermoduric microorganisms to avoid the risk of compromising the shelf life and technological potential of pasteurized milk.

Key words: lipolytic, milk spoiler, proteolytic, thermotolerant

INTRODUCTION

Pasteurization is an efficient process for eliminating all pathogenic microorganisms, thus providing microbiologically safe milk for consumption and reducing the amount of deteriorating microorganisms (Sørhaug and Stepaniak, 1997; Lewis, 2003; Knight et al., 2004). However, the thermal processing of raw milk is not sufficient to eliminate all the microorganisms capable of causing deterioration. Thermoduric microorganisms resist the pasteurization process of milk and use components, such as proteins and fat, as nutrient sources, causing organoleptic changes and a reduced product shelf life (Hull et al., 1992; Huck et al., 2007).

Thermoduric microorganisms can influence the shelf life of pasteurized milk (Durak et al., 2006; Huck et al., 2007), together with the enzymatic activity of the proteases and lipases produced by spoilage microorganisms—mainly psychrotrophs—in raw milk (Fairbairn and Law, 1986; Sørhaug and Stepaniak, 1997; Marchand et al., 2008) and the effect of endogenous proteases, such as plasmin (Bastian and Brown, 1996; Murphy et al., 2016), the type of packaging (Petrus et al., 2010), and the contaminants acquired after pasteurization.

Endospore-forming microorganisms, mainly in the genera *Bacillus* and *Paenibacillus*, are often described as being responsible for reducing the shelf life of pasteurized milk (Huck et al., 2007; Ranieri et al., 2009, 2012). However, other nonsporulated microorganisms can resist pasteurization (Buehner et al., 2014; Ribeiro Júnior et al., 2017), such as *Streptococcus* and *Enterococcus* species (Delgado et al., 2013).

In Brazil, the poor quality of raw milk on farms and the deficiencies in the processing and packaging of pasteurized milk reduce the shelf life of milk to only a few days. Microorganisms capable of resisting the pasteurization process in vegetative forms are of major importance in the dairy industry in that they give Brazilian pasteurized milk a short shelf life. However, little is known about the identity and proteolytic and lipolytic potential of the thermoduric microbiota in Brazilian milk, and it is difficult to determine the origin of and

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control the milk contamination by these microorganisms in dairy farms.

In contrast to most Brazilian dairy farms, some Brazilian dairy regions are the gold standard in milk quality and productivity. The municipality of Castro, which is in the southern region of the Paraná state, is an example of this gold standard. There are properties that produce large daily volumes and have a large number of implemented technical and hygiene practices (Ribeiro Júnior et al., 2015). The animals have a great capacity for milk production, and the microbiological quality of the milk meets international standards ($<10^5$ cfu/mL; FDA, 2009), presenting the possibility of producing pasteurized milk with a long shelf life (Ribeiro Júnior et al., 2015).

From the identification of thermoduric microorganisms that are potentially milk proteolytic and lipolytic, it is possible to determine their origin under Brazilian conditions, establish specific strategies for the control of this microbiota in raw milk, and, consequently, increase the shelf life of milk after pasteurization. This process would also preserve the integrity of the raw milk constituents, increase the industrial potential in the production of dairy products, and reduce the technological problems in UHT milk and dairy products (Murphy et al., 2016). Thus, the aim of this study was to quantify the thermoduric microorganisms in milk of high microbiological quality produced in the southern region of Brazil to evaluate the spoilage potential of these microorganisms as well as their diversity and identity as a preliminary step for determining the source of raw milk contamination in Brazilian farms and developing control practices.

MATERIALS AND METHODS

Twenty samples of raw refrigerated milk were collected and evaluated from the bulk milk tanks of 20 different dairy farms in the region of Castro in Paraná, Brazil. This municipality was recently recognized by the Brazilian federal government as the Brazilian capital of milk. The description of properties has been previously reported by Ribeiro Júnior et al. (2015).

Of these milk samples, 95% had total bacterial counts $<10^5$ cfu/mL, with a mean of 1.5 ($\pm$3.4) $\times$ 10^4 cfu/mL (Ribeiro Júnior et al., 2018). In closed and continuous flow, the raw milk was precooled before being stored in a bulk tank at 4°C. The samples were collected in bulk tank immediately after the morning milking. The milk of the afternoon milking of the previous day to the collection was already in the tanks. The samples were collected aseptically, refrigerated, and sent to the Animal Origin Products Inspection Laboratory at the State University of Londrina, where they were immediately processed.

Thermoduric bacteria were enumerated after heating milk (5 mL) to a temperature that stimulates pasteurization (62.8 $\pm$ 0.5°C for 30 min), followed by immediate refrigeration at 10°C, in accordance with Frank and Yousef (2004). After treatment, serial dilutions of the samples were performed up to 10^{-3} in sterile peptone (0.001%) saline (0.85%) solution, and duplicates were surface plated (0.1 mL) on plate count agar (Oxoid, Basingstoke, UK). Plates were incubated at 35 $\pm$ 1°C for 48 h.

All isolated colonies obtained on the dilution plates used for counting were spiked on milk agar (Acumedia, Baltimore, MD) supplemented with 10% reconstituted milk powder solution (10%) and tributyrin agar (Himedia, Mumbai, India) supplemented at 1% with tributyrin (Himedia) for their proteolytic and lipolytic potential, respectively (Hantsis-Zacharov and Halpern, 2007). These plates were incubated under the same conditions recommended for thermoduric enumeration (Frank and Yousef, 2004).

The isolates that had deteriorating activity were grown in brain heart infusion broth to extract their genomic DNA by boiling according to Ribeiro Júnior et al. (2016b). Extraction products were subjected to partial amplification of the 16S rRNA gene using primers 27f (5′-GAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-GGYTACCTTGTTACGACTT-3′; Osborne et al., 2005). The reactions were carried out with final amounts of 50 ng of template DNA, 100 nmol of each nitrogen base, 1× buffer, 75 mmol/L of $MgCl_2$, 20 pmol/L of each primer, and 2.5 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), with a final volume of 50 μL. The amplification conditions were as follows: 1 cycle of initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min; and 1 final extension step at 72°C for 10 min.

To avoid redundancy in the sequencing reactions, the partial 16S rRNA gene amplicons obtained in the reactions described above were subjected to RFLP for hierarchical genetic grouping from the restriction profiles with the enzymes Cfo I (Promega, Madison, WI), *Taq*I (Ludwig Biotech, Santa Maria, Brazil), Rsa I (Invitrogen), and *Hae*III (Invitrogen) using the protocols described by the manufacturers.

After the restriction reactions, the products were electrophoresed on a 2% agarose gel for 60 min at 70 V. The gels were stained with ethidium bromide (0.2 mg/L) and imaged. The images of each restriction pro-